Elastin-based silver-binding proteins with antimicrobial capabilities

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**ELASTIN-BASED SILVER-BINDING PROTEINS WITH
ANTIMICROBIAL CAPABILITIES**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING
2013

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ABBREVIATIONS

2xYT	Two times yeast/tryptone medium
2 or 3-D	2 or 3-Dimension
AgNPs	Silver nanoparticles
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DSS	Disuccinimidyl suberate
ECM	Extracellular matrix
<i>E. coli</i>	Escherichia coli
ELPs	Elastin-like proteins
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
HRTEM	High resolution transmission electron microscopy
LCST	Lower critical solution temperature
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization - time of flight mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NHS ester	N-hydroxysuccinimider ester
Ni-NTA	Nickel- nitrilotriacetic acid
OD	Optical density
PBS	Phosphate-buffered saline
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid)
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TB	Terrific broth

ABSTRACT

Elastin-based biomaterials have gained popularity in tissue engineering and regenerative medicine due to their well-defined chemical and physical properties. In particular, elastin-based materials have tremendous potential as wound dressing materials due to their elasticity and biocompatibility. Yet, elastin-based materials with antimicrobial properties have not been developed to date. In this work, we constructed novel elastin fusion proteins bearing AG3 silver-binding motifs (GPG-AG3) and their thin films. In the presence of NaCl, GPG-AG3 proteins self-assembled to form aggregates with diameters of about 100 nm. Further incubation in silver nitrate yielded silver nanoparticles on the surfaces of the protein particles and thin films. The silver nanoparticles obtained in our work had diameters of 20 – 25 nm from protein aggregates solution and $471 \text{ nm} \pm 178 \text{ nm}$ on protein films. The effect of varying NaCl concentrations and pH on the size and morphology of silver particles was examined.

The nucleated silver nanoparticles exhibited antibacterial properties when tested against gram-negative *E. coli*. In both 2xYT agar plates and culture media containing 20 $\mu\text{g/mL}$ of synthesized silver, or silver-coated protein films, *E. coli* growth was completely inhibited. Silver-coated GPG-AG3 films were able to support mammalian cell growth for up to 3 days, but displayed significant cytotoxicity after 7 days.

In summary, we have developed GPG-AG3 proteins with the ability to nucleate antibacterial silver particles. In our future work, we will focus on the fabrication of silver nanowires using GPG-AG3 templates. The antibacterial properties and cytotoxicity of silver nanowires will also be examined *in vitro*.

1. INTRODUCTION

1.1. Background

Wound infection remains a serious problem in the clinic due to the difficulty in countering the multitude of fast-evolving pathogens in the environment. Silver is one of the most effective antimicrobial agents due to its broad range of antimicrobial activities against viruses, bacteria and some eukaryotic micro-organisms [1]. Particularly, silver-based wound dressings have been used as the first line of defense against bacterial contamination in wound management [2]. Recently, silver nanoparticles have been explored as antimicrobial agents because of their large surface area to volume ratios and physicochemical properties. As a result, silver-based nanomaterials have emerged as one of the most promising materials for wound treatments.

While silver nanomaterials are widely used in chronic wound treatment due to their strong antibacterial properties, there are several limitations that have yet been resolved. One main challenge is the propensity of silver nanoparticles to aggregate in solution, leading to subsequent loss of bactericidal effect of silver nanoparticles over time [3]. More importantly, long-term exposure to silver, especially uncoated silver nanoparticles, may have significant cytotoxic effects in humans. As a result, researchers have developed methods to modify the surfaces of silver nanoparticles to limit particle surfaces contact directly to cellular components. These modifications have successfully reduced the toxicity of silver nanoparticle exposure in human cells [4].

1.2. Problem statement

To date, much of the attention has been devoted to produce stabilized silver-based materials using chemical routes such as nanofibrous alkylthiol derivatives or poly-(vinylpyrrolidone) polymers coupled with silver nanoparticles [5]. Nevertheless, these products encounter problems of high cost, lengthy synthesis steps and inadequate biocompatibility.

Recently, the discovery of silver-binding peptides has led to the development of new silver synthesis methods [6]. Biomimetic approaches using these peptides have gained widespread popularity due to their mild and environmentally friendly synthesis conditions. Silver nanoparticles have been successfully synthesized using peptide-based approaches [7]. In addition, silver-binding peptides have also been immobilized onto 2-D substrates such as PLA [8], PLGA [9] to serve as antibacterial biomaterials in biomedical applications. However, toxic organic solvents were often used in the production of these materials, rendering them less desirable for implementation in the clinic. In view of the current challenges, new synthesis methods that can lead to the efficient synthesis of safe and functional silver-based materials are desired.

1.3. Hypothesis, objectives and scope

1.3.1. Biomimetic synthesis of silver using fusion proteins as templates

We recognize the need for new methods in the production of silver-based biomaterials. Ideally, these methods should have low production costs, simple to perform and require mild conditions. Following the work of Naik and coworkers, we wondered if a facile route could be devised for the synthesis of silver-based biomaterials by using fusion proteins as templates. We hypothesize that fusion proteins containing both structural domains as well as silver-binding sequences could serve as templates for biomimetic synthesis of silver.

We will test this hypothesis in the first portion of the work. First, we will design and construct novel fusion proteins with the ability to bind silver under physiological conditions. The elastin-like sequence (GPG), derived from tropoelastin, will be incorporated due to its ability to undergo self-assembly, triggered by a change in salt concentration [10], pH and temperature [11]. In addition, the known biocompatibility of elastin will render the overall material suitable for use in the body [10]. Separately, the AG3 domain, discovered by Naik and co-workers [12], has been shown to nucleate silver readily in solution under mild physiological conditions. Figure 1 shows the proposed amino acid sequence of the elastin-like fusion proteins containing silver-binding domains (GPG-AG3).

Figure 1: Amino acid sequence of elastin silver-binding fusion protein GPG-AG3. The GPG-AG3 fusion protein comprises of repetitive pentapeptide Proline-rich domains (□) for coacervation (named **P** domains), Glycine-rich domains (■) for self-assembly into cross-β structures (named **G** domains) and **AG3** silver-binding domain (■) for nucleation of silver. The protein also contains lysine residues (■) for cross-linking and a 6x-His tag (■) for protein purification using Ni-NTA.

We expect GPG-AG3 proteins self-assemble to form aggregates via hydrophobic interactions of the cross-β structures. Subsequently, the AG3 domains exposed on the surface of the protein aggregates will interact with silver ions present in the silver nitrate solution by reduction of silver ions at the interface between silver and AG3 domain. As a result, silver nucleation will occur on the surface of the GPG-AG3 proteins. Figure 2 shows the hypothesized mechanism of the synthesis route.

Figure 2: A schematic of the proposed route of silver synthesis using GPG-AG3 fusion protein templates.

To confirm the working mechanism in our synthesis route, GPG-AG3 fusion proteins will be incubated in the presence of NaCl and silver nitrate over time. We will determine the presence of particles through electron microscopy and presence of elemental silver via energy dispersive X-ray spectrum. Finally, antibacterial properties of the silver synthesized in our work will be evaluated using gram-negative bacteria cultures.

1.3.2. Effect of templating on silver synthesis

We noted from literature, that silver nanoparticles synthesized using silver-binding peptides were 5 – 50 nm in size, with shapes ranging from spheres to hexagonal [12, 13]. In contrast, studies where identical silver-binding peptides were immobilized onto 2-D substrates produced mostly spherical silver nanoparticles, with sizes ranging from 100 – 200 nm [14] and in another report, 1 – 4 μm particles were observed [7]. The striking differences in shape and sizes obtained due to the presentation of silver-binding peptides led us to wonder if templating indeed played a role in determining the overall shape and size of the silver nanoparticles.

To better understand the effect of templating (i.e., 2-D vs 3-D) on the shape and size of the nucleation of silver, we propose to prepare two forms of GPG-AG3 templates. We will use GPG-AG3 self-assembled aggregates as 3-D templates and chemically crosslinked GPG-AG3 thin films as 2-D substrates. The GPG-AG3 proteins will be crosslinked by reacting the NHS esters of the DSS crosslinker with the primary amines found on the lysine residues within the GPG-AG3 protein backbone (Fig. 1). The size and shape of the silver particles prepared on both templates will be characterized.

Separately, parameters of the synthesis conditions (NaCl concentration and pH) will be varied to examine their effects on the resulting morphologies of the silver nanoparticles formed on GPG-AG3 thin films. We will also study the relationships between silver particle morphologies and their antibacterial capabilities using gram-negative bacteria. Finally, we will also evaluate the cytotoxicity of the silver-bound GPG-AG3 thin films using mammalian cell culture *in vitro*.

1.4. Novelty

In this work, we proposed a novel synthesis strategy to produce silver-based biomaterials. Specifically, we reported a novel elastin-like silver-binding fusion protein (GPG-AG3) with the ability to nucleate silver in physiological conditions. In addition, we showed the feasibility of using fusion proteins to fabricate silver-containing thin film scaffolds with antibacterial properties. Taken together, outcomes of this work will lead to the development of novel silver-based biomaterials with potential uses in antibacterial wound dressings and biomedical implant coatings.

We examined the effect of templating (2-D vs. 3-D) on the biomimetic synthesis of silver. Silver particle size and morphologies prepared using both types of GPG-AG3 templates were characterized. In addition, the role of NaCl and pH on the synthesis of silver using GPG-AG3 thin films were also examined. A systematic correlation between the silver nanoparticle morphologies and their resultant antibacterial properties was performed. Our findings provided insights on the critical process parameters for the general biomimetic fabrication of silver-based materials.

2. LITERATURE REVIEW

2.1. Tropoelastin

Tropoelastin is the precursor of elastin consisting of alternating hydrophobic and hydrophilic domains (Fig. 3). The hydrophobic domains are highly repetitive motifs; most of residues are non-polar Proline, Valine and Glycine. The elastic properties of elastin are determined by these hydrophobic domains [15].

On the other hand, hydrophilic domains have high content of Lysine and Alanine residues, which are used for cross-linking, giving rise to the overall stability and insolubility of the elastin protein [16].

Figure 3: Map of human tropoelastin domains [17].

2.1.1. Proline-rich domains

The hydrophobic, or Proline-rich domains, are encoded by exons 2, 9, 16, 18, 20, 22, 24, 26, and 33 in the tropoelastin gene of human. The high content of Proline generally contributes to the rigid structure of the hydrophobic domains. However, these domains have the ability to self-assemble into higher order structures (coacervation). Coacervation is an essential step for the maturation of tropoelastin to form elastin, and is strongly dependent on

the content of hydrophobic residues and on their relative positions in the tropoelastin molecule [18].

The coacervation of tropoelastin occurs reversibly at a phase transition temperature, known as lower critical solubility temperature (LCST). At temperatures below the LCST, the tropoelastin chains form random coils in aqueous solution via hydrophilic hydration. Contrary, at temperatures above the LCST, tropoelastin chains adopt conformations to exclude water molecules. The phase transition temperature depends on factors such as concentration of NaCl or pH of solution [10]. Other soluble elastin derivatives such as α -elastin and elastin-derived polypeptides also exhibit similar phase transition behaviors.

The intrinsic coacervation behavior of the hydrophobic domains of tropoelastin has been exploited to create novel materials. For instance, Reguera *et al.* developed elastin-like polymers $[(VPGVG)_2(VPGEG)(VPGVG)_2]_{15}$ with the ability to undergo coacervation. In their work, they showed that nanoporous films could be obtained by controlling the pH of the surrounding medium (Fig. 4) [19].

Figure 4: Tropoelastin-like materials exhibiting coacervation. (A) Schematic showing the aggregation of elastin-like polymers in response to pH stimuli. (B) AFM image of resulting nanoporous film. [19]

2.1.2. Glycine-rich domains

On the other hand, the hydrophobic Glycine-rich domains are derived from exons 3, 5, 7, 11, 28 and 30 in human tropoelastin gene. Unlike the Proline-rich hydrophobic domains, the Glycine-rich domains possess high flexibility due to the rich content of Glycines. As a result, the Glycine-rich domains have the ability to self-assemble into amyloid-like fibrils [18].

When Proline residues in the hydrophobic domains were mutated to Glycines, extended β -sheet conformations resulting in the formation of fibrils were observed. Hence, the propensity of amyloid fibers formation is largely dominated by the ability of an elastin polypeptide sequence to adopt the cross- β structure [18].

The amyloid-forming abilities of the Glycine-rich domains have also been utilized in the design of novel hydrogels in tissue engineering. In particular, Bochicchio and coworkers have designed the exon 28 pentapeptide XGGZG motifs (X, Z: Val, Leu) that have the ability to self-assemble into amyloid fibers in solution (Fig. 5) [17].

Figure 5: Amyloid-forming exon 28 pentapeptides. (A) A possible amyloid formation pathway. (B) A coexistence of very long polymorphic fibers in ordered aggregates [20]

2.2. Elastin-like proteins (ELPs)

Elastin is a type of extracellular matrix (ECM) protein, found commonly in connective tissues, such as skin, lung, heart and blood vessels. Elastin provides elasticity and resilience to tissues, allowing them to stretch and deform [21]. Elastin also binds fibrillin, a large glycoprotein, to form networks of elastic fibers in the ECM. Elastin and elastin-like proteins (ELPs) have been shown to promote cell proliferation and cell adhesion [22]. Due to their mechanical properties, elastin is widely used in drug delivery and tissue engineering in combination with growth factors for wound dressings or dermal substitutes applications [23].

2.3. Elastin co-block polypeptides

Elastin-like recombinant proteins have been developed, and have been shown to possess mechanical properties similar to that of native elastin [15]. Both hydrophobic and hydrophilic domains confer native tropoelastin the unique ability to undergo self-assembly under physiological conditions [24]. Using recombinant methods, Sugawara and coworkers successfully constructed novel elastin-derived “double-hydrophobic” block polypeptides, which comprised of both Proline-rich and Glycine-rich domains (abbreviated **GPG**, Fig. 6). [25]

Figure 6: Schematic of the elastin-derived co-block polypeptides GPG. The Glycine-rich domains are denoted in green and Proline-rich domains are denoted in orange.

In their work, an assembly model of GPG protein into nanofibers was proposed (Fig. 7). In aqueous environment, disordered GPG proteins were dehydrated and converