Exploring Bacterial Interactions of Expanded-PTFE in the Presence of PVP-coated Silver Nanoparticles, Peptides P753 and P359 in 2D and 3D Models

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ABSTRACT

Tissue engineering is recognized as an avenue to improve treatment for various diseases. Expanded-PTFE (ePTFE) has gained popularity as a tissue scaffold to develop a vascular graft for generating blood vessels in cardiac surgeries. However, grafts with antimicrobial properties for efficient functioning are desired. In this study, grafts were prepared using Silver PVP-coated nanoparticles and peptides p359 and p753 and were assessed for antibacterial activity against *Escherichia coli* in 2D/3D models. The 2D models were carried out by determining MIC of these compounds through the broth dilution method in 96 well plates. MTT assay was done using HEK 293 cells to determine toxicity of these compounds to cells. The MIC of *E. coli* to Ag PVP was 150 µg/mL and 25 µg/mL for P359/P753. The Kirby disk diffusion assay and plate counts supported inhibition. MTT assay showed these compounds were non-toxic to cells up to 100 µg/mL for Ag PVP NPs, and 50 µg/mL for P753 and P359 each. In the PTFE 3D model, PTFE was further treated with Low temperature plasma (LTP) to generate cell-friendly functional groups for enhanced cell attachment. PTFE scaffolds were incubated with these compounds overnight and seeded with 20,000 cells the following day on both untreated and treated LTP-treated PTFE. After 24 hours, the cells were infected with Bacteria at MOI 1:10 and inhibition was investigated using microscopy, plate counts and live/dead assay. We observed efficient bacterial inhibition in 3D system without cell death and complemented these findings with SEM. When *E. coli* is treated in the presence of Ag PVP NPs, P753 and P359, a down-regulation of gene expression was observed within *E. coli* genes KatA, MurE, RecN, and RecA. These results exhibit that these compounds are antibacterial agents that are viable to use in vascular grafts.

Keywords: antimicrobial peptide, Ag-PVP, bacterial inhibition, scaffold, 3D cell culture

1 INTRODUCTION

Worldwide, cardiovascular disease (CVD) is responsible for more total fatalities than strokes, lower respiratory infections, and chronic obstruction pulmonary disease (COPD) every year. As such, there has been an interest in exploring different treatment options and expanding on those that have been established. One of these established treatments that are currently being expanded upon is employing bypass surgery using vascular grafts, serving as an alternative to bypass surgery using autologous veins. A scaffold material of choice that has shown promise in mimicking the native vessel wall of vascular tissue is expanded polytetrafluoroethylene (ePTFE). However, what has not been clear is to what extent it responds to specific bacteria, specific antibacterial agents, and specific embryonic cells. In this study, human embryonic kidney 293 (HEK 293) cells are loaded with ePTFE and evaluated for their response against E. coli and with polyvinylpyrrolidone (PVP) coated silver nanoparticles (Ag PVP NPs), peptide p753 (P753), and peptide p359 (P359) Both silver nanoparticles and antimicrobial peptides have been shown to exhibit biocidal activity, with the latter having both prokaryotic and eukaryotic origins.

2 METHODS

2.1 Minimum Inhibitory Concentration

The minimum inhibitory concentrations of Ag PVP NPs, P753, and P359 against *E. coli* were assessed in non-tissue culture treated 96-well plates. Lysogeny broth (LB) was loaded with each antibacterial agent and underwent two-fold serial dilution with a 10 mg/mL stock solution of each antibacterial agent. These serial dilutions resulted in concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL for peptides p753 and p359. For Ag PVP NPs, these concentrations were 200 µg/mL, 175 µg/mL, 150 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, and 3.125 µg/mL. A negative control for each antibacterial agent was also used. *E. coli* with a cell density of 1 x 10^5 cfu/mL was loaded into each well after the addition of the antibacterial agent. Resazurin dye was employed as a colorimetric assay to assist in determining bacterial growth in response to varying concentrations of the antibacterial agents. After the addition of all materials,

the plates underwent four minutes of shaking before being subjected to an overnight incubation period at 37 ℃.

2.2 HEK 293 Cell Viability via MTT Assay

To assess for non-toxic concentrations of the antibacterial agents Ag PVP NPs, P753, and P359 in HEK 293 cells, the MTT assay was employed. In a 96 well tissue culture plate, HEK 293 cells were plated with a seeding density of 15,000 cells per well and loaded with 100 µL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12). The plate then underwent a 24-hour incubation period with 5% CO_2 at 37 °C. Following incubation, Ag PVP NPs, P753, and P359 were added to the cells at varying concentrations via a 1 mg/mL stock solution with no serial dilution taking place. For Ag PVP NPs, the concentrations employed were 100 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, and 5 µg/mL. For P753 and P359, the concentrations employed were 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL, and 2.5 µg/mL. A negative control for all agents were also used. Following addition of the antibacterial agents, the plate underwent a 24-hour incubation period once more with 5% CO_2 at 37 °C. 20 µL of MTT dye were added to each well following incubation and was incubated for a period of 4 hours with 5% CO_2 at 37 ℃ in the absence of light. 100 µL of dimethyl sulfoxide (DMSO) were added to each well following incubation and was incubated for a final period of 60 minutes. At the end of the incubation, the plate was analyzed for optical density with a measurement wavelength of 570 nm and a reference wavelength of 630 nm.

2.3 Kirby-Bauer Disc Diffusion Susceptibility Test

To assess for the sensitivity of *E.coli* to Ag PVP NPs, P753, and P359, the Kirby-Bauer Disc Diffusion Susceptibility Test was employed. 40 µL of *E. coli* with a density of 1 x 10^6 cfu/mL were streaked on nutrient agar plates. Two filter paper discs were saturated first with 10 µL of LB before adding a fixed volume of antibacterial agent to each disc. All extracted volumes of each antibacterial agent originated from a 10 mg/mL stock solution. For one disc, 20 µL of each antibacterial agent was adsorbed and added to a region on nutrient agar plate. For the other, disc, 10 µL of the same antibacterial agent was adsorbed and added to a region on a nutrient agar plate. An Amoxicillin/Clavulanic Acid-30 (AmC 30) disc was also added to the plate as a positive control. Each nutrient agar plate was incubated for 24 hours at 37 °C. Following the incubation period, the zone of inhibition was measured with a ruler.

2.4 Three-Dimensional Cell Culture Model

e-PTFE scaffolds were evaluated as a surface for growth in HEK 293 cells and *E. coli* separately, as well as both organisms together. All three configurations were incubated at 37 °C in 5% CO_2. *E. coli* with a density of 1 x 10^6 cfu/mL were plated with various concentrations of Ag PVP NPs, P753 and P359 and loaded with e-PTFE in a non-tissue culture treated 96 well plate. The medium of choice used to grow *E. coli* was LB. HEK 293 cells with a seeding density of 15,000 were plated with no bacteria, the same concentrations of the agents, and loaded with e-PTFE in a tissue culture treated 96 well plate. The medium of choice used to grow HEK 293 cells were DMEM X. *E.coli* and HEK 293 cells with the aforementioned densities were plated together with the same concentrations of the agents and loaded with e-PTFE in a tissue culture treated 96 well plate. The medium of choice used to grow HEK 293 cells and *E.coli* together was DMEM X**.** After the incubation, the configuration(s) of microbes with bacteria-loaded scaffolds were analyzed with immunofluorescence microscopy using the Live/Dead assay. Additionally, the configuration(s) of microbes with HEK 293 cell-loaded scaffolds were stained with DAPI and were analyzed with immunofluorescence microscopy.

2.5 Scanning Electron Microscopy

To assess for the effects of the compounds on the surface of *E. coli*, scanning electron microscopy (SEM) was employed. Bacteria with a density of 1 x 10^6 cfu/mL were treated with the compounds and fixated with glutaraldehyde. Samples were then processed with OsO_4 and underwent EtOH washes of the following percent concentrations: 30, 50, 70, 90, 100. After the final EtOH wash, 50 µL of sample was extracted and placed on SEM stubs and dried overnight. The stubs were then coated with gold and were inserted into the SEM for analysis.

2.6 quantitative-Polymerase Chain Reaction

To assess for select *E. coli* gene expression when treated with the compounds, quantitative-Polymerase Chain Reaction (q-PCR) was employed. Bacteria with a density of 1 x 10^6 cfu/mL were treated with the compounds and had their RNA extracted with the Qiagen RNeasy mini kit. cDNA was then synthesized using the extracted RNA and was mixed with forward and reverse primers of the genes of interest, SYBR Green Master Mix, and nuclease-free water in a PCR-specific 96 well plate. Before being analyzed, the plate underwent a brief 10 second shaking period.

2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

To assess for the total protein of *E. coli* treated with the compounds, the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) was employed. Protein was extracted from *E. coli* with the Pierce™ BCA Protein Assay Kit and Diluted Albumin (BSA) standards were prepared. 25 µL of each standard and sample were pipetted into a non-tissue culture treated 96 well plate, with one well for each standard and sample. 200 µL of the working

reagent (WR) were added to each well and underwent shaking for 30 seconds. The plate was then incubated at 37 °C for 30 minutes and the absorbance was read at a measurement wavelength of 562 nm. A standard curve was prepared to assess final protein sample concentrations. 30 µg of each protein sample was mixed with 3-5 µL of Lomant's Reagent. The sample was then cooked at 100 °C for 3 minutes before being loaded into the gel. After samples were loaded, electrophoresis ran for an hour, initially at 120 V for 45 minutes and then 125 V for 15 minutes. The gel was then extracted and went through a staining and destaining process. Pictures of the gel were taken using the Bio-Rad ChemiDoc XRS.

3 RESULTS AND DISCUSSION

3.1 Minimum Inhibitory Concentration

Significant bacteria inhibition were observed, with the MIC of *E. coli* treated with Ag PVP NPs, P753 and P359 being 100 µg/mL, 12.5 µg/mL, and 12.5 µg/mL, respectively. These values were supplemented with Rezasurin dye, which served as a colorimetric assay. A violet color indicated bacterial inhibition, while a pink color indicated bacterial non-inhibition.

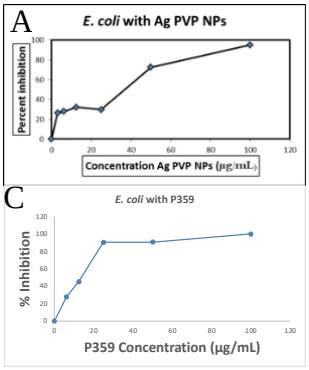

Figure 1: MIC Charts of *E. coli* when treated with A) Ag PNP NPs B) P753 C) P359

3.2 HEK 293 Cell Viability via MTT Assay

HEK 293 cell viability was assessed to be non-toxic in concentrations up to 100 µg/mL for Ag PVP NPs and 50 µg/mL for P753 and P359. 80% of HEK293 cells were found to be alive when subjected to Ag PVP NPs, 83% for P359, and 76% for P753.

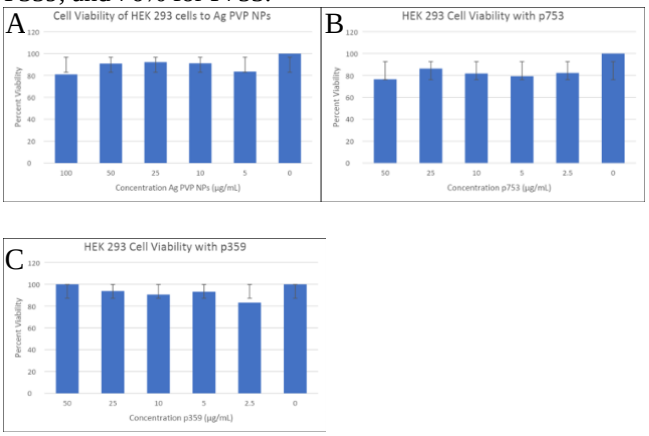

Figure 2: HEK 293 Cell Viability at Varying Concentrations of A) Ag PVP NPs B) P753 C) P359

3.3 Kirby-Bauer Disk Diffusion Susceptibility Test

The growth of *E. coli* was observed to have been successfully inhibited by Ag PVP NPs, P753 and P359, as exhibited by the Kirby-Bauer Disk Diffusion Susceptibility Test. Zone of inhibition values are recorded in Table 1.

Table 1: Zone of inhibition values for *E. coli* in response to different compounds.

Compound	Zone of Inhibition (mm)
AmC 30 #1	15
P753 25 µg/mL	11
P359 µg/mL	12
AmC 30 #2	15
Ag PVP NPs 100 µg/mL #1	16
Ag PVP NPs 100 µg/mL #2	14

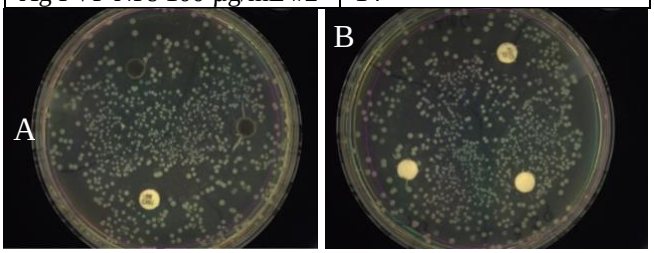

Figure 3: Kirby-Bauer Disk of *E.coli* treated with A) Ag PVP NPs B) P753(bottom left) & P359 (bottom right).

3.4 HEK 293 Cell Culture Imaging

e-PTFE was illustrated to be employed as a surface for growth for HEK 293 cells with different treatments (Figure 4). Visualized under a bright field filter & DAPI staining through immunofluorescence microscopy.

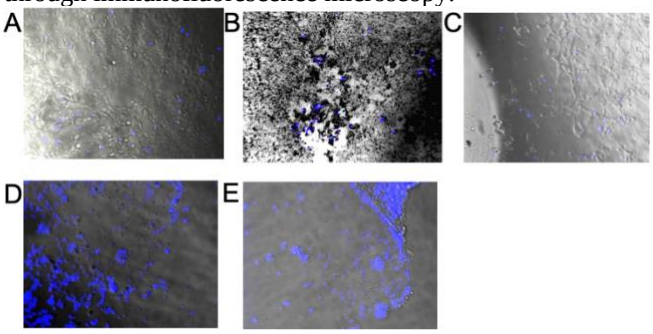

Figure 4: HEK 293 cells grown on e-PTFE treated with A) No compound, B) 100 µg/mL Ag PVP NPs, C) 25 µg/mL p753, D) 25 µg/mL p359, E) 12.5 µg/mL p753

3.5 Live/Dead Bacterial Viability Assay

When *E. coli* is treated with Ag PVP NPs, P753 and P359, all reagents appear to exhibit inhibited growth of the bacterium under the concentrations outlined. Both a Live/Dead Assay and DAPI staining were employed to visualize the results through immunofluorescence microscopy. As shown in Figure 5 under Pictures D and L, P359 is observed to inhibit bacterial growth in the presence of the bacterium as well as HEK 293 cells. Pictures C and K show inhibition by P753, and Picture B shows inhibition by

Ag PVP NPs. Due to the abundant presence of bacteria in Pictures B-D, HEK 293 nuclei could not be observed under DAPI staining. Pictures E-H show that HEK 293 cells are able to grow when cultured with the compounds.

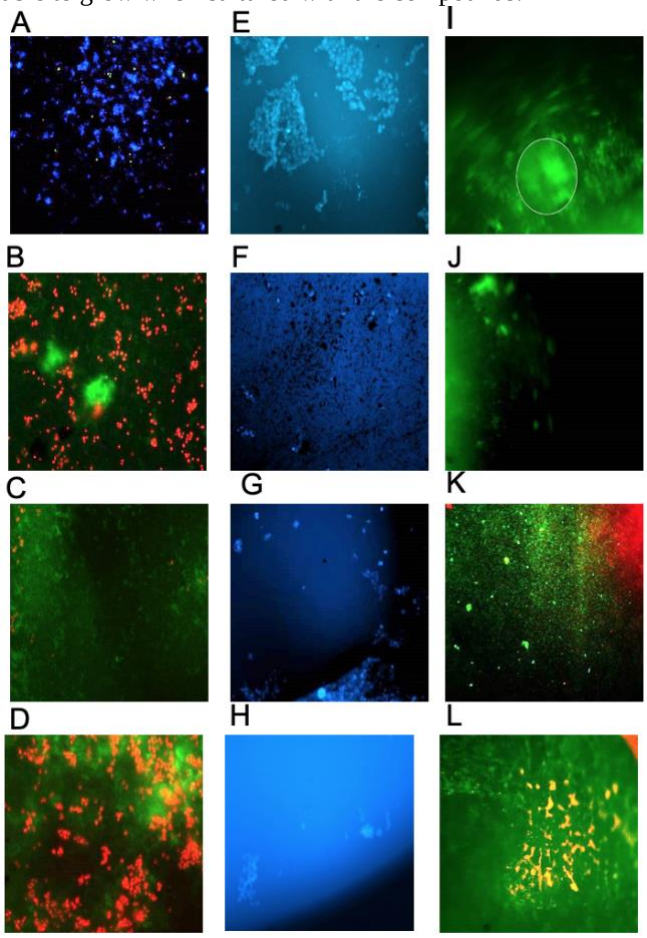

Figure 5: e-PTFE cultured with *E. coli*, HEK 293 cells, and compound treatment. A-D) *E. coli* + HEK 293, E-H) HEK 293, I-L) *E. coli*; A/E/I) Control, B/F/J) 100 µg/mL Ag PVP NPs, C/G/K) 25 µg/mL P753, D/H/L) 25 µg/mL P359

3.6 Scanning Electron Microscopy Analysis

E. coli was treated with the compounds and examined for their surface under SEM in Figure 6. Throughout different regions in pictures A-C where *E. coli* are not clumped, the compounds do not appear to affect the structure of *E. coli*, but do appear to have inhibited growth in some instances.

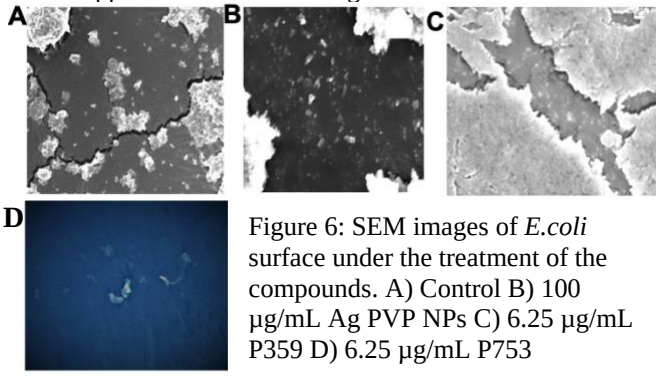

Figure 6: SEM images of *E.coli* surface under the treatment of the compounds. A) Control B) 100 µg/mL Ag PVP NPs C) 6.25 µg/mL P359 D) 6.25 µg/mL P753

3.7 Gene Expression Analysis

Through q-PCR, it was assessed that for certain *E. coli* genes, RecA(Homologous Recombination), RecN(DNA Repair), KatA(Catalase), and MurE(Nucleotide/ATP Binding) all appeared to be down-regulated when treated with select concentrations of specific compounds.

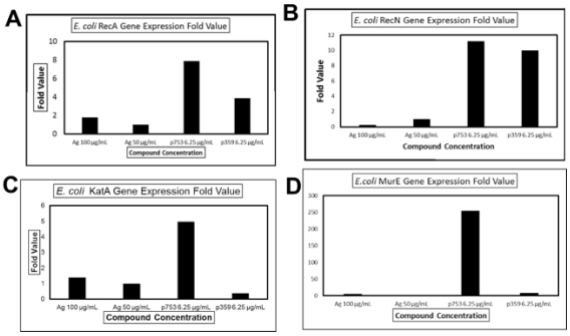

Figure 7: *E. coli* gene expression fold values for various genes treated with the compounds. A) Rec A B) RecN C)KatA D)MurE

3.8 *E. coli* Protein Molecular Weight

In order to assess for the molecular weight of *E. coli* proteins treated with the compounds, an SDS PAGE was performed. Several proteins of various molecular weights can be observed in Figure 8.

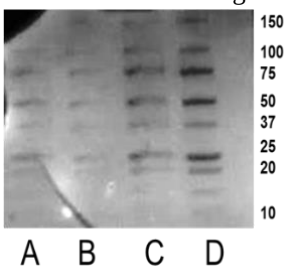

Figure 8: Protein molecular weight of *E. coli* treated with the compounds. A) 100 µg/mL Ag PVP NPs B) 6.25 µg/mL P753 C) 6.25 µg/mL P359 D) 10-150 kDa ladder

4 CONCLUSION

In the present study, Ag PVP NPs, P753, and P359 all appear to be effective and safe antimicrobial compounds when employed at proper concentrations. Further, the compounds do not appear to exhibit biocidal activity against HEK 293 cells when used in conjunction with e-PTFE. These observations may allow for e-PTFE and the compounds to be employed as a viable and antimicrobial vascular tissue graft.

5 ACKNOWLEDGEMENTS

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