Application of Antiviral Polyoxometalates to Living Environments—Antiviral Moist Hand Towels and Stationery Items

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Abstract: Safe, secure, and environmentally friendly active substances should be developed. VB (virus block) refers to an antibacterial/antiviral mixture of two kinds of polyoxometalates (PMs), i.e., K_{11}[H_{2}[(VO)_{3}(SbW_{9}O_{33})_{2}]]·27H_{2}O (VB2) and α-Na_{2}[SbW_{9}O_{33}]^{9-} (VB3), and polyhexamethylene biguanide (PHMB). VB was demonstrated to exert antiviral effects on cultured cells. The effects were maintained even in hygiene products or solids. The antiviral effects were analyzed by reverse transcription–polymerase chain reaction (RT–PCR), and the results were correlated with TCID50, potentially eliminating the need for handling infectious viruses. VB was demonstrated to be extremely effective (up to 99.99% inhibition) in cultured cells, with antibacterial/antiviral effects maintained in VB-containing hygiene products. VB was applied to solids, demonstrating their high applicability and versatility. VB withstands high temperatures regardless of materials because its effects are enhanced by more frequent contact with viruses and bacteria due to the increased surface area of the compound.

Keywords: polyoxometalate; influenza virus; SARS-CoV-2; norovirus; MRSA; Bacillus cereus; Pseudomonas aeruginosa

1. Introduction

As the spread of the new coronavirus affects social life worldwide, vaccines and antiviral agents should be immediately developed. In the future, living environments should be improved as a preventive measure. As coronavirus has an envelope structure, alcohol and hypochlorous acid are extensively used to some effect [1,2]. However, the frequent use of antibacterial agents has recently caused resistant bacteria (e.g., methicillin-resistant Staphylococcus aureus (MRSA)). Antiviral agents may have adverse effects when frequently applied to mutable RNA viruses [3,4]. Therefore, safe, secure, and environmentally friendly active substances with different mechanisms of action should be developed in the long run.
Polyoxometalates (PMs) are transition metal-oxide clusters, a series of hundreds of compounds created by substituting metal elements and modifying their three-dimensional structures [5]. Their biological activities have become predictable based on their three-dimensional structures [5]. Of the PMs with antitumor, antiviral, antibacterial, or other activities, antiviral PMs have been investigated regarding their activities and mechanisms of action [6,7]. Of note, PM-1002 (VB2; K_{11}H[(VO)_{3}(SbW_{9}O_{33})_{2}]·27H_{2}O) and PM-19 (K_{7}[PT_{2}W_{10}O_{40}]·6H_{2}O) were effective against RNA and DNA viruses, respectively [6–8]. Additionally, the synergistic effects of PMs combined with oxacillin on MRSA have been demonstrated [9]. In addition, antibacterial/antiviral mixtures were developed by adding antibiotic oxacillin and antibacterial agent Irgasan to three PMs [10,11]. However, its marketing was prohibited in the United States because oxacillin and Irgasan may cause resistant strains. Therefore, oxacillin and Irgasan were replaced by PHMB, and the antiviral activities of minimal formulations (VB2, VB3, and PHMB) were evaluated. The two PM compounds, VB2 and VB3, are the major components for exerting antiviral efficacy, with low cytotoxicity (VB2: IC_{50} > 200 M) and a broad viral spectrum (particularly RNA-based viruses) [8,10]. The stability study of the PM solution also showed no change in conformation at 50 °C for 12 weeks [10].

In the present study, the effects of a new version of VB on infected cells, hygiene products (in solutions), and solids (materials for stationery items) are investigated. Plaque and TCID50 assays are routinely employed to measure viral titers. However, to establish an evaluation assay free of infectivity and biohazard in culture equipment, RT–PCR was performed with bacterial and viral RNA sequences, and its correlation with TCID50 was examined. In addition, an affinity assay was performed using Biacore X (Biacore, Uppsala, Sweden) because the affinities between virus particles and active compounds may be reflected in antiviral activities [12].

