Safety, formulation, and in vitro antiviral activity of the antimicrobial peptide subtilosin against herpes simplex virus type 1

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Abstract

In the present study the antiviral properties of the bacteriocin subtilosin against Herpes simplex virus type 1 (HSV-1) and the safety and efficacy of a subtilosin-based nanofiber formulation were determined. High concentrations of subtilosin, the cyclical antimicrobial peptide produced by *Bacillus amyloliquefaciens*, were virucidal against HSV-1. Interestingly, at non-virucidal concentrations, subtilosin inhibited wild type HSV-1 and aciclovir-resistant mutants in a dose-dependent manner. Although the exact antiviral mechanism is not fully understood, time of addition experiments and western blot analysis suggest that subtilosin does not affect viral multiplication steps prior to protein synthesis.

Poly(vinyl alcohol) (PVOH)-based subtilosin nanofibers with a width of 278 nm were produced by the electrospinning process. The retained antimicrobial activity of the subtilosin-based fibers was determined via an agar well diffusion assay. The loading capacity of the fibers was 2.4 mg subtilosin/g fiber, and loading efficiency was 31.6%. Furthermore, the nanofibers with and without incorporated subtilosin were shown to be nontoxic to human epidermal tissues using an in vitro human tissue model.

Taking together these results subtilosin-based nanofibers should be further studied as a novel alternative method for treatment and/or control of HSV-1 infection.

Keywords

subtilosin; bacteriocin; antiviral; nanofiber

Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2, respectively), are serious human pathogens. HSV-1 is often associated with orofacial infections and encephalitis, whereas HSV-2 has been found to be the most frequent cause of genital herpes, the major cause of genital ulcers worldwide [9, 40, 52]. However, in the past decade several studies have identified HSV-1 as the causative agent of at least half of the new genital herpes episodes [4, 21]. HSV infections persist during the lifetime of the host, generally establishing latent infections [17, 18]. In addition, it has been shown that genital herpes represents a risk factor for the transmission of human immunodeficiency virus (HIV), increasing the risk of contagion by two- to threefold [26, 27, 31].

Nowadays, the agents most commonly used for the management of herpetic infections are the nucleoside analogues acyclovir (ACV), penciclovir (PCV) and their respective prodrugs valacyclovir and famciclovir. The triphosphate forms of these analogues selectively inhibit the viral DNA polymerase (DNA pol) activity. After more than three decades of ACV administration, drug-resistant HSV isolates are rarely found in immunocompetent subjects (0.1–0.7%) but more frequently recovered from immunocompromised patients (4–14%) [12, 34, 36, 47]. HSV resistance to ACV generally arises as a result of mutations in viral thymidine kinase and in a lesser number from mutations in the viral DNA pool [19, 38]. The pyrophosphate analogue foscarnet (FOS) is often used in the management of resistant HSV infections. However, FOS resistance is rising rapidly and its use is reserved to the cases where other drugs fail because it is more toxic and less bioavailable [11, 33]. Considering the incidence of HSV infections, the reported emergence of ACV, PCV and FOS resistant mutants and that it has been recently shown that antiviral treatment of herpetic infection fail to reduce HSV and HIV transmission [10, 29] there is a need for new antiherpetic compounds with different mechanisms of action. Antimicrobial peptides are produced by a variety of organisms including insects, fungi, Gram-positive and Gram-negative bacteria, and many of them have been reported as viral inhibitors [1, 28]. For example, cationic peptides like -defensins, hecate, and synthetic derivatives of magainins are active against HSV in vitro replication [6, 22] while melittin, a 26 amino acid amphipathic peptide isolated from the venom of the European honeybee *Apis melliphera*, inhibits the replication of both HIV and HSV [2, 6, 51]. Several antimicrobial peptides ribosomally produced by bacteria, collectively called bacteriocins, have been described for the members of the genus *Enterococcus* [7]. Wachsman *et al.* were the first to describe an antiviral peptide produced by *E. faecium* CRL35. The peptide, 3.5 kDa in size, inhibited late stages of the HSV-1 and HSV-2 multiplication cycle [51]. In addition, Serkedjieva *et al.* described a 5.0 kDa-peptide, produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* with activity against influenza virus [37].

Based on the documented history of bacterially-produced peptides, we chose to investigate the novel bacteriocin subtilosin, for such characteristics. Subtilosin is a 3.4 kDa, cyclical peptide [32] produced by both *Bacillus subtilis* [5] and *B. amyloliquefaciens* [42] with proven antimicrobial activity against a variety of human pathogens, including the bacterial vaginosis-associated *Gardnerella vaginalis*. Sutyak *et al.* further established that subtilosin has potent spermicidal activity and is nontoxic to human vaginal tissues [41]. Due to its range of activities and its safety for human use, subtilosin was an obvious target for investigation into its antiviral properties. Furthermore, we have chosen to examine the feasibility of subtilosin's incorporation into a nanofiber-based delivery system, considering the successful use of this method with other bacteriocins [16, 24, 25]. Here, we report for the first time the antiviral activity of the anionic antimicrobial peptide subtilosin, and its safety for use in a novel nanofiber delivery system.

Materials and Methods

Compounds

Subtilosin was isolated and purified from cultures of the producer strain, *Bacillus amyloliquefaciens* KATMIRA1933, according to the protocols previously described by Sutyak *et al.* [41]. All subtilosin solutions were prepared in sterile ddH$_2$O and maintained at 4°C until use. Acyclovir and foscarnet were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Drug stock solutions were prepared in dimethyl-sulfoxide (DMSO) with a final concentration of 0.1% and diluted with maintenance medium (MM) consisting of minimum essential medium (MEM) (Gibco, Carlsbad, CA, USA) with 2% inactivated fetal bovine serum. Poly(vinyl alcohol) (PVOH, M$_w$=61 kDa, Sigma-Aldrich) was chosen as a carrier polymer due to its high biocompatibility [16]. Trypticase™ soy broth (TSB), trypticase™ soy agar (TSA), agar and yeast extract were purchased from Becton, Dickinson and Company (Sparks, MD, USA). Yeast extract was added into TSB and TSA in the amount of 0.6% as a nutritional supplement to improve microbial growth. The indicator organism *Micrococcus luteus* ATCC 10240 was chosen due to its high sensitivity to bacteriocins and its widespread used as a bacteriocin-sensitive indicator strain by academia and industry [35].

Cells and viruses

African green monkey kidney (Vero) cells were grown as monolayers in MEM (Gibco) supplemented with 5% inactivated fetal bovine serum and 50 μg/mL of gentamycin. HSV-1 strain F (tk+) was obtained from the American Type Culture Collection (Rockville, MD, USA). HSV-1 strain Field (tk deficient) was kindly provided by Dr. G. Andrei (Rega Insititue, Leuven, Belgium). Virus stocks were prepared in Vero cells.

