Genetically Engineered Phage Fibers and Coatings for Antibacterial Applications

by

JOAN Y. MAO

Submitted to the Department of Materials Science and Engineering in partial fulfillment of the requirements for the degree of Master of Science in Materials Science and Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Abstract

Multifunctionality can be imparted to protein-based fibers and coatings via either synthetic or biological approaches. Here, we demonstrate potent antimicrobial functionality of genetically engineered, phage-based fibers and fiber coatings, processed at room temperature. Facile genetic engineering of the M13 virus (bacteriophage) genome leverages the well-known antibacterial properties of silver ions to kill bacteria. Predominant expression of negatively-charged glutamic acid (E3) peptides on the pVIII major coat proteins of M13 bacteriophage (or phage) enables solution-based, electrostatic binding of silver ions and subsequent reduction to metallic silver along the phage length. Antibacterial fibers of micrometer-scale diameters are constructed from such E3-modified phage, via wet-spinning and glutaraldehyde-crosslinking of the E3-modified phage. Silverization of the free-standing fibers is confirmed via energy-dispersive spectroscopy (EDS) and inductively-coupled plasma atomic emission spectroscopy (ICP-AES), showing ~0.61 μg/cm of silver on E3-Ag fibers. This degree of silverization is threefold greater than that attainable for the unmodified M13-Ag fibers. Conferred bactericidal functionality is determined via live-dead staining and a modified disk-diffusion (Kirby-Bauer) measure of zone of inhibition (ZoI) against Staphylococcus epidermidis and Escherichia coli bacterial strains. Live-dead staining and ZoI distance measurements indicate increased bactericidal activity in the genetically engineered virus fibers attached to silver. Coating of Kevlar fibers with E3 viruses exhibits antibacterial effects, as well, with relatively smaller ZoIs attributable to the lower degree of silver loading attainable in these coatings. Such antimicrobial functionality is amenable to rapid incorporation within fiber-based textiles to reduce risks of infection, biofilm formation, or odor-based detection, with the potential to exploit the additional electronic and thermal conductivity of fully silverized fibers and coatings.

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1 Introduction

The search for antimicrobial textiles began as far back as the 1940s, during World War II. Cotton fabrics used for tenting, tarpaulins, and truck covers were susceptible to rotting from microbes infesting and growing within the fibers, especially in wet and humid conditions. Military efforts to collect data on fungi, yeast, and other microbes in tropical regions led to the development of the first fungicides and treatments for cotton textiles [1]. Today, antibacterial fibers have many more applications beyond protection from fabric degradation. They are relevant as suture and wound dressing materials to prevent infection, industrial filters to clean water, sportswear to prevent sweat odor, and even as vehicles for drug delivery. The vast majority of such antimicrobial fibers involve polymers in some capacity, either woven into another textile or assembled as nonwoven mats. Often, natural or synthetic polymers are grafted or chemically conjugated to antimicrobial agents such as antibiotics [16], quaternary ammonium moieties [17], phenols [18], and halogenated compounds [19]. However, due to the reaction selectivity of the base polymer, additional or multiple functionalities can be difficult to impart upon such fibers.

Viruses require bacterial hosts for parasitic replication of the viral genome, but also present an intriguing and contradictory opportunity to engineer multifunctional, antibacterial fibers. Manipulation of the viral DNA enables the potential for multifunctionalization of the virus surface via controlled expression of different peptides on the outer capsid proteins of the virus coat. It has been shown previously that genetically engineered virus fibers can be fabricated via wet-spinning of a particular virus, the M13 bacteriophage ("phage" for short), into a glutaraldehyde-crosslinking solution to bind gold [20]. Here, this technology is modified and leveraged to create fibers and coatings useful for antibacterial applications. A novel method for creating bactericidal fibers is presented in this thesis, by functionalizing a genetically-engineered scaffold of phage with silver particles, which have been well-known to exhibit strong biocidal properties. These silverized phage fibers are characterized and tested for antibacterial activity. Finally, proof-of-concept experiments are demonstrated on Kevlar fibers, showing that bactericidal properties can be imparted onto synthetic polymer fibers
via a post-processing coating of crosslinked phage.

This thesis is organized as follows: Chapter 2 provides a brief literature review of other antimicrobial agents and fibers, as well as some background bacteria and the M13 bacteriophage as a functionalizable template; Chapter 3 describes the fabrication methods of phage fibers and coatings in detail; Chapter 4 presents the results of antibacterial testing and discusses the findings; finally, Chapter 5 elucidates the contributions and conclusions derived from the research, in addition to potential ideas for future work. (*This document contains material currently under review for publication in Advanced Functional Materials.*)
2 Literature Review

The first section of this chapter expands on competitive antibacterial fiber technologies to establish background for comparison against other methods in Chapter 4, Results and Discussion. The second section of this chapter describes how our viruses function as biological templates and reviews previous work utilizing this concept.

2.1 Definition of bacteria

Before reviewing antibacterial agents, perhaps we should investigate the enemy momentarily: what are bacteria? Classified as prokaryotes, bacteria are unicellular microorganisms with a broad range of morphologies, including spirals, rods, spheres, etc. They do not contain a nucleus or membrane-bound organelles [53]. The bacterium cell membrane is encased in the cell wall, which can be classified as two types: Gram-positive and Gram-negative (based on the cell’s appearance after Gram staining) [51]. The cell wall of Gram-positive bacteria is thick, containing many layers of peptidoglycan and teichoic acids. Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan surrounded by a second lipid membrane containing lipopolysaccharides and lipoproteins [52]. In research, antibacterial agents are commonly tested against examples of both Gram-positive and Gram-negative strains, such as Staphylococcus epidermidis (Gram-positive) and Escherichia coli (Gram-negative).

The term ‘antibacterial’ has a very broad definition colloquially; generally, it can often mean either bactericidal (causing bacterial death) or bacteriostatic (inhibiting bacterial growth) [53]. Here, we use it to signify bactericidal properties. The terms bactericidal, antimicrobial, biocidal and antibacterial are all used interchangeably in this thesis.
2.2 Antibacterial agents incorporated into fibers

The enormous number of current antibacterial agents and fabrication methods for fibers prohibits an exhaustive literature review here; however, the most common and well-cited technologies are enumerated. Silver, in particular, will be discussed at length, due to its relevance in this work. Technical challenges of fabrication and potential commercial viability are briefly mentioned; however, comparisons of bactericidal efficacies are not broached. No general conclusions on this topic can be offered here, as an agent’s efficacy depends on many experimental factors and conditions [49]. It can be assumed that all of the following are broad spectrum biocidal agents, with the notable exception of specific antibiotics.

2.2.1 Drugs

Antibiotic-conjugated fibers have not yet gained widespread clinical usage, but researchers have identified the main applications of such fibers as materials for wound dressings, drug delivery controlled release systems, and possibly even prosthetics or tissue scaffolds [2, 3]. The incorporation of antistaphylococcal antibiotics, such as ciprofloxacin, into polymers is most frequently studied for these purposes. Methods include trapping the drug in a polymer mat via solvent-based casting [47], textile dyeing processes [23, 24], and various other chemical reactions that impregnate the polymer backbone with drug monomers [25]. Various synthetic polymers have been attempted as scaffolds, such as polyurethanes [47], polyesters [24], and polyethylene terephthalate [23]. While broad spectrum antibiotics exist, the specificity of polymeric synthesis reactions limits possible drug candidates for conjugation. Another obstacle of drug candidacy is that high reaction temperatures and volatile solvents may limit the potencies of some drugs.

2.2.2 Polycations

Synthetic polycations such as quaternary ammonium compounds [4], guanidine polymers [5], and phosphonium derivatives [6] all display positive charges that bind to and disrupt the negatively-charged cell membrane of microbes, resulting in lysis of the cell. Polymeric
biocides have the potential for particularly high antibacterial activities due to the high local density of active groups in the vicinity of polymer chains. However, antimicrobial properties can vary greatly among even polycations of similar structure, possibly because molecular weight and polydispersity play roles in the biological function of these polymers [4]. Researchers are still investigating structure-activity relations of polycations. In fiber form (or bound to other textiles), none of the aforementioned polymers have yet been employed in consumer products, though they have industrial application as additives in water treatment and sanitization. Commercialization could be hindered by scale-up issues, cost, or simply not-yet-perfected processing routes.

The most commercially ubiquitous polycation thus far has been the natural polymer chitosan. Industrially, chitosan is derived from the deacetylation of chitin, a positively-charged polysaccharide found in the shells of crustaceans. In nature, chitin protects shrimp and crabs from microbial infection. Because chitosan is natural, biodegradable, and biocompatible (thus producing no immunological response), it is better suited for biomedical applications than other synthetic polycations [7]. It is often used as the carrier polymer in drug conjugation as well [15], because its charge enhances the transport of polar drug molecules across epithelial surfaces. Chitosan also clots blood quickly, making it highly desirable as a wound dressing material [8], one of its primary commercial uses. Other applications include chitosan in antibacterial baby clothing [9] and in food packaging films [26].

2.2.3 Phenols

The biocidal mode of action in phenols begins with the inhibition of fatty acid synthesis in bacterial cells. Without these lipids, bacteria cannot build membranes and perform normal cell functions, leading to cell death [11, 12]. Phenolic compounds can be derived from natural botanical extracts or synthesized in the laboratory. Triclosan, short for 5-chloro-2-(2,4-dichlorophenoxy)-phenol, is the most commercialized phenolic agent, commonly found in household products such as handsoaps, mouthwashes, dishwashing detergents, and toothpastes. Textiles such as bedsheets and mattresses are finished with Triclosan through lam-
ination processes [13]. Because of its ubiquity, bacteria have begun to develop resistance towards Triclosan, and alternatives are required [14]; e.g., incorporating both Triclosan and silver. Today, many consumers are wary of overexposure. Furthermore, Triclosan is toxic to aquatic life, and bioaccumulation of the compound in rivers and streams is damaging the Earth’s ecosystems [27].

2.2.4 N-halamines

N-halamines are compounds in which one halogen atom is bonded to a nitrogen atom from cyclic or acyclic imide, amide, or amino groups [28]. It is thought that positive halogens from N-halamines transfer to cell receptors and destroy enzymatic and metabolic cell processes. The main attraction of this chemistry is its capability for regeneration. This mechanism consumes halogen atoms, but repeated halogenation treatments can refresh antibacterial function [29]. N-halamine precursors (radical initiators, such as hydantoin monomers) are often incorporated via textile finishing processes. Fabrics (cellulose and polyesters have been tested) [30] are dipped into a solution of these precursors, then patted down to control the amount of chemical deposition, then dried and cured at temperatures above 100°C. Initiation and graft polymerization can occur during the drying step [30]. Chlorination treatment of chitosan has also been found to transform chitosan amino groups into N-halamines [29].

2.2.5 Silver

Since ancient times, materials containing silver ions and compounds have been well-known to exhibit strong antibacterial functionality. Silver is hypothesized to bridge thiol groups between proteins, binding active enzyme centers and eventually ceasing cellular metabolism in micro organisms [31]. The mode of biocidal action is only partially understood; dynamic, real-time evidence has not confirmed the mechanism. Previous research has suggested that Ag⁺ kills bacteria by disrupting DNA replication [32] or phosphate uptake pathways [33]. Along with chitosan and triclosan, silver is already highly commercialized in fibers, primarily in athleticwear (e.g., ski clothing manufactured from Ciba Tinosan SDC, which uses
silver as an antimicrobial agent), but also as wound dressings in the biomedical industry [50].

One of the early common methods of incorporating silver into fibers was through oxidation and carbonization into activated carbon fibers [34, 35, 36]. Activated carbon fibers (ACF) are microporous, providing a large surface area for both bacterial adhesion and chemical reactions to occur. The process usually requires hours of oxidation, then carbonization under nitrogen gas at 940°C. Subsequently, the carbonized fiber is activated with steam and immersed into silver ion precursors such as silver nitrate. Finally, the resulting fiber is heated and decomposed to obtain ACF-Ag [34].

More recently, silver nanoparticles have been integrated into many types of textiles, including wool via the pad-dry-cure textile process [37] (described above in the N-halamines section), cotton via graft polymerization [38], and polyesters via corona (air plasma) treatment [39].

Hollow fibers loaded with silver are also a topic of biocidal fiber research. Hollow fiber membranes have the added advantage of more surface area to volume ratio, facilitating greater silver loading [40]. These fibers are produced using dry-wet spinning, a process similar to that used for ACF-Ag fibers referenced above. Researchers have reported 0.19 wt% silver loading in polyacrylonitrile fibers [41].

In a similar vein, again utilizing optimal surface area to volume ratio, researchers have fabricated polymer fibers encapsulating silver via electrospinning to create nanofiber dimensions [42, 43, 44]. While this method is a good alternative to dry-wet spinning polymer processes, large volumes of fibers are difficult to produce.

### 2.3 Viruses as biotemplates

In this thesis, we employ a virus called M13 bacteriophage (phage, for short) to biotemplate silver. M13 bacteriophage is a well-studied filamentous virus that reproduces in *E.*
coli non-lytically. The virus poses no risk to mammalian cells. M13 is composed of five different, modifiable capsid proteins that can be independently altered. Directed modification of all M13 capsid proteins is possible, by cloning into the full M13 genome or phagemid (phage/plasmid hybrid) vectors. The vast majority of these is major capsid gene VIII protein (pVIII), which forms a helical-pitched cylinder that is 7 nm in diameter. The pVIIIIs contribute the most to M13’s wild-type length of 880 – 920 nm. Previously, various capsid proteins on M13 have been genetically engineered to bind to gold [20], cobalt [45], zinc sulfide [46], silver [21] and other inorganic materials. M13’s programmable protein functionalities offer great potential for hetero- and multifunctional structures, e.g., multifunctional phage fibers.
3 Fabrication of Phage Fibers and Coatings

We aim to optimize the antibacterial properties of our phage-based fibers by employing a genetically engineered glutamate-rich phage (E3), shown in Fig. 1A, which expresses three glutamate and one aspartate amino acid at the N-terminus of the gene VIII protein. Continuous fibers of μm-scale diameter and cm-scale length were extruded via the extrusion method described by Chiang et al [20]. To explore the application of these crosslinked phages as a post-processing coating for fibers of relatively greater mechanical strength[20], Kevlar fibers were coated with phage solution and crosslinked with glutaraldehyde. After crosslinking, both pure phage fibers and Kevlar composite fibers were plated with silver by electroless deposition, using silver acetate as a silver ion precursor and subsequently reducing with sodium borohydride. This electroless deposition and reduction is referred to as silverization of the phage fiber. Figures 1B-D illustrate the three types of fibers constructed: silverized fibers comprising wildtype M13 phages (M13-Ag, Fig. 1B), silverized fibers comprising genetically engineered E3 phages (E3- Ag, Fig. 1C), and silverized composite phage-coated Kevlar fibers (Kevlar-E3-Ag, Fig. 1D). The following sections describe the methods summarized above in detail.
Figure 1: (A) Schematic depiction of single M13 bacteriophage with DNA sequence engineered (gVIII, orange) to express three negatively-charged glutamates on the body coat proteins (pVIII, orange ovals). This E3 sequence can electrostatically attract silver ions (Ag+) [21]. In this study, phage were glutaraldehyde-crosslinked upon extrusion to produce fibers comprising (B) pVIII-altered E3 and (C) wildtype M13 phage that attract Ag+ to a lesser degree. (D) Kevlar fibers were also coated with crosslinked E3 phage. On all fibers, bound Ag+ ions were reduced to metallic particles in situ via sodium borohydride.

3.1 Large-scale phage amplification

Phage amplification is a vital step to creating phage fibers, as mechanically robust fibers require a very high concentration of phage (10^{15}-10^{16} plaque forming units (pfu)/mL). Phage that have been genetically engineered to display large peptides or positively-charged peptides may not reproduce quickly in sufficient concentrations; additionally, mutations may be more likely to occur. We attempted large-scale amplification of E4 phage (genetically engineered with 4 glutamates instead of 3) prior to E3, but were unsuccessful in producing high enough phage concentrations to spin fibers.

Amplification begins with shaking an overnight culture of E. coli (strain ER2738 from New England Biolabs) with tetracycline antibiotic (see Appendix A for a detailed protocol of large-scale phage amplification). The overnight culture is then diluted into Luria-Bertani (LB) broth at a 1:100 volumetric ratio. Typically, 3 liters of diluted culture will produce
approximately 120-180 μL of phage at the correct concentration for crosslinking, which translates to 0.5-1 m of phage fiber if no phage is wasted. The diluted E. coli culture is then infected with either E3 or M13 phage. Ideally, 500 μL-1 mL of phage at $10^{11}$-$10^{12}$ pfu/mL is added to each liter of culture for infection. However, we have found that lower infection concentrations of phage are sometimes successful. After infection, the cultures are agitated aerobically at 225 RPM at 37°C for 18+ hours. Sequencing results have shown that E3 does not mutate over this period of time. We endeavored to produce enough phage with shorter growth cycles, but discovered a minimum of 12 h of agitation was necessary.

Subsequent to phage reproduction, E. coli are pelleted out with a centrifuge (see Step 10 in Appendix A). Phage are then precipitated out from the resulting solution with PEG-NaCl overnight, and separated from the LB via centrifugation. After resuspension of the phage in Milli-Q water, residual bacteria are pelleted out for a second time from the resuspended solution. Following this step, it is essential to consolidate the supernatant phage solution into as few centrifuge bottles as possible to avoid loss of phage. (At this point, we typically combine solutions into two 50-mL Falcon tubes, each holding 25 mL of phage solution.) A second overnight precipitation cycle with PEG-NaCl is then performed, and the phage is pelleted again via centrifugation. We then resuspend the phage pellets in 2-3 mL total of Milli-Q water.

At this stage, the phage is still not yet concentrated enough for wet-spinning, but this concentration is ideal for longer term storage at 4°C. We have noticed that storing phage suspensions at these high concentrations leads to rapid separation and an overall "goopiness" in its constitution. A day prior to spinning the fibers, the stored solution is centrifuged once more to remove any last remnants of bacteria that tend to alter the viscosity of the solution. Then 500 μL of the supernatant phage is concentrated into about 30 μL of Milli-Q water through pelleting and resuspension. These volumes are not exact: this process is somewhat of an art; we might resuspend in more or less than 30 μL depending on the size of the phage pellet that was achieved. After this last concentration step, the phage solution appears completely opaque and a pearlescent white. The final phage solution is stored overnight at 4°C.
Standard titering was performed to confirm phage concentrations (see Appendix B).

3.2 Wet-spinning

Before extrusion, the concentrated phage is spun in a microcentrifuge at no more than 5000 RPM for 5 seconds to de-gas the suspension, as bubbles create noncontinuous fiber strands. (Faster speeds or longer centrifugation times result in phage pellet formation.) The phage solution is then slowly pipetted into the bottom of a 1 or 3 mL BD Luer-Lock syringe 15 μL at a time. (Luer-Lock syringes are necessary because pressure build-up in the syringe can often discharge the needle.) During pipetting, the plunger is simultaneously pulled back slowly to receive the phage; additionally, an air space is left between the plunger and phage solution to avoid contact between the phage and plunger.

A 33 gauge needle is inserted onto the end of the syringe, and phage solution is extruded vertically into an 8 vol% glutaraldehyde solution (Sigma-Aldrich) by a syringe pump, producing approximately 2-3 cm per microliter of phage suspension (see Fig. 2 for a photo of the fiber spinning setup). Previous procedures published by Chiang et al. [20] indicate a 1-2 vol% glutaraldehyde solution produces the most mechanically robust fibers comprised of gold-binding phage, but repeated experiments did not confirm as such for E3 phage for reasons unknown. It is possible that a different genetically modified phage might result in different crosslinking dynamics. It is unclear whether the speed of extrusion affects the quality of the fibers; furthermore, the speed set with the syringe pump controller is not the actual speed of extrusion: back-pressure in the syringe and the viscosity of the phage solution significantly affect the real speed. Clogging of the needle also plays a role in how quickly fiber is spun, as well as affecting the diameter of the fiber. Severe clogging of the needle can result in very thin fibers. After extrusion, fibers are incubated in glutaraldehyde solution for 20 min, and then rinsed in Milli-Q water.
3.3 Silverization

Using forceps, fibers were then individually transferred to a bath of 5 mM silver acetate (Sigma-Aldrich) and incubated for 30 s. Subsequently, fibers were transferred to a bath of 5 mM sodium borohydride (EMD Chemicals, Inc.) and incubated for another 30 s before being rinsed with Milli-Q water. Finally, the fibers were extracted with the forceps and suspended in air (held with forceps) to dry for 2-3 min, until fibers no longer adhered to surfaces through moisture.

3.4 Kevlar coating

Kevlar strands were dipped into a pool of highly-concentrated phage solution ($10^{15} - 10^{16}$ pfu/mL in Milli-Q water) and drawn out slowly with forceps, allowing the phage to adhere to the Kevlar via van der Waals forces. The coated Kevlar was immersed in 8 vol% glutaraldehyde solution and then silverized with same method described above for the phage
fibers.

Another method of coating the Kevlar with phage was attempted by dispensing 15 μL of the highly-concentrated phage solution onto a petri dish, then placing the Kevlar fiber in the solution, and dispensing 15 μL more of the phage solution on top of the fiber. Glutaraldehyde was then poured slowly into the petri dish to crosslink the phage. However, when attempting to extract the newly-coated Kevlar fiber with forceps, the phage had formed thick films on top of and below the Kevlar fiber instead of an annulus around the fiber. (This might be a method of producing high-concentration phage films.)
4 Results and Discussion

Following the successful fabrication of silverized phage fibers, we characterized the fibers to examine the extent of silver loading. Antimicrobial activity was then quantified through several experiments, with a focus on comparisons between M13-Ag and E3-Ag fibers to highlight the importance of genetic engineering. Finally, this technology was leveraged as post-processing coatings on Kevlar fiber. These composite silverized Kevlar-phage fibers were similarly characterized and tested for biocidal potency.

4.1 Characterization of silverized virus fibers

We employed scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS) to characterize the relative degree of silverization on M13 (unmodified) and E3 (modified) phage fibers (Fig. 3A, B). Successful genetic manipulation is evidenced by the comparative heights of the AgLa peaks from E3-Ag and M13-Ag in the energy-dispersive spectroscopy (EDS) analyses (Fig. 3C) with the higher peak indicating greater silver loading on E3-Ag fibers. EDS is a method of elemental analysis in which a sample is bombarded with charged particles, causing it to emit X-rays characteristic of certain elements. Greater intensities of these X-rays signify a higher concentration of the element present in the sample. Inductively-coupled plasma atomic emission spectroscopy (ICP-AES) was employed to determine the silver mass per length on these fibers. In ICP-AES, the sample is atomized with argon gas into hot plasma, causing the light wavelengths characteristic of its elements to be emitted. Our results showed 0.61±0.072 μg of silver was loaded per cm of E3-Ag fibers as compared to only 0.19 ±0.024 μg/cm for M13-Ag (Fig. 3D). Amplification of the negative surface charge of phage fiber via E3 genetic modification thus results in approximately three times the degree of silver loading.
Figure 3: Comparison of degree of silverization on E3 (engineered) and M13 (wildtype) phage fibers. (A) Scanning electron microscopy (SEM, left) backscattering and energy-dispersive spectroscopy (EDS, right) indicate dense, uniform adhesion of Ag particles to E3 phage fibers, whereas images in (B) demonstrate lower binding affinity to the unmodified M13 phage fibers. (C) EDS analysis of Ag_La peaks (arbitrary units) and (D) inductively-coupled plasma atomic-emission spectroscopy quantify increased degree of silverization on engineered E3 phage fibers. EDS spectra shifted vertically for clarity. Scale bar = 50 μm.
4.2 Antibacterial activity of silverized virus fibers

4.2.1 Live-dead staining

The bactericidal effects of this increased silver loading were visualized by fluorescence-based live-dead staining. *E. coli* (2x10^7 colony-forming units (cfu)/mL, ATCC 700728) were stained using the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, L13152). Fibers approximately 3-4 cm in length were incubated in 15 μL of this *E. coli* culture under agar for 2 h at 25°C in the dark. Cells were then visualized via fluorescence microscopy (Olympus IX51); intact cell membranes (live cells) were indicated by green fluorescence, and broken membranes (dead cells) were indicated by red fluorescence. Cell counts were performed using ImageJ software. During this 2 h time span, E3-Ag fibers killed virtually all *E. coli* bacteria (99±0.98%) within a 300 μm distance from the fiber (Fig. 4A,C). However, M13-Ag fibers proved less potent, displaying only a 74±14% bacterial inhibition ratio within the same perimeter (Fig. 4B,C).
Figure 4: Bactericidal activity (in contrast to growth inhibition) of phage fibers is confirmed via live-dead staining of *E. coli*. Bacteria within a 300 μm distance from the fiber edge of the (A) M13-Ag fiber and (B) E3-Ag fiber are shown after incubating fibers in a culture of *E. coli* (OD₆₀₀ = 0.1) under agar at 37°C for 140 min. Live bacteria are indicated as green, and dead as red; (examples indicated by arrows). Fiber edge is demarcated by yellow dashed lines. Scale bar = 100 μm. (C) Live-dead cell counts for replicate experiments confirm a significantly higher percentage of dead cells near the E3-Ag phage fibers. *Statistically significant by student t-test, n = 3 per phage fiber, p = 0.04.*
4.2.2 Modified disk diffusion experiments

Bactericidal activity of these fibers was also characterized for longer time spans in modified disk diffusion (Kirby-Bauer) tests. Bactericidal activity was evidenced by zones of inhibition (ZoIs) where colony growth was prevented around the fibers. Fibers approximately 3-4 cm in length were incubated in 15 μL of either *E. coli* or *S. epidermidis* (ATCC 14990) cultures under agar for 20 h at 37°C. Fibers were challenged with both low (2x10^7 cfu/mL) and high (>10^9 cfu/mL) densities of bacteria culture. ZoI distances were measured from the micrographs using ImageJ software; distances were taken perpendicularly from the center of the fiber to the first visible bacterial colony. Unsilverized phage fibers (M13 or E3 fibers) did not exhibit any antibacterial ZoIs (Fig. 5A, B), confirming that these phage fibers are not inherently antibacterial to the *E. coli* or *S. epidermidis* strains considered herein. In contrast, silver ion leaching from the silverized fibers prevented colony growth, creating a zone of inhibition (ZoI) around the fibers (Figs. 5C-F). ZoI sizes (measured perpendicularly from the center of the fiber to the first sign of bacteria colony growth) are qualitatively larger for E3-Ag fibers (Fig. 5C, E) as compared to M13-Ag fibers (Fig. 5D-F). It is commonly accepted that quantitative conclusions cannot be drawn from these modified disk diffusion tests (e.g., mathematical relations between ZoI size and silver loading on or ion diffusivity from the M13-Ag and E3-Ag fibers), as environmental and medium conditions are difficult to standardize in replicate bacteria incubations and the silver particles may be inhomogeneously distributed on the fibers [22]. However, the overall larger ZoIs surrounding E3-Ag fibers indicate stronger antibacterial potency in the genetically engineered fibers. This comparison corroborates the live-dead stain findings relevant to the earlier time points of bacteria-fiber interactions.
Figure 5: Antibacterial activity is characterized by zones of inhibition (ZoI) of *E. coli* growth (demarcated by red dashed line) in modified disk diffusion tests. Unsilverized fibers comprising (A) E3 phage and (B) M13 phage show no ZoIs, confirming that phage fibers are not inherently bactericidal. (C - F) For both phage fiber types, ZoIs are reduced at higher bacteria concentrations. However, for a given bacteria concentration, ZoIs are smaller for the genetically engineered E3-Ag phage fibers as compared to wildtype M13-Ag phage fibers. (G) summarizes these trends among replicate experiments (n = 4). Scale bar = 2 mm.

Exposing the fibers to higher bacteria concentrations (>10⁹ cfu/mL) resulted in decreased ZoI sizes, as demonstrated in comparing Figs. 5C and 5E for E3-Ag fibers (and Figs. 5D and 5F for M13-Ag fibers). Despite 20 h of incubation in extremely high-density bacteria cultures, the silverized fibers were still able to maintain minimal ZoIs. The disk diffusion trends over replicate experiments (n = 4 for each condition) are summarized graphically in Fig. 5G.

Modified disk diffusion tests with *S. epidermidis* cultures at OD₅₄₀ = 0.08 (~8x10⁷ cfu/mL) gave similar results in the comparison of E3-Ag (Fig. 6B) and M13-Ag fibers (Fig. 6D), supporting our expectation that silverized fibers are equally effective against these Gram-positive and Gram-negative species. Again, unsilverized E3 (Fig. 6A) and M13 (Fig. 6B) fibers did not inhibit any bacteria growth against *S. epidermidis*, as was the case against *E. coli*. 
Figure 6: Antibacterial activity is characterized by zones of inhibition (ZoI) of *S. epidermidis* growth (demarcated by red dashed line) in modified disk diffusion tests. (A) Unsilverized E3 and (C) M13 fibers do not exhibit any biocidal activity and thus have no zones of inhibition. (B) E3-Ag and (D) M13-Ag display comparable ZoI sizes against *S. epidermidis* as with *E. coli* in Fig. 5. Scale bar = 2 mm.

4.2.3 Kinetic experiments

In order to examine antibacterial activity of the fibers at time points between 2 and 20 h, we incubated fibers in cultures of *E. coli* and monitored bacteria growth via UV-Vis spectrometry. Calipers were used to measure out 13 mm lengths of fiber, which were then placed in 250 µL of *E. coli* culture medium in a 96-well plate. Over a period of 20+ hours, the covered plate was incubated and agitated at 37°C under ambient atmosphere. UV-Vis absorption measurements at $\lambda = 600$ nm were acquired approximately every 90 min before and during log-phase growth (the fastest period of growth in which the cell population is doubling constantly, characterized by the log-shaped region of the kinetic curves in Fig. 7), then less frequently after the bacteria population saturated. The initial concentration of *E. coli* was varied in several experiments, resulting in different dynamics: Fig. 7A shows the kinetics for fibers initially in a culture concentration of $OD_{600} = 0.07$ (2.1x10^7 cfu/mL), Fig. 7B in $OD_{600} = 0.1$ (3x10^7 cfu/mL) culture, and Fig. 7C in $OD_{600} = 0.18$ (5.6x10^7 cfu/mL) culture. The initial culture concentration affects the kinetics of bacteria growth and killing. Both E3-Ag and M13-Ag fibers halted all cell replication at sufficiently low bacteria density.
(Fig. 7A). However, at slightly higher bacteria densities corresponding to the initiation of log-phase growth, E3-Ag fibers inhibited growth slightly more than M13-Ag fibers, though neither phage fiber was able to stop growth completely (Fig. 7B). The divergence between the E3-Ag and M13-Ag kinetic curves in Fig. 7B corroborates our results in both the live-dead staining and zone of inhibition tests: here, too, the genetic engineering of E3-based fibers confers stronger biocidal potency than wildtype-based fibers. When E. coli were already growing at log-phase pace, neither E3-Ag nor M13-Ag fibers inhibited bacteria growth (Fig. 7C). Interestingly, the results in Fig. 7 do not directly correlate with the ZoI sizes seen in Fig. 5. For instance, a bacteria density of 3x10⁹ cfu/mL as seen in Fig. 5E-F would result in kinetic curves showing no growth inhibition similar to Fig. 7C, though small zones of inhibition are still visible in Fig. 5E-F. This discrepancy is likely due to the different experiment formats (which may affect the diffusion mechanism of silver ions), as well as the different volumes of culture and lengths of fiber.

4.2.4 Human perspiration challenge

To simulate a more realistic microbial challenge demanded of fibers incorporated within antimicrobial textiles, E3-Ag and M13-Ag fibers about 13 mm in length were immersed in 250 μL of bacteria cultured from perspiration swabbed from the human axilla (underarm) for 2 h at 37°C. The initial culture concentration was estimated to be about 10⁷ cfu/mL based on growth time and the probability of culturing mostly S. epidermidis cells [10]. Fibers were then extracted with forceps and incubated under agar for 20 h at 37°C. Adhesion of viable bacteria and subsequent colony growth was observed on M13-Ag fibers in micrographs (Fig. 8A), but E3-Ag fibers remained uncontaminated (Fig. 8B). This demonstrates the capacity of E3-Ag fibers to resist adhesion of viable bacteria in challenges relevant for wearable applications that require minimal risk of bacterial infection or associated odor production.
Figure 7: The kinetics of biocidal action were examined over 20+ h through UV-Vis absorption spectrometry measurements of fibers incubated in *E. coli* cultures of varying initial concentrations: (A) $\text{OD}_{600} = 0.07$ (2.1x10^7 cfu/mL), (B) $\text{OD}_{600} = 0.1$ (3x10^7 cfu/mL), and (C) $\text{OD}_{600} = 0.18$ (5.6x10^7 cfu/mL). $n = 3$ for each type of fiber and initial condition. ($\text{OD}_{600}$ = optical density measured at 600 nm.)

4.2.5 Other examinations of biocidal activity

Prior to any bactericidal activity tests outlined in the previous sections, we attempted to detach adhered cells from the virus fiber after exposure to bacteria culture and colonize them to ascertain their viability. To do so, we attempted a modified sonicator assay based on a procedure described by Kadouri and O’Toole[48]. Similar to the kinetics experiments, *E. coli* were grown to 2x10^7 cfu/mL, and fibers (13 mm in length) were incubated in the culture. Incubation occurred without agitation and at room temperature to slow further growth of bacteria (because higher bacteria densities would require serial dilutions in subsequent steps to visualize distinct colonies). The fibers were then transferred into clean LB and sonicated...
Figure 8: Adhesion of viable bacteria from normal human axilla (underarm) perspiration to phage fibers. Phage fibers comprising (A) E3-Ag and (B) M13-Ag were incubated in a culture of bacteria present in normal human perspiration (OD$_{540}$ = 0.04) for 2h at 37°C, then extracted and incubated under agar for 20 h at 37°C. Colony growth was observed on the M13-Ag fibers, but not on E3-Ag fibers. Scale bar = 200 μm.

at 40% amplitude for 8 s using a probe sonicator (Sonics VCX 130 PB). Should there be any bacteria adhered to the fibers, sonication would free them from attachment. However, these sonicator conditions are not aggressive enough to break open cell membranes; thus, live adhered bacteria would remain live [48]. After sonication, the supernatant was plated onto LB-agar plates: ~200 μL of supernatant were pipetted onto the agar plate containing 4-6 glass beads, which spread and disseminated the solution across the plate when agitated manually. (Optical density measurements of the supernatant could estimate the amount of bacteria in the solution, but might also be measuring absorbances from dead bacteria as well as live.) These plates were incubated at 37°C for 20 h. Colonies were then counted by the naked eye to quantify how many live bacteria was adhered to the fiber. If no colonies grew, this would indicate either no bacterial adhesion or dead bacteria. Conclusions can only be interpreted through comparison to negative and positive control plates.

Results of these experiments were inconsistent and not repeatable. Colony counts ranged from 0 to full-blown lawns for every type of fiber and control plate. One difficulty of the procedure was that droplets of bacteria culture typically adhered onto the tweezer tips, and this was often transferred into the clean LB along with the fiber, contaminating the LB with
bacteria that had not been previously adhered to the fiber. Additional washes in clean LB did not seem to rectify the problem. Aspirating solutions in and out of the wells instead of transferring the fiber itself avoided the use of forceps, but bacteria adhered to the well walls instead.

Ultimately, modified disk diffusion tests and live-dead staining confirmed biocidal activity, exhibiting the potency of silver-leaching that extends visibly far enough beyond the fiber (precluding the condition of adhesion). Further exploration of this sonicator assay was not pursued.

4.3 Application to Kevlar as a coating: characterization and antibacterial tests

Upon the successful construction of biocidal phage fibers, we considered whether modification of this phage processing could confer antimicrobial functionality to a mechanically robust base fiber as a coating. As a proof of practical application, we chose to cover individual Kevlar fibers with our genetically engineered E3 phage, and silverize the composite fiber. Here, Kevlar strands were dipped into a pool of highly-concentrated phage solution (10^{15}-10^{16} pfu/mL in Milli-Q water) and drawn out with forceps. The coated Kevlar was immersed in 8 vol% glutaraldehyde solution and then silverized with same method described above for the phage fibers.

These composite Kevlar-E3-Ag fibers have potential use in preventing odor-based detection in protective clothing, as well as reducing the risk of infection to surface skin wounds exposed to soiled clothing. SEM and EDS of surface chemical composition confirmed successful silverization of the Kevlar fibers (Fig. 9A-C).

Again, modified disk diffusion tests were used to characterize the bactericidal capability of the coatings. Under moderate bacteria concentrations (2x10^7 cfu/mL), the Kevlar-E3-Ag exhibits more limited antibacterial potency, exhibiting smaller ZoIs (Fig. 9E-F) than
Figure 9: Relative degrees of silverization on (A) unmodified Kevlar and (B) Kevlar coated with crosslinked E3 phage are characterized in SEM/EDS maps. Scale bars = 10 μm. (C) Corresponding EDS spectra of E3-Ag phage-coated Kevlar and unmodified Kevlar, characterized via Ag L edge peaks (arbitrary units). Spectra shifted vertically for clarity. Modified disk diffusion tests conducted on (D) unmodified Kevlar, (E) Kevlar-E3-Ag with *E. coli*, and (F) Kevlar-E3-Ag with *S. epidermidis* indicate clear zones of inhibition (ZoIs, red dashed lines) in bacteria growth around only the phage-coated fibers. Scale bars = 100 μm.

the pure phage fibers in Fig. 5. This reduced potency is attributable chiefly to less E3 phage-and thus less silver-loading-in the composite E3-Ag-coated Kevlar fiber compared to a pure E3-Ag phage-fiber. The E3-Ag coating, which is approximately 3-4 μm in thickness around the Kevlar fiber core of 9.3 μm diameter (Fig. 9B), decreases the functionalizable E3 volume as compared to the pure E3-Ag phage-fibers of ~40-100 μm diameter. Using our ICP-AES measurements of Ag content for this glutaraldehyde-crosslinked E3-Ag and these average fiber diameters and coating thickness, we estimate silver loading to be 0.10 μg/cm of composite fiber (see calculations below). This six-fold reduction in silver loading as compared to the pure E3-Ag fibers (0.61 μg/cm) resulted, as expected, in smaller ZoIs under comparable bacteria challenges.
Calculations:

For E3-Ag fibers:

\[
\frac{\text{Mass of silver loaded (from ICP data)}}{\text{Volume of E3 phage}} = \frac{0.61 \mu g}{1.53 \times 10^7 \mu m^3} = 4.0 \times 10^{-6} \mu g/\mu m^3
\]

For Kevlar-E3-Ag fibers:

Volume of E3 phage in 1 cm of Kevlar-E3-Ag fiber = \([128.6-67.9] \mu m^2] \times [1 \text{ cm}] = 6.07 \times 10^5 \mu m^3

\[
\frac{\text{Mass of silver loaded}}{\text{length of Kevlar-E3-Ag fiber}} = \frac{6.07 \times 10^5 \mu m^3}{\text{cm}} \times \frac{\mu g}{4.0 \times 10^{-6} \mu m^3} = 0.1 \mu g/cm
\]

This is about 1/6 of the degree of silverization measured in E3-Ag fibers (~0.61 \mu g/cm) according to ICP-AES data.

4.4 Discussion and comparison

Although these phage fibers and coatings perform as viable biocides, this method still presents certain limitations from a practical standpoint; these challenges are common to other antiseptic fiber technologies. Large quantities of phage fibers are difficult to manufacture at the research scale due to the high concentration of phage required \(\left(10^{15}-10^{16} \text{ pfu/mL}\right)\). Given the large volumes of bacteria required to yield such concentrations, large-scale synthesis of phage fibers would require a continuous bioreactor, as is used in industrial-scale phage production. In addition, controllable release and distribution of silver particles would be a desirable feature to integrate into this design.

However, besides the future prospect of multifunctionality, these phage fibers already demonstrate several advantages over alternate current fiber production techniques. First, the silver leaching results in relatively rapid bactericidal action (<2 h exposure, as evidenced by
live/dead stains), which is beneficial over other fiber platforms that require prolonged hours of contact before cell death occurs. Second, phage fiber processing can be performed in ambient conditions, unlike many polymerization methods that demand high temperatures and inert atmospheres, as described in the literature review in Chapter 2. Textile finishing processes such as the pad-dry-cure method require high temperatures and harsh chemicals, as well as oxidation/plasma treatment and radical initiation steps (in graft polymerization). Third, the extrusion, crosslinking, and silverization can be completed in less than an hour for continuous fibers of tens of centimeters in length, whereas chemical processing times can sometimes span hours or even days. Thus, the relative ease of wet-spining and electroless plating of these Ag-phage fiber processing presents several advantages over synthetic antimicrobial polymer fiber platforms, considering the potency and efficacy of the imparted antimicrobial functionality. Further, this material platform offers the potential to combine multifunctional antimicrobial strategies (e.g., controlled release of expressed functional groups) and distinct functionalities (e.g., electrical conductivity) via further genetic manipulation or post-processing of E3 phage fibers.
5 Contributions and Future Work

In summary, I have successfully synthesized phage-based fibers that exhibit bactericidal activity against *E. coli*, *S. epidermidis*, and perspiration subcultures when functionalized with silver. My results show that genetic engineering of the phage facilitates significant silverization (> 600 ng/cm of fiber) and, correspondingly, strong antibacterial effects. Examining bacteria cells under short time scales demonstrated the rapid bactericidal action of silver ions; bacterial cells within 300 μm of the fiber are killed within a 2 h exposure. Even under continued, 20 h contact with bacteria concentrations as high as 10⁹ cfu/mL, these silverized phage fibers were capable of inhibiting cell growth in the immediate vicinity, as demonstrated by the modified Kirby-Bauer tests of zones of inhibition. This genetically engineered fiber also inhibits bacterial attachment and growth after undergoing a 2 h immersion in bacteria cultured from human perspiration, under challenges relevant to antimicrobial textile applications. Silver-functionalized, phage-coated Kevlar also displayed bactericidal activity, though to an extent reduced by the lower volume of the silverized phage as compared to pure phage-fibers. Potential applications of these fibers and coatings include medical applications such as wound dressings, military applications to diminish armor odor, as well as commercial textiles such as athleticwear. Despite some upscaling limitations, the relatively facile and low-toxicity processing of these fibers presents certain advantages over other synthetic polymer-based biocidal textiles. Moreover, the successful integration of antimicrobial functionality within a biological scaffold shows promise for further genetic engineering toward the design of multifunctional fibers. For instance, we might be able to develop a flame-retardant antibacterial fiber: we might display a peptide that binds phosphorus (a known flame-retardant agent) on the *geneIII* protein while still displaying the negatively-charged E3 peptides along the *geneVIII* proteins. With five modifiable proteins comprising the M13 bacteriophage, each capable of binding to a different material, we have ample possibilities for developing multifunctional fibers.

Future work should be aimed at more effective methods of coating mechanically robust fibers such as Kevlar with phage, post-processing. The main limitation to imparting bacte-
ricidal functionality onto these fibers is attaching enough phage for sufficient silverization. Currently, it is difficult to even uniformly coat the Kevlar completely with phage. The van der Waals forces facilitating phage adhesion to the Kevlar are weak; covalent or electrostatic bonds might result in a stronger, thicker coating of phage. However, Kevlar is notoriously difficult to chemically functionalize due to its satisfied (and thus inert) chemical structure. It might be possible to coat a layer of epoxy or some other synthetic polymer over Kevlar before depositing the phage solution to assist in bonding. Multilayer depositions of phage and silver might also increase the extent of silver loading. It is possible that during the second deposition of phage, the first layer of silver particles might leach out into the glutaraldehyde solution, but the crosslinking process is rapid enough for the loss of silver to be minimal. The high density of the second layer of phage would also slow down the diffusion of the silver into the glutaraldehyde.

It would also be useful to characterize the *distribution* of silver particles on the virus fibers. In my estimation, the same number of silver particles are not evenly plated onto the fiber in replicate silverization processes, which hinders standardized antibacterial testing. This is an observation noted visually from the change in color of the phage fibers during silverization which sometimes does not occur consistently throughout the length of the fiber. Prior to silverization, the phage fiber is white in color (in solution; slightly yellow in color when dried), but after electroless plating, the phage fiber turns black. Areas that did not load as much silver appear lighter in color. (The black color likely indicates that these are not silver nanoparticles, but larger clusters of silver.)

Finally, an alternative, degradable crosslinker could be substituted in place of glutaraldehyde to create antibacterial, biodegradable sutures. This crosslinker should dissolve over time under physiological conditions and be biocompatible.
6 Appendix A:

Large-scale phage amplification

DAY 0:

1. Prepare overnight *E. coli* culture (OC) in a 250 mL autoclaved Erlenmeyer flask: 35 mL LB, 35 µL of 1000x tetracycline solution, 1 colony of ER2738. Shake culture aerobically with flask covered loosely with foil overnight at 37°C in air, 225 RPM.

2. Add 25 g of LB into a 2 L Erlenmeyer flask using a funnel, then add water up to 1L mark. Repeat 2x for a total of 3 L. Cover each flask with foil and autoclave.

3. Take out LB and let cool.

DAY 1:

4. Add 1 mL of tetracycline to each flask of LB. Dilute 10 mL of overnight culture into each LB flask and shake in 37°C rotary shaker in Belcher lab (or in incubator in Van Vliet chem bay) for 2 hours at same conditions as step 1.

5. Add 500 µL- 1 mL of 10^{12} pfu/mL phage into flask.

6. Place flask in 37°C incubator for 15-30 min.

7. Shake flask in 37°C shaker for 18+ hours. Autoclave 4 one-liter centrifuge bottles.

DAY 2:

8. Divide contents of each flask into 4 disposable, BD Falcon 225 mL blue conical centrifuge tubes.

9. Balance the four tubes with their casings.

10. Centrifuge at max speed (or any speed above 5,000 RPM) using tabletop Beckman Spinchron centrifuge for 20 minutes to pellet out bacteria. Repeat for all solutions.

11. Pour divide supernatant into the 4 autoclaved centrifuge bottles (about 675 mL each) and add 1/6 volume PEG-NaCl* (about 135mL) into each bottle. Invert and store at 4°C
overnight.

*PEG-NaCl: 200 g PEG 8000 + 146 g NaCl + 1 L H₂O, autoclaved

**DAY 3:**

12. Balance the 4 centrifuge bottles.

13. Spin phage-PEG/NaCl solution in Beckman Avanti J-20 XP centrifuge at 13500 RCF (not RPM!) with rotor ID JLA 8.1000 for 20 minutes to pellet out phage.

14. Carefully pour out supernatant and resuspend (vortexing allowed) phage into ~10 mL Tris-buffered saline solution (TBS) or Milli-Q H₂O for each bottle.

15. Combine the solutions from all three bottles into two 50mL Falcon tubes. Balance them.

16. Spin at 10,000 RPM in Sorvall XA-20 with rotor ID SLA-3000 for 15 min. to re-pellet remaining bacteria.

17. Carefully pour supernatant into a new 50 mL Falcon tube and add 1/6 volume PEG-NaCl (~3.33 mL for 20 mL of supernatant).

18. Invert and store at 4°C overnight or on ice for several hours.

**DAY 4:**

19. Re-balance tubes if necessary and spin at 13,500 RCF in Sorvall XA-20 centrifuge with rotor ID SLA-3000 for 20 minutes to pellet out phage.

20. Carefully discard supernatant and resuspend phage in 1mL TBS or H₂O for each Falcon tube. Transfer into an Eppendorf tube and store in 4°C.

21. Before using phage, spin down again at 10,000 RPM in the tabletop microcentrifuge for 15 min. to pellet out any remnants of bacteria. Aspirate out supernatant and transfer to a new Eppendorf.
Appendix B: Determining Phage Concentration via Titering

1. Prepare titer culture: 10 mL LB, 10 μL 1000x tetracycline solution, 1 colony of ER2738 in an autoclaved 250 mL Erlenmeyer flask. Agitate aerobically at 225 RPM, 37°C, until optical density at 600 nm is 0.5, which should take 3.5-5 hrs depending on age of bacteria on streaked plate.

2. Melt agarose top* in microwave and aliquot 3-4 mL of liquid agarose top into each BD 14 mL round-bottom tube. Place loosely-capped tubes in any oven at 50-55°C and let agarose top temperature equilibrate for >30 min.

*Agarose top: 5 g Bacto-Tryptone + 2.5 g yeast extract + 2.5 g NaCl, 0.5 g MgCl₂ • 6H₂O + 3.5 g agarose + 500 mL H₂O, autoclaved

3. Place unsealed LB-agar plates with tetracycline/IPTG/XGAL into 37°C incubator upside-down to warm for >30 min.

4. Prepare serial dilutions (10⁻¹, 10⁻³, 10⁻⁵...10⁻¹⁵ or whatever estimated phage concentration might be) in TBS or H₂O of phage solution in Eppendorf tubes.

5. Dispense 185 μL of titer culture into new Eppendorf tubes, one for each dilution to be plated. Infect each bacteria solution with 10 μL of diluted phage solution. Let solution sit for no more than 5 min.

6. In sterile biosafety cabinet (not used for eukaryotic cell culture), plate each phage-infected bacterial solution: first, remove agarose top tubes from oven and let cool for about 1 min but without letting the agarose solidify; transfer phage-infected bacterial solution to the agarose top; gently vortex; pour bacteria/agarose over pre-warmed plate; and let cool for a few minutes. Repeat for each phage dilution.

7. Incubate unsealed plates overnight in 37°C incubator and count blue plaques to determine concentration (multiply the count by the dilution of the plate to obtain the concentration in plaque forming units).
References


[51] H. C. Gram, Fortschritle der Medizin 1884, 2, 185.