The antiviral effects of the new version VB were demonstrated on cultured cells and maintained even in hygiene products and solids. RT–PCR for detecting RNA from viruses (e.g., severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and norovirus) and bacteria (e.g., MRSA) was established, demonstrating the high affinity of the new version VB for the viral and bacterial components. The application of the VB compound to living environments may have preventive effects on infections.

2. Materials and Methods

2.1. VB (Virus Block, Registered™)

VBs are the names of three mixtures with antimicrobial antiviral activity, including two polyoxometalates (PM)—K_{11}H[(VO)_{3}(SbW_{9}O_{33})_{2}]·27H_{2}O (VB2) and α-Na_{2}[SbW_{9}O_{33}]^{9−} (VB3)—and polyhexamethylene biguanide (PHMB). The mixing ratio of VB was VB2:VB3:PHMB = 9.71:87.35:2.94 (wt%) dissolved in ultrapure water and used for experimentation (International Publication No. WO2019/230210). Each of VB2 and VB3 was prepared by dissolving the crystallized material in an aqueous solution after being crushed with a milk beak. When used in molded articles for stationery, a pore-sized, 45-µm filter was also prepared. Two PM compounds (VB2 and VB3) were synthesized according to the methods described in a previous study [13]. PHMB was purchased as 20% aqueous solution or crystalline from Funakoshi. These raw materials were prepared in an FSX in-house laboratory and used as VB.

2.2. Virus, Bacteria, and Their Constituents

Influenza virus A (A/WS/33; N1H1; VR-825) and B (B/GL/1739/54; VR-103), quantitative synthetic norovirus G1 (I) RNA (VR-3234SD), methicillin-resistant Staphylococcus aureus (MRSA; BAA-1026), Bacillus cereus (13061), and Pseudomonas aeruginosa (10145) were purchased from the American Type Culture Collection (ATCC; VA, USA). Influenza virus A was subjected to a one-step growth experiment with Madin–Darby canine kidney (MDCK) cells. Other components were used as templates for RT–PCR. Influenza A virus, Matrix 2 protein (AP78163), SARS-CoV-2 (COVID-19) S1 protein, Fc Tag
(SIN-C5255), and norovirus GII.4 VP1 VLPs (OPNB00044-100UG) were purchased from Funakoshi to be subjected to affinity assays for VB using Biacore X (Biacore, Upppsala, Sweden).

2.3. Cell Cultures and Virus Infection for One Step Growth

MDCK cells were maintained at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium (MEM) containing 10% fetal bovine serum (Carson, CA, USA), penicillin (100 U/mL), streptomycin (100 µg/mL), and kanamycin (10 µg/mL). MDCK cells were used for virus infection (35-mm dish) and an assay of TCID₅₀ (96-well plate). The virus infection was induced, as described previously [14]. MDCK cells were infected with Influenza virus A at an MOI of 0.01. After adsorption of virus for 30 min at 4 °C, cells were washed with phosphate-buffered saline (PBS) to remove unattached virus particles and then incubated in a maintenance medium containing VB at 37 °C.

2.4. VB-Contained Moist Hand Towel and Wet Sheet

VB-containing moist hand towels and wet sheets, manufactured and distributed by 22 hand towel manufacturers in partnership with FSX Inc., were randomly selected for the experiment.

2.5. Molding Materials for Stationery Items

Molded products (90 × 50 × 1 mm), prepared by adding VB into various materials (polycarbonate (PC), polypropylene (PP), acrylonitrile butadiene styrene (ABS), thermoplastic styrenic elastomer (TPS), polybutylene terephthalate (PBT), polyoxymethylene (POM), and polyester elastomer (TPC)) at 0–0.5% by weight for manufacturing ballpoint pens at Mitsubishi Pencil Co. Ltd. were subjected to the experiment (Figure S1).

2.6. Measurement of Antiviral Effects

2.6.1. Infectivity Titers by TCID₅₀ Assay

MDCK cells (100 µL, 50,000 cells/mL) were seeded onto a 96-well plate, and, 24 h later, the old culture medium was aspirated, followed by washing with PBS. Subsequently, 100 µL of a sample serially diluted with 2% FBS-MEM was added, and the culture was continued for 2 days. The culture supernatant was removed by aspiration, followed by washing with PBS. Then, 100 µL of Cell Counting Kit reagent [15] was added, and, 1 h later, the absorbance was measured at 450 nm using a microplate reader (SYNERGY/HT, BioTek, Japan). After serial dilution, 50% inhibition was calculated from each absorbance value, and TCID₅₀ was determined from the dilution rate. TCID₅₀ relative to the virus control is indicated as Delta TCID₅₀.

2.6.2. Relative Levels of Viral RNA by RT–PCR

For viral RNA extraction, Trizol® reagent (ThermoFisher Scientific Co. Ltd.) was used, and the work was carried out according to the protocol. Viral solution or the culture medium (0.2 mL) was added to a 1.5 mL test tube after adding 1 mL of Trizol. Chloroform (0.2 mL) was added thereto, stirred, and then centrifuged (the centrifugal conditions were all 4 °C and 12,000 rpm, 15 min below). After centrifugation, the upper layer section was transferred to a new 1.5 mL test tube, 0.5 mL of isopropyl alcohol was added to it, stirred, and then centrifuged. After removing the upper layer, 1.0 mL of ethanol was added, mixed by inversion, and then centrifuged. The upper layer was removed, and 50 µl of ultrapure water (RNase-free) was added to the precipitate and completely volatilized with ethanol to serve as a total RNA sample. A portion of this sample was used to quantify RNA levels by NanoDrop (ThermoFisher Scientific). Total RNA (500 ng) was used as a template for PCR and cDNA synthesis by reverse-transcription reactions (reverse transcriptase; RT reactions) and a one-step RT–PCR, in which quantitative PCR could be performed in one tube, was performed using the Luna Universal One-Step RT–PCR Kit. The PCR device was comprised of a Takara machine and Thermal Cycler Dice Real-Time System II. As one system, Luna Universal One-Step Reaction Mix (2x), 10 µL,
Luna WarmStart RT Enzyme Mix (20×), 1 µL forward primer (10 µM), 0.8 µL reverse primer, and 0.8 µL template RNA were used, and nuclease-free water was adjusted to 20 µL. The reaction was carried out at 95 °C for 30 s for 1 cycle, and at 95 °C for 5 s and 60 °C for 30 s for 50 cycles. For RT–PCR, primers for various targets were used (Table 1) [16–21].

Table 1. Primers for PCR.

| Target                          | Primer                      | References |
|---------------------------------|-----------------------------|------------|
| Influenza virus, AH1            | Inf-AH1 Forward; Reverse    | [16]       |
| Influenza virus, AH3            | Inf-AH3 Forward; Reverse    | [16]       |
| Influenza virus, B              | Inf-B Forward; Reverse      | [16]       |
| Herpes Simplex virus Type1      | HSV-1 Forward; Reverse      | [17]       |
| Herpes Simplex virus Type2      | HSV-2 Forward; Reverse      | [17]       |
| Noro virus                      | NoroGI-SK Forward; Reverse  | [18]       |
| NoroGI-SK                       | Forward; Reverse            | [18]       |
| Meticillin-resistant Staphylococcus aureus (MRSA) | MRSAX-xsa325-F Forward; Reverse | [19] |
| MIRSA-mecv574-R Reverse        | MIRSA-mecv519-R Reverse     | [19]       |
| MIRSA-mecv511-R Reverse        | MIRSA-mecv492-R Reverse     | [19]       |
| Emetic Bacillus cereus          | serus-casTM Forward; Reverse| [20]       |
| Pseudomonas aeruginosa          | Pseudo-Ps2 Forward; Reverse | [21]       |
| Gram-positive bacteria          | Gramposi-Gp Forward; Reverse| [21]       |
| Gram-negative bacteria          | Grammego-Gn Forward; Reverse| [21]       |

Legend: R; A, G, N; A, C, G, T, H; A, T, C, Y; C, T, M; A, C.

2.7. Experiment Design

2.7.1. Antiviral Effects of VB on Cultured Cells (One-Step Growth)

After the adsorption of viruses on MDCK cells for 30 min, the culture was continued with VB-containing culture medium and the culture medium was collected for TCID$_{50}$ assay.

2.7.2. Direct Inactivating Effects of VB on Virus Particles

Each virus solution ($6 \times 10^6$ PFU/mL), dissolved in 2% FBS–MEM, was mixed with VB (final concentrations: 0, 0.1, 1, 10, 100, and 300 g/mL) at a ratio of 1:1, followed by incubation at 37 °C for 1 h. The effects of VB were examined based on virus infectivity titers (TCID$_{50}$) and the relative numbers of virus molecules (RT–PCR).

2.7.3. Biacore Assay of Bimolecular Affinity between VB (VB2) and Virus Component Proteins

The binding affinity and kinetics of VB (VB2) and virus protein were measured using surface plasmon resonance with a BIACORE X (Biacore, Uppsala, Sweden) instrument containing a CM5 sensor chip. Virus proteins were immobilized onto each of the flow cells using NHS/EDC amine coupling chemistry. Flow cells were activated for 7 min, with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M 3-(N,N-dimethylamino)-propyl-1-ethylcarbodiimide at a flow rate of 20 µL/min. VBs (VB2) as
ligands were injected over each of the flow cells for 2–3 min, which resulted in a surface density of 1000–1500 resonance units (RU). Surfaces were blocked with a 7-min injection of 1 M ethanolamine–HCl at pH 8.5. The running buffer used for kinetic experiments contained 10 mM HEPES at pH 7 and 150 mM NaCl (HBS-N) purchased from BIACORE. All experiments were performed at 25 °C at a flow rate of 20 µL/min. Each analysis cycle consisted of (i) the serial dilution of each analysis being injected for 2 min over both flow cells, the Logan-immobilized and reference cells; (ii) a 3-min stabilization period; (iii) the regeneration of the Logan surface with a 30 s injection of 30 mM NaOH; (iv) a 3-min stabilization period. The signal was monitored as the ligand-immobilized flow cell minus the reference cell. The data from the buffer blank was subtracted from all of the sample runs. The normalized data were then simultaneously fit to a 1:1 binding with a mass transport model using steady-state affinity analysis in BIA Evaluation 3.1 software to obtain Kass and Kdiss parameters.

2.7.4. Negative Test of Commercially Available Hygiene Products by Quantitative RT–PCR with Viral and Bacterial RNA

Commercially available hygiene products (VB-containing moist hand towels and wet sheets) were subjected to extraction with purified water (10 mL/g). General bacteria and fungi were examined by a plate culture method using CompactDry “Nissui” TC and YM (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). Moist hand towel manufacturers in Japan were under instruction from the Ministry of Health, Labour, and Welfare. General bacteria count should be <100,000 colonies per sample, and no fungi should be detected [22].

2.7.5. Antiviral Effects on Molding Materials for Stationery Items

The virus solution (3 mL) was added to a sterile bag (Unipack; AS ONE Corporation, Osaka, Japan) containing each molded product, followed by incubation at 37 °C. Samples (0.2 mL each) were collected. Some samples were subjected to RT–PCR after RNA extraction and the others to TCID50 assay.

2.7.6. Experiment and Statistical Analysis

All the experiments were performed at the Research Organization of Biological Activity (Tokyo, Japan), Mitsubishi Pencil Co. Ltd. (Tokyo, Japan), Central Research Laboratories, Yokohama University of Pharmacy (Yokohama, Japan), and Narita Animal Science Laboratory Co. Ltd. (Chiba, Japan).

The mean and SD is shown for each experimental test result. Significant differences between groups were determined using Student’s t-test or one-way analysis of variance. A significance level (p-value) of under 0.05 was considered to compare the means.

3. Results

3.1. Antiviral Effects of VB on Cultured Cells

The antiviral effects of VB, added immediately after viral adsorption, on the one-step growth of the virus were examined. Serial changes were observed with VB (100 and 300 µg/mL; [Figure 1a]). The effects of VB at various concentrations were examined at 24 h by Delta TCID50 assay and relative quantitative RT–PCR, which reflects the number of virus particles ([Figure 1b,c]). VB strongly suppressed virus growth by four orders of magnitude (99.99% at 300 µg/mL) in a concentration-dependent manner ([Figure 1a]). The decrease in virus molecules was well correlated with the changes in TCID50 in a concentration-dependent manner ([Figure 1b,c]).
The decrease in virus molecules was well correlated with the changes in TCID50 in a concentration-dependent manner (Figure 1b,c).

**Figure 1.** Inhibitory effects of virus block (VB) on the one-step growth of influenza virus. (a) Serial changes in viral load in culture medium. (b) Concentration-dependent response to the viral load at 24 h; TCID50 assay. (c) Concentration-dependent response to the viral load at 24 h; relative quantitative RT–PCR. *p < 0.001 relative to the virus control.
3.2. Direct Inactivating Effects of VB on Virus Particles

As revealed by viral infectivity (TCID$_{50}$; Figure 2a) and the relative number of virus molecules (RT-PCR; Figure 2b), VB inactivated virus particles in a concentration-dependent manner. As the maximum effect, TCID$_{50}$ changed by one order of magnitude with 1/1000 reduction in virus molecules. Both TCID$_{50}$ and RT-PCR changed similarly in a concentration-dependent manner.

![Graph 2a](image1.png)

![Graph 2b](image2.png)

**Figure 2.** Direct inactivating effects of VB on viruses. (a) TCID$_{50}$ assay; (b) relative quantitative RT-PCR. * $p < 0.001$ relative to the virus control (VB, 0 $\mu$g/mL).
3.3. Biacore Assay of Bimolecular Affinity between VB (VB2) and Virus Component Proteins

The affinity of VB for viral component proteins was analyzed using Biacore. The analysis was conducted for each component because VB consists of three components. The results of VB2 are shown in Table 2. In addition, VB3 showed comparable affinity (data not shown). PHMB had extremely high binding properties but little dissociation. Therefore, the viral protein of the ligands was damaged during regeneration, precluding kinetic analysis. VB2 also showed a comparably high affinity for SARS-CoV-2 S1 protein of the same coronavirus species and GII.4 VP1 VLPs of a nonenveloped norovirus virus.

Table 2. Binding affinity of VB to virus compartments by BIACORE.

| Ligands              | Analytes  | KD (M)   |
|----------------------|-----------|----------|
| Influenza A virus    | VB(VB2)   | 3.6 x 10^{-9} |
| Matrix 2 protein     | Acyclovir | No response |
| SARS-CoV-2           | VB(VB2)   | 8.1 x 10^{-9}  |
| Norovirus GII.4 VP1 VLPs | VB(VB2) | 6.4 x 10^{-9}  |

3.4. Negative Test of Commercially Available Hygiene Products by Quantitative RT–PCR with Viral and Bacterial RNA

As shown in Figure 3, quantitative RT–PCR was established for several viral and bacterial species. Amplification patterns are shown for each RNA after 5-fold dilution. Influenza virus (RNA virus), HSV (DNA virus), and a nonenveloped norovirus were subjected to the experiment. In addition, MRSA, Bacillus cereus, and Pseudomonas aeruginosa, which cause problems in living environments, and Gram-positive and -negative bacteria among general bacteria were detected. By quantitative RT–PCR and plate culture method (for general bacteria and fungi), viral molecules and bacteria were tested in commercially available hygiene products (3 samples each from 22 manufacturers). No virus was detected in any of the samples, while general bacteria were detected in small amounts for each manufacturer, although the detection level was below the reference value (100,000 colonies/sample), and no fungi were detected (Figure S1).

3.5. Antiviral Effects on Molding Materials for Stationery Items

In the present study, VB, which had been used in solutions, was applied to solids. First, molded products were made from several materials with VB dissolved at different concentrations (Figure S2). Their antiviral effects were examined (Figure 4a). VB had antiviral effects on typical materials, PC and PP, in a concentration-dependent manner, although the effects were less potent than those in solutions. The effects were enhanced with an increase in the surface area by reducing the particle size of the crystal (Figure 4b). In addition, the effects, which appeared within an hour of examination, continued (Figure 4c,d). To examine the effects of VB on various materials, PC was used to confirm the reproducibility, with five additional materials (ABS, TPS, PBT, POM, and TPC) for examination (Figure 5). Each material showed the antiviral effects of VB in a concentration-dependent manner. The effects did not differ with the materials. VB had antiviral effects when added at <0.3%. The relative viral molecules were reduced to 1/100 for solids, resulting in Delta TCID_{50} of one order of magnitude.
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Figure 3. Standard curves generated from serial dilution of RNA from virus (a) and bacteria (b).
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Figure 4. Effects of VB on molded products. (a) Dependence of VB concentration. (b) Effects of the particle size of VB crystal. (c) Time required for the onset of VB effects in polycarbonate (PC). (d) Time required for the onset of VB effects in polypropylene (PP). * $p < 0.001$ relative to VB, 0 $\mu$g/mL or normal (b).

Figure 5. Effects of VB on molded materials. (a) TCID$_{50}$ assay; (b) relative quantitative RT–PCR.
4. Discussion

“Antibacterial/antiviral products” are attracting considerable social attention. Safe and secure active substances that do not induce resistant strains are needed.

Among conventional PM compounds, those with specific transition metal elements and certain three-dimensional structures have been found to have antiviral activity [5]. Of these, VB1(VOSO₄), VB 2, and VB 3 were combined with antibiotic oxacillin and antibacterial agent Irgasan to develop antibacterial/antiviral mixtures as broad-spectrum antiviral and antibacterial agents [10]. As in the United States, the use of oxacillin and Irgasan has been prohibited in European countries because of their adverse effects on the bacterial purification of sewage. In the present study, a new version VB was prepared by adding PHMB in addition to a PM compound (patent number 6739772). The antiviral effects of VB were demonstrated in cultured cells (Figure 1a–c). As the maximum effect, TCID₅₀ changed by four orders of magnitude (99.99% inhibition). The effects were observed in a concentration-dependent manner (Figure 1b,c). Additionally, the direct inactivating effects occurred in a concentration-dependent manner (Figure 2a,b).

In the cultured cells, virus molecules decreased and TCID₅₀ changed by four orders of magnitude each, suggesting the usefulness of quantitative RT–PCR in evaluation. However, as direct inactivating effects, TCID₅₀ decreased by only one order of magnitude, although the number of virus molecules decreased by four orders of magnitude. Thus, about 1000 viral RNA molecules correspond to one infectious titer. In addition to the direct inactivation, VB inhibits virus adsorption and invasion by binding to the surfaces of living cells. Furthermore, VB significantly influences TCID₅₀ because of its inhibitory effects on cell surfaces upon the secondary infection of virus particles released during the primary infection.

A high affinity for the Matrix 2 protein, i.e., the surface protein of the influenza virus, was demonstrated (Table 2). Indeed, the high affinities of PM compounds for virus receptors on cell surfaces have also been demonstrated in HSV infection, suggesting that these compounds act in a similar way on the influenza virus.

High affinities were also demonstrated for the SARS-CoV-2 S1 protein of the same coronavirus species as the influenza virus and GII.4 VP1 VLPs of a nonenveloped norovirus. Therefore, VB may act as a broad-spectrum antiviral agent. This may be explained by the fact that VB mainly acts on the cell surface.

We examined whether the effects of the hygienic products containing VB, which showed high antiviral activity in cultured cells, were maintained. For this purpose, a quantitative RT–qPCR was established for viral and bacterial component RNAs because of the difficulties of handling infectious viruses in terms of equipment and throughput. Representative viruses and bacteria, with a significant impact on the human body in the living environment, were selected (six viral and five bacterial species in Figure 3). In addition, general bacteria and fungi were simultaneously examined by the plate culture method according to the manufacturing standards for hygiene products. Commercially available products were randomly collected for examination in cooperation with 22 manufacturers of VB-containing products. As shown in Figure S1, >300 colonies of general bacteria, detected in a petri dish, corresponded to the reference value (30,000/sample). All the samples met the reference value, with no fungi detected, and were negative for viral and bacterial RNA molecules. Safety can be examined to some extent because infectivity can be negated if no gene molecule is detected. In the evaluation of antiviral effects, the results of quantitative RT–PCR were correlated with the TCID₅₀ assay.

VB has only been used in solutions. In the present study, its application to solids was examined. In the conventional use of VB in solutions, the crystals of VB are ground for dissolution in water. In the present study, the application of VB to PC and PP was examined with ground crystals (Figure 4a). To produce such molded products, VB was added at <300 °C, which caused no problem with stability (e.g., thermal decomposition; see personal communication). Although the concentration-dependent effects of VB were observed, the effective concentration was determined to be 0.35%. VB is added at 0.01% if the effective concentration of VB to act on cultured cells is determined to be 100 µg/mL.
Additionally, VB needs to be added to solids at a high concentration. This may be determined by the surface area where VB comes into contact with viruses. Therefore, ground crystals were finely granulated through a 45-µm mesh, enhancing the effects by 30–50% (Figure 4b). In the future, the effects of VB may be further enhanced by increasing the surface area. The effects, which already appeared within an hour of examination, continued for 12 h (Figure 4c,d). Several types of molded materials were also examined (Figure 5). VB showed comparable effects in a concentration-dependent manner, regardless of the materials. The effects in solids were inferior to those in solutions. However, unlike in test tubes, viruses are not concentrated in one place in a living environment. The effects of viruses and bacteria attached to stationery items on the human body can be eliminated if they become noninfectious there (Figure 6). In the future, the applicability and versatility of VB should be examined for various daily necessities, besides stationery items, to further expand the scope of application.

**Figure 6.** Effects of VB on the applicable materials.

**5. Conclusions**

In the evaluation of antiviral effects, the results of quantitative RT–PCR were correlated with TCID$_{50}$, potentially eliminating the need for handling infectious viruses.

The effects of the antibacterial/antiviral VB compound were examined in new formulations. The VB compound was extremely effective in cultured cells (up to 99.99% inhibition), demonstrating that the antibacterial/antiviral effects were maintained in VB-containing hygiene products. The application of VB to solids was examined, demonstrating its high applicability and versatility based on the enhanced
effects through more frequent contact with viruses and bacteria due to the increased surface area of the compound, applicability to any material, and processability due to heat resistance.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2076-3417/10/22/8246/s1, Figure S1: Negative testing of commercial hygiene products, Figure S2: Stationary fitting materials.

**Author Contributions:** All authors contributed to this work. K.D., K.F., H.S., Y.O., S.S., and A.T. designed the research. Moist hand towels and wet sheets were prepared by FSX members. The stationary materials were prepared by Mitsubishi Pencil members. K.D., K.F., H.S., Y.K., M.S., Y.S., and K.T. carried out the experiments and analyzed the results. All authors interpreted the results and designed the research strategy. K.D., K.F., and H.S. prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

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