Cell cytotoxicity assays

To determine the cytotoxic concentrations of the compounds, confluent monolayers of Vero cells were grown in tissue culture plates for 48 h and exposed to various concentrations of the compounds. After 48 h of incubation, cell viability was examined by the ability of the cells to cleave the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) with the mitochondrial enzyme succinate dehydrogenase, yielding a quantifiable blue product (formazan) [15]. The precise MTT procedure has been previously described [43, 50]. The CC$_{50}$ was defined as the compound concentration that reduced Vero cells viability by 50%, calculated by regression analysis.

Virucidal assay

HSV-1 tk+ and tk deficient preparations were incubated with 200 μg/mL of subtilosin or with MM (control) for 0, 15, 45 and 90 min at 37°C. Then, aliquots were taken and diluted in a serial fashion in MM in order to infect Vero cell monolayers grown in 24-well culture plates to test virus survival using a plaque assay. Plaques were counted exclusively in wells where subtilosin concentration was less than 0.2 μg/mL, a concentration 50 fold lower than the concentration needed to inhibit virus multiplication in 50% compared to untreated infected cultures). To assay the effect of subtilosin over the viral particles at antiviral concentrations, HSV-1 was incubated with subtilosin at concentrations ranging from 6 to 100 μg/mL or MM for 60 min at 37°C. Then, aliquots were taken and Vero cells were infected to test virus survival using a plaque assay.

Virus yield reduction assay

Antiviral activity was evaluated by the virus yield reduction assay. For that purpose, Vero cells grown in 24-well culture plates for 48 h were individually infected with the two strains

of HSV-1 at a multiplicity of infection (m.o.i.) of 1 PFU/cell. After 1 h of adsorption at 37°C, the infective viral particles were removed and cells were covered with MM (control) or MM containing various concentrations of subtilosin. After 24 h of incubation at 37°C, the supernatants were harvested and cell-free virus yields were determined by a plaque assay on Vero cell monolayers. The antiviral activity was expressed as the EC_{50} (50% effective concentration), i.e. the compound concentration required to reduce plaque formation by 50% after 72 h compared with the untreated infected cultures. The EC_{50} values were calculated by plotting percentages of inhibition versus different concentrations of each compound.

Effect of time of addition of subtilosin on HSV-1 production

Subtilosin at a concentration of 50 μg/mL was added to confluent monolayers of Vero cells either at 1, 3, 5 or 8 h post-infection (p.i.) with HSV-1 (m.o.i. = 1). Cultures were further incubated at 37°C until 24 h p.i. and extracellular virus infectivity was quantified by plaque assay on Vero cells.

Western-blotting

Monolayers of confluent Vero cells were infected with HSV-1 (m.o.i. = 1) or left non-infected (control), and cultures were incubated at 37°C for 1 h to allow virus internalization. After removing the inoculum, monolayers were covered with MM alone or containing different concentrations of subtilosin, and incubated at 37°C for 24 h. Immediately after, cells in duplicate wells were lysed with Laemmli sample buffer (Bio-Rad, CA, USA) with 5% β-mercaptoethanol. Samples were heated for 2 min in boiling water before loading onto 10% acrylamide gels. Following electrophoresis, the resolved proteins were transferred to a PVDF membrane (Perkin Elmer Life Sciences, Inc., Waltham, MA, USA) in a dry system (LKB Multiphor II, Pharmacia, Sweden). Glycoprotein gD was revealed with mouse anti-gD (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and a peroxidase anti-mouse immunoglobulin G (Promega, Madison, WI, USA) as secondary antibody. ERK1 used as a loading control was revealed with rabbit anti-ERK1 (Santa Cruz Biotechnology Inc.) and a peroxidase anti-rabbit immunoglobulin G (Promega) as secondary antibody. Chemiluminescence was visualized using a chemiluminescence kit (Perkin Elmer Life Sciences).

Preparation of antimicrobial fibers

PVOH (1.5 g) was dissolved in 10 mL Millipore water and heated at 80°C for 8 h. After cooling, the PVOH solution was blended with a purified 4.6 mg/mL subtilosin solution for a final concentration of 0.12 g/mL PVOH and 0.9 mg/mL subtilosin. The PVOH+subtilosin solution was then transferred into a 10 mL plastic syringe with a blunt-end metal needle (O.D. 1.27 mm). The syringe filled with stock solution was mounted onto a syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA). Figure 1 displays the schematic diagram of the electrospinning facility. A high-voltage external electric field was applied to the polymer solution under which a Taylor cone [53] formed at the needle end. As electrostatic repulsion on the liquid surface surpassed the surface tension, a polymer liquid jet was then erupted from the Taylor cone. After experiencing jet whipping [39] and solidification, polymeric fibers were deposited onto the grounded aluminum foil collector. The whole process was maintained at the condition of 15 kV for voltage supply, 0.2 mL/h for feeding rate, and 10 cm for needle-to-collector distance. As a negative control, 0.12 g/mL PVOH solution without subtilosin was electrospun by the same procedure as above. After 5 h of processing, the fibrous mat was directly peeled off from the grounded collector covered with aluminum foil. Fibers were then dried under vacuum for 4 h prior to further use.

Morphology characterization of antimicrobial nanofibers

Surface images of the antimicrobial PVOH fibers with and without subtilosin were collected with a commercial Nanoscope IIIa Multi-Mode AFM (Veeco Instruments, Plainview, NY, USA) equipped with a J scanner, which was operated in tapping mode using a silicon cantilever. The scanned images were obtained at the scan size of 5×5 μm and 50×50 μm. The scan frequency was set at 0.1 Hz. The section analysis embedded in the software Nanoscope 3.0 was utilized to calculate the fiber diameter distribution.

Assessment of subtilosin loading capacity and efficiency via well diffusion assay

The evaluation of subtilosin loading capacity and efficiency was conducted by agar plate well diffusion inhibition assays [13]. All nanofiber mats were sterilized by direct exposure to UV light (257 nm) in a biosafety cabinet (Forma Class II, A2 Biological Safety Cabinet, Thermo Fisher Scientific, Pittsburgh, PA, USA) for 10 min per side prior to use. Twenty mg of PVOH+subtilosin fibers were cut with sterile tweezers, immersed in 200 μL sterile ddH$_2$O, and kept at 4°C for 24 h. Twenty mg of PVOH fibers were also dissolved as a negative control to eliminate the possibility of PVOH inhibition. An overnight culture of *M. luteus* in TSB (c. 10^9 CFU/mL) was diluted 100-fold by blending with 55°C soft agar (TSB supplemented with 7 g/L agar). Then, 4 mL of the soft agar containing *M. luteus* was transferred onto the surface of a TSA plate. After 30 min of solidification, a sterile glass pipette (approx. 5 mm diameter) was used to create wells in the agar plate. 50 μL of purified subtilosin, PVOH+subtilosin fibers (24 h water solution), negative control and their double dilutions were injected into the wells in duplicate. After 24 h incubation at 37°C, minimum inhibitory concentration (MIC) was defined as the lowest concentration to form a visible inhibition circle on the *M. luteus* growth layer. Tests were repeated three times.

Skin cytotoxicity assays

The safety of nanofiber mats with and without incorporated subtilosin for use on human skinwas tested on a cultured, reconstituted human epidermal model. The EpiDerm (NHEK) tissue model (MatTek Corporation, Ashland, MA, USA) consists of normal, human-derived epidermal keratinocytes that are cultured to form a highly differentiated model of the epidermis, and allows for *in vitro* testing of a compound's irritancy and cytotoxicity. All EpiDerm tissues were maintained at 4°C upon receiving and prior to use. The standard method developed by MatTek, and later outlined by Frasch *et al.* [20], was followed. Briefly, all tissues were preincubated in room temperature culture medium at 37°C with 5% CO$_2$ for 1 h prior to use. Nanofiber mats containing 12% PVOH or 12% PVOH+subtilosin were sterilized by direct exposure to UV light in a biosafety cabinet for 10 min per side, and then cut to size using a sterilized size 5 cork borer (Thermo Fisher Scientific). These nanofiber "discs" were applied onto the surface of the pre-conditioned tissues with light pressure to ensure that the disc came in full contact with the tissue. Positive (50 μl Triton-X) and negative (50 μl sterile ddH$_2$O) controls and all test compounds were assayed in triplicate. The tissues were exposed to the test compounds for 24 h at 37°C with 5% CO$_2$. After exposure, the tissues were washed with PBS, transferred into MTT assay buffer, and further incubated for 3 h at 37°C with 5% CO$_2$. After a second wash with PBS, the tissues were submerged in isopropanol and held at room temperature overnight to allow for extraction of the formazan dye from the tissues. The following day, the optical density of the formazan-isopropanol mixture from each tissue was measured in triplicate at 570 nm. Cell viability was then calculated as a percentage of the average negative control tissues. Tests were performed at least twice.

Results

Antiviral activity: subtilosin inhibits HSV-1 multiplication

To assess the antiviral activity of subtilosin, we first determined the subtilosin's concentration that reduced cell viability to 50% as compared to the control (CC_{50}) by using the MTT method. A virus yield inhibition assay was then performed to determine cell-free infectivity and the selectivity index (SI), i.e. the relationship between CC_{50} and EC_{50} values. For comparative purposes, the inhibitory effect of ACV and FOS was also assayed. Between 12 and 100 μg/mL, subtilosin inhibited HSV-1 replication in a dose-dependent manner (Figure 2) and a remarkably strong inhibition of virus replication was obtained with 200 μg/mL subtilosin. At a concentration of 100 μg/mL, subtilosin inhibited replication in a 99% and at 12 μg/mL, a concentration 26 fold lower than the CC_{50}, subtilosin still inhibited approximately 90% of HSV-1. A 50% of inhibition was found with subtilosin at a concentration of 9.6 μg/mL. Lower concentrations of the compound did not display considerable antiviral activity. The SI value for subtilosin against HSV-1 tk+ was 33, while the values for ACV and FOS were 957 and 14, respectively. On the other hand, subtilosin rendered a SI value of 31 against tk-deficient HSV-1, while the SI value for ACV was only 10.3 (Table 1).

Since it has been reported that microbicidal peptides such as defensins may inactivate HSV on direct contact [30] and because we found an unusually high inhibition at 200 μg/mL, a virucidal assay was performed to determine if subtilosin at this concentration produces a direct effect on the viral particle. To that purpose, viral suspensions were incubated with subtilosin 200 μg/mL for 15, 45 and 90 min. Then, aliquots were taken, diluted in MM and used to infect Vero cell monolayers to test virus survival using a plaque assay. The incubation of viral suspension with subtilosin for 15 min at 37°C resulted in an inhibition of the viral titer of 99%. After 45 min of treatment, the reduction of viral infectivity was higher than 99.99%, indicating that subtilosin is a potent virucidal agent active against tk-positive and tk-deficient (data not shown) HSV-1 (Figure 3).

To establish if subtilosin's inhibitory activity at low concentration was due to its direct effect on the viral particle, we performed a similar experiment at concentrations below 200 μg/mL. Treatment of viral suspensions for 1 h at 37°C with 6 to 100 μg/mL of subtilosin did not reduce viral infectivity (Figure 4), indicating that the reduction of the viral titers at these concentrations (as seen in Figure 2) were only due to antiviral activity.

In order to determine if subtilosin interferes with the initial steps of the viral multiplication cycle at a non-virucidal concentration, a time of addition experiment was performed. For that purpose, 50 μg/mL of subtilosin was added to HSV-1 infected Vero cells at different times after infection and at 24 h p.i., cell free infectivity was determined. As it depicted in Figure 5, when subtilosin was present starting from 1 h, 3 h, 5 h or 8 h p.i., virus yields were reduced by approximately 2 log, suggesting that subtilosin's antiviral activity is not due to an inhibition of early stages of viral multiplication.

To further characterize the inhibitory action of subtilosin, Vero cells were infected with HSV-1 and treated with different concentrations of the peptide ranging from 25 to 100 μg/mL. Afterwards, a polyacrylamide gel electrophoresis followed by a Western blot analysis was performed to determine if subtilosin reduces viral protein synthesis. As shown in Figure 6, viral protein gD synthesis was not affected by the different concentrations used in this experiment, suggesting that the antiviral mechanism of subtilosin does not interfere with this or previous steps in the viral multiplication cycle.

Formulation

Nanofiber morphology—Figure 7 shows the tapping mode-atomic force microscopy (TP-AFM) images of antimicrobial PVOH fibers. Relatively straight fibers were formed from the pure PVOH solution (Figure 7A), and an individual PVOH fiber's diameter was 567 nm (Figure 7B) as calculated by the section analysis embedded in the Nanoscope software. Addition of subtilosin affected the fiber's morphology, resulting in thinner fibers from the electrospinning process (Figure 7C). An individual PVOH+subtilosin fiber diameter was determined to be 278 nm (Figure 7D). This phenomenon is likely due to subtilosin's interaction with PVOH, resulting in weakened entanglement between the PVOH polymer chains, shifting the critical entanglement concentration of PVOH solution toward a higher value and subsequently reducing fiber diameter.

Loading capacity and loading efficiency—Loading capacity was defined as the amount of subtilosin released from a gram of subtilosin+PVOH nanofiber. Loading efficiency was defined as the ratio of loading capacity to the amount of subtilosin used in the blended solution to produce 1 g of nanofiber. First, the MIC of subtilosin against *M. luteus* was determined by well diffusion assay as 60 mg/L. The 4-fold dilution of a 20 mg nanofiber per 200 μL ddH$_2$O solution, equivalent to 25 mg fiber/mL ddH$_2$O, was the lowest dilution that retained antimicrobial activity, establishing it as the MIC. The concentration of subtilosin released from 25 mg of fiber in 1 mL of sterile ddH$_2$O was equal to 60 μg/mL. Thus, the loading capacity was calculated to be 2.4 mg subtilosin per gram of fiber.

Since the water acting as a solvent in the subtilosin+PVOH solution was evaporated and dried during the electrospinning and vacuum processes, 1 g of nanofiber was assumed to be primarily composed of PVOH. Since 8.3 mL of 0.12 g/mL PVOH solution was required to form 1 g of fiber, the amount of subtilosin used in the blended solution was 7.6 mg. Thus, loading efficiency was calculated to be 31.6%. The loss of subtilosin may be the result of the high voltage applied [23] or the difficulty of subtilosin release from encapsulation.

Nanofibers cytotoxicity—The safety of subtilosin-based nanofibers for use on human skin tissues was tested using the in vitro EpiDerm model. After 24 h of exposure, the tissues exposed to the negative control (ddH$_2$O) and PVOH fibers retained 100% viability. Tissues exposed to the PVOH+subtilosin fibers retained an average of 98.5% viability in comparison to the negative control. There was zero viability for the tissues exposed to the positive control, Triton-X.

Discussion

Antimicrobial peptides have shown to act against viruses in many different ways. Most of them exert their activity by directly inactivating the viral particle or inhibiting early events in virus multiplication cycle [28]. For example, the peptides MCP-1 and MCP-2, the human neutrophil peptide (HNP)-1 and brevinin-1 inactivate viral particles [14, 30, 54]. On the other hand, dermaseptins, lactoferricin and the polyphemusin analogue T22 seem to interfere at the level of virus-cell interface, thus blocking viral entry [3, 8, 44]. However, other antimicrobial peptides exert their antiviral activity by preventing virus multiplication, as melittin and cecropin which were shown to inhibit the HIV replication by suppressing viral gene expression [48]. Other bacteriocins such as CRL35 and ST4V, isolated from *Enterococcus faecium* and *Enterococcus mundii* respectively, inhibited the HSV-1 and HSV-2 replication in a dose-dependent manner [46, 49, 51]. In this work, we studied subtilosin's activity against HSV-1. We found that subtilosin at a non-cytotoxic concentration of 200 μg/mL inactivates HSV-1 viral particles. The incubation of viral suspension with 200 μg/mL of subtilosin for 15 min was enough to reduce infectivity by

99% and after 45 min of treatment the reduction of viral infectivity was higher than 99.99%. Interestingly, at lower non-virucidal doses, subtilosin inhibits HSV-1 multiplication cycle in a dose dependent manner. At subtilosin's concentration of 100 μg/mL, 99% inhibition of HSV-1 multiplication was achieved. Remarkably, 12 μg/mL subtilosin, a concentration 26 fold lower than the CC_{50}, caused more than 90% inhibition of HSV-1. In addition, subtilosin was equally active against HSV-1 tk+ and tk-deficient strains and rendered higher SI values than FOS, raising the possibility for using subtilosin against ACV-resistant mutants. It was previously mentioned that antimicrobial peptides generally display virucidal activity or interfere with early events in viral multiplication cycle. Interestingly, subtilosin is able to inactivate viral particles and to inhibit viral multiplication, although in different concentrations. Furthermore, it does not seem to affect any viral multiplication step up to viral protein synthesis as shown by the time of addition of subtilosin experiment and Western blot analysis, suggesting that it may interfere with late stages such as viral formation and release. Taking into account that some studies suggest that subtilosin binds to lipid membranes, that high concentrations of the compound may result in membrane permeabilization [45] and that viral formation and release are viral steps particularly sensible to membrane structure, future analysis will be conducted to elucidate the precise mechanism underlying the antiviral activity of subtilosin.

Considering the incidence of HSV infections and the issues regarding the clinical approved antiherpetic drugs, such as the reported emergence of ACV, PCV, and FOS resistant mutants and that it has been recently shown that the available antiviral treatment of herpetic infections fail to reduce HSV and HIV transmission because they do not prevent subclinical reactivations of the virus [10, 29], novel treatment methods are of critical importance. One possible alternative is the use of subtilosin in a nanofiber-based formulation. As we demonstrated, subtilosin is readily encapsulated into PVOH fiber mats, which may be directly applied onto areas of infection. Moreover, nanofibers both with and without incorporated subtilosin were demonstrated to be fully safe for human use. Heunis and Dicks [25] have demonstrated the feasibility of incorporating a variety of different types of antimicrobials into nanofibers, and proven that the fibers retain antimicrobial activity against bacterial pathogens such as methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*.

Conclusion

In this report, the natural, safe antimicrobial peptide subtilosin was shown to have potent virucidal and antiviral activity against HSV-1. Preliminary research on the safety of subtilosin in a novel nanofiber formulation indicates that the formulation has potential for use in human applications. Further research will be conducted to elucidate the molecular mechanism underlying the antiviral activity of subtilosin, to optimize the nanofiber formulation and to assay the activity of subtilosin nanofibers against this and other *Herpesviridae* family members.

Acknowledgments

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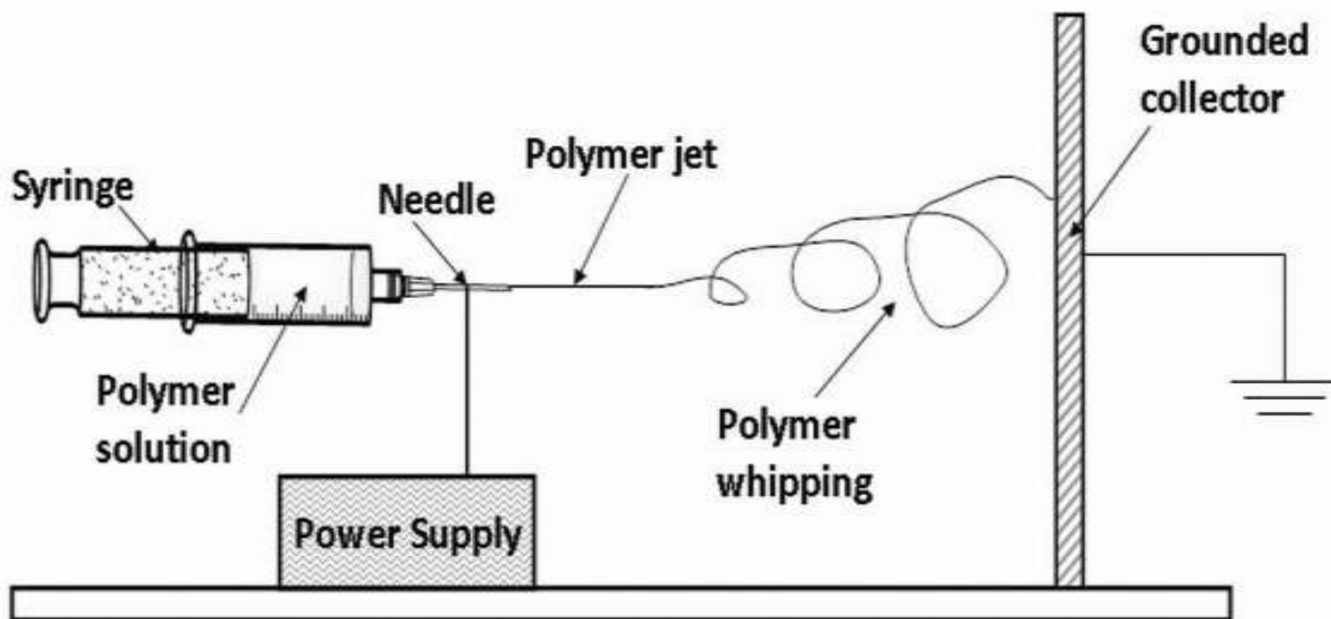
Figure 1. Schematic diagram of nanofiber formation through the process of electrospinning
A PVOH and subtilosin mixed solution (final concentrations: 0.12 g/mL PVOH and 0.9 mg/mL subtilosin) was loaded into a plastic syringe, which was then mounted onto a syringe pump. During the electrospinning process, a liquid polymer jet is ejected at the end of the needle when a high-voltage electric field is applied to the polymer solution. The liquid polymer undergoes jet whipping and solidification, and the resultant polymer fibers are then deposited onto the grounded aluminum collector surface. The whole process was maintained at the condition of 15 kV for voltage supply, 0.2 mL/h for feeding rate, and 10 cm for needle-to-collector distance. As a negative control, a 0.12 g/mL PVOH solution without subtilosin was electrospun by the same procedure as above. Fibers were collected from the grounded surface after 5 h processing.
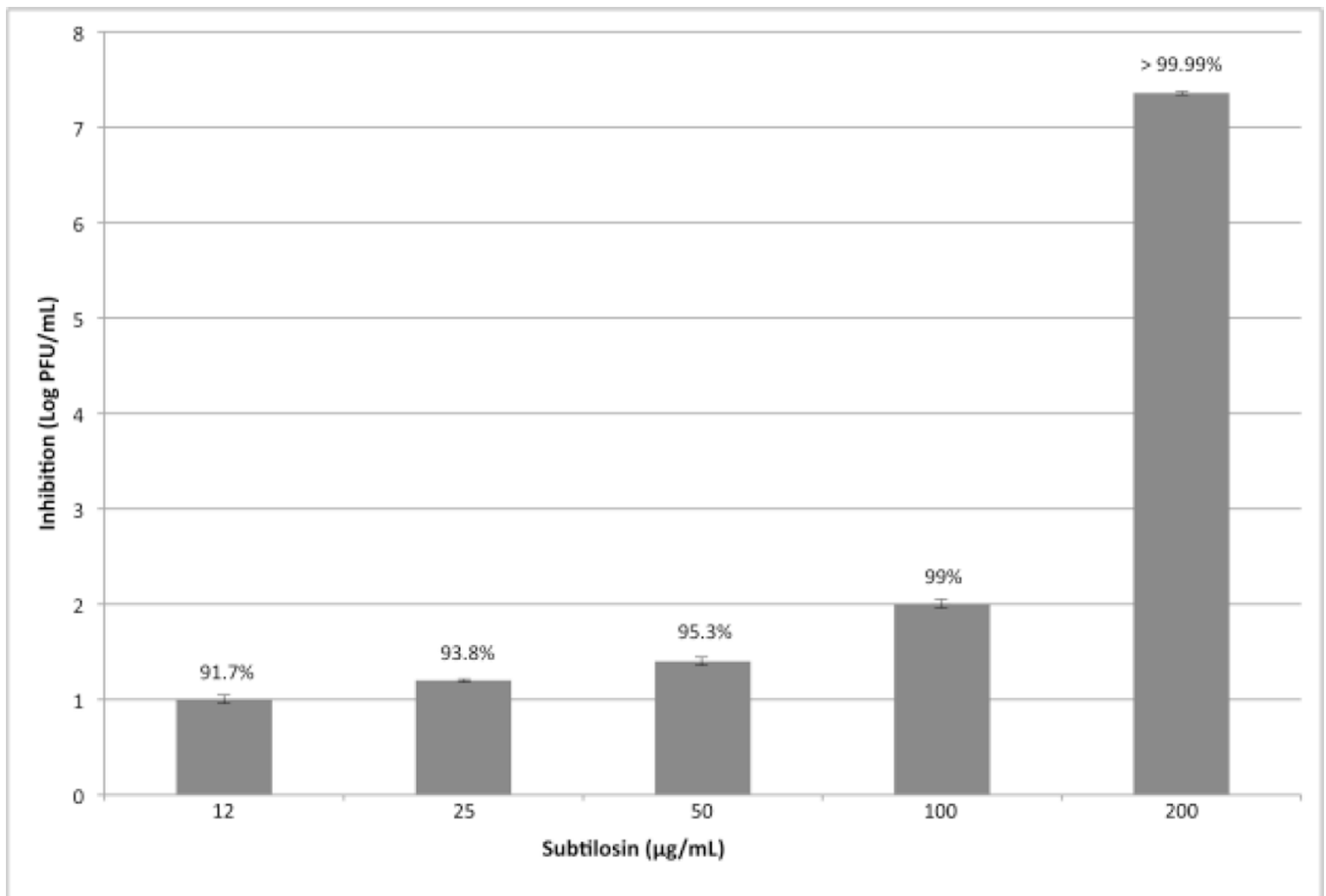
Figure 2. Subtilosin inhibits HSV-1 replication in a dose-dependent manner
Vero cells infected with HSV-1 tk+ (m.o.i.= 1) were incubated with different concentrations of subtilosin for 24 h. Then, supernatants were harvested and cell free virus yields were determined by a plaque assay. Values are means ± SD of the log reduction in HSV-1 titers for each concentration compared to no drug. Data are from three independent experiments, where each titration was carried out in duplicate. Percentages of inhibition of the different treatments are expressed in each column.
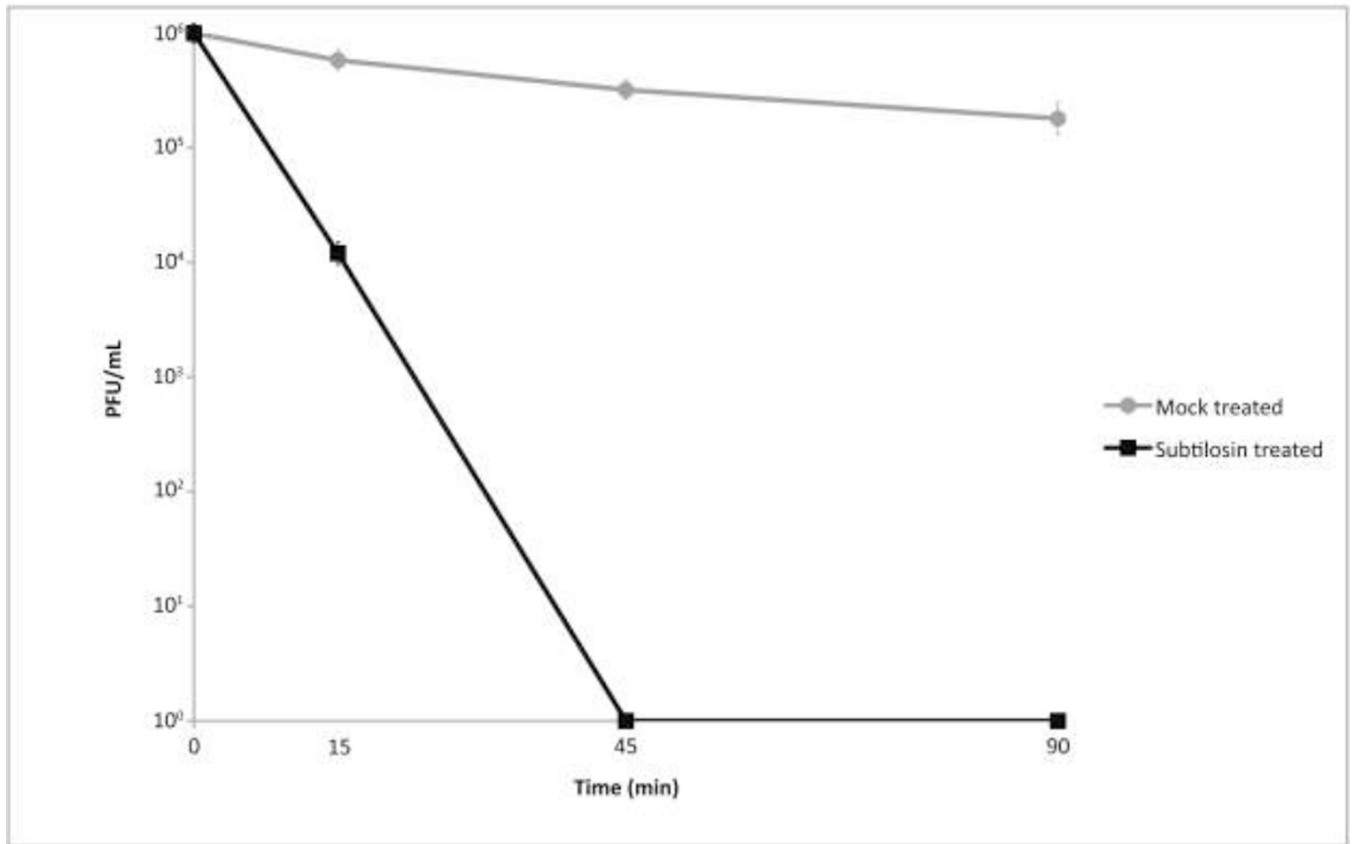
Figure 3. Subtilosin inactivate HSV-1 particles at a concentration of 200 μg/mL
HSV-1 tk+ and tk deficient (data not shown) were incubated with subtilosin at a
concentration of 200 μg/mL or with MM for 0, 15, 45 and 90 min at 37°C. Then, aliquots
were taken and Vero cells were infected to test virus survival using a plaque assay. Values
are means of HSV-1 tk+ infectivity ± SD from three independent experiments, where each
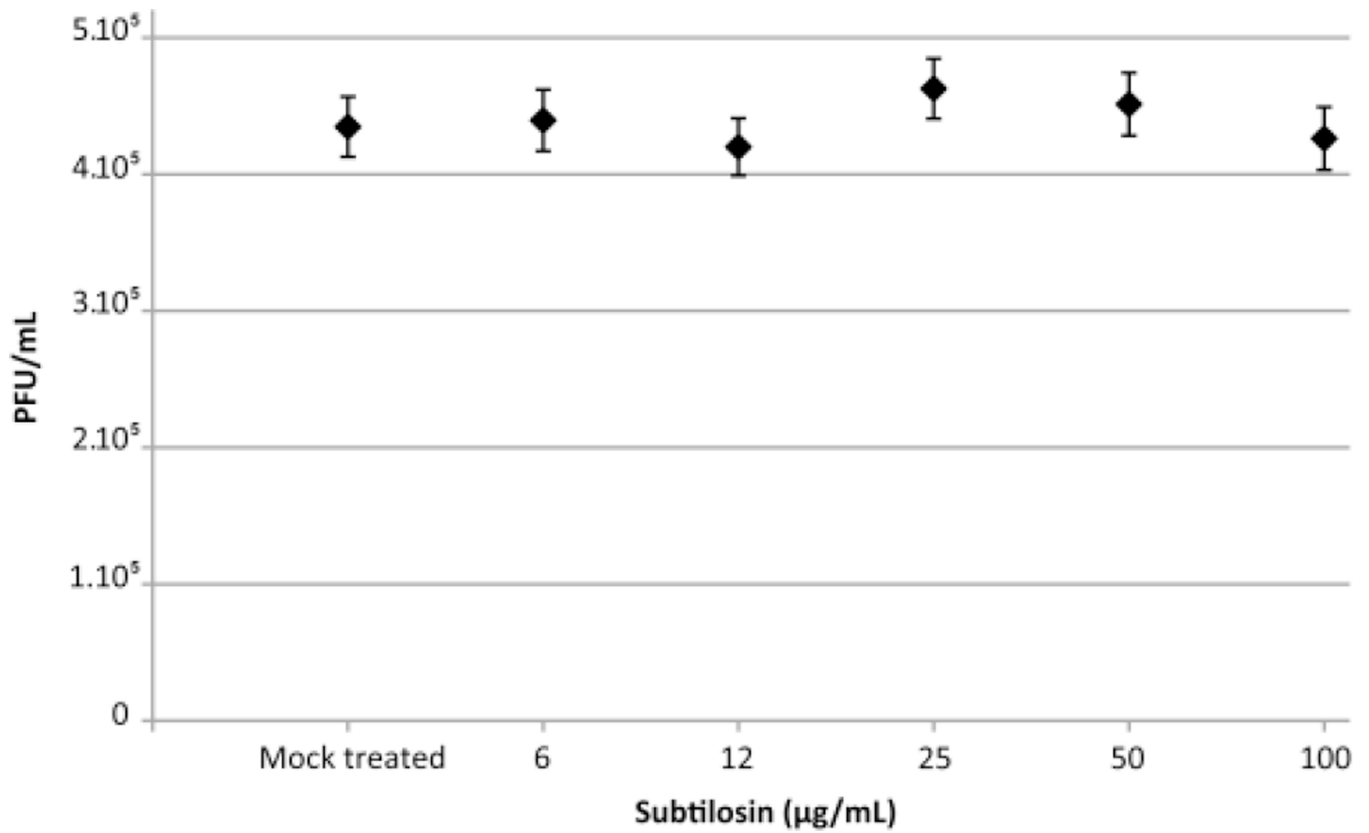titration was carried out in duplicate.

Figure 4. Low concentrations of subtilosin do not produce a virucidal effect against HSV-1
HSV-1 tk+ was incubated with subtilosin at concentrations ranging from 6 to 100 μg/mL or
MM for 60 min at 37°C to assay virucidal activity of subtilosin at concentrations that
display antiviral activity. Then, aliquots were taken and Vero cells were infected to test virus
survival using a plaque assay. Values are means ± SD from three independent experiments,
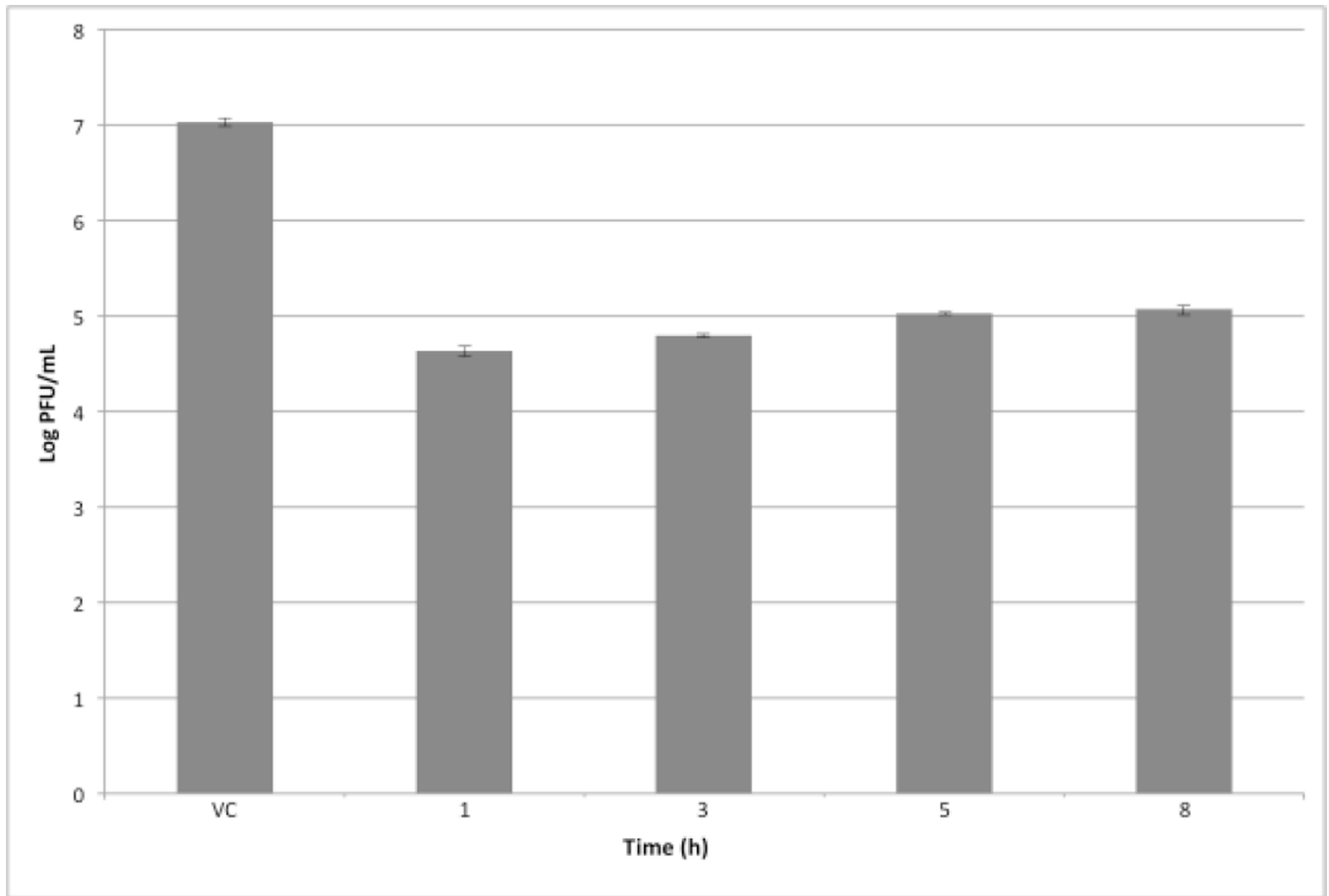where each titration was carried out in duplicate.

Figure 5. Effect of time of subtilosin addition on HSV-1 production
Vero cells infected with HSV-1 (m.o.i. = 1) were incubated with MM (VC) or MM containing subtilosin 50 μg/mL added either at 1, 3, 5 or 8 h p.i. Cultures were further incubated at 37°C until 24 h p.i Then, cell free virus yields were determined by plaque assay. Values are means ± SD from three independent experiments, where each titration was carried out in duplicate.
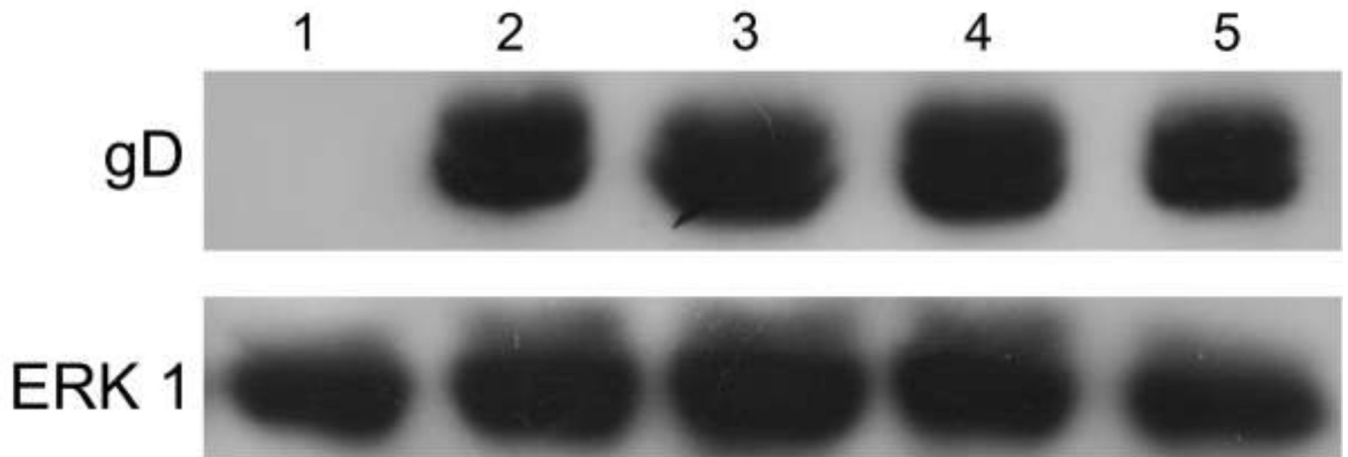
Figure 6. Viral protein gD synthesis is not affected by subtilosin treatment
Vero cells infected (Lanes 2–5) or not (Lane 1) with HSV-1 tk+ at a m.o.i. of 1 were incubated at 37°C for 1 h to allow internalization. Afterwards, the inoculum was discarded and the monolayers were covered with MM or MM containing subtilosin at different concentrations (Lane 2: virus control; lane 3: 25 μg/mL; lane 4: 50 μg/mL; lane 5: 100 μg/mL) and incubated at 37°C. At 24 h p.i. cell lysates were collected and subjected to Western blotting and glycoprotein gD was analyzed with an antibody against gD. To verify equal loading, Western blotting was performed with an antibody to ERK1. Data are from one of two different experiments.
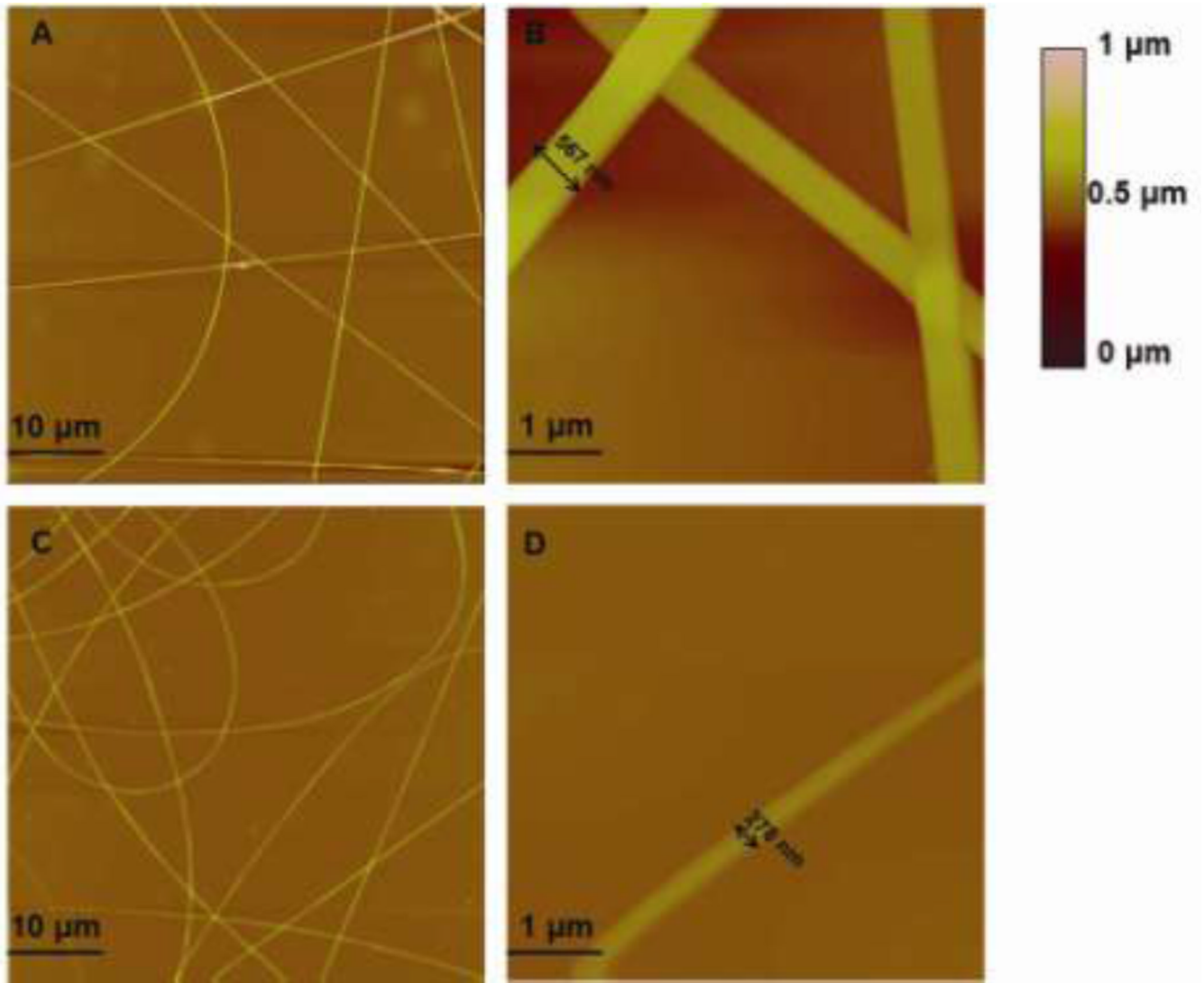
Figure 7. Surface morphology of polyvinyl alcohol-based nanofibers with and without incorporated subtilosin
Tapping mode-atomic force microscopy (AFM) images of PVOH electrospun fibers including (A) 50 μm×50 μm height image of PVOH electrospun fibers; (B) 5 μm×5 μm height image of PVOH electrospun fibers (C) 50 μm×50 μm height image of PVOH +subtilosin electrospun fibers; (D) 5 μm×5 μm height image of PVOH+subtilosin electrospun fiber.

Table 1

Cytotoxicity and antiviral activity of Subtilosin, Aciclovir (ACV) and Foscarnet (FOS) against HSV-1.

Compound (MW)	Structural Formula	IUPAC name	Virus	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
Subtilosin * (3398.9)		-	HSV-1 tk+	314	9.6	33
			HSV-1 tk deficient	314	10	31
FOS (300.04)		phosphonoformic acid trisodium salt hexahydrate	HSV-1 tk+	210	15	14
			HSV-1 tk deficient	-	-	-
ACV (225.2)		9-(2-hydroxyetoxymetyl) guanine	HSV-1 tk+	67	0.07	957
			HSV-1 tk deficient	67	6.4	10.4

CC_{50} compound concentration required to reduce cell viability by 50%, as determined by the MTT method.

EC_{50}: compound concentration required to reduce virus yield by 50%.

SI (selectivity index): ratio CC_{50} / EC_{50}.

*
Structural formula of subtilosin reproduced from Marx et al. [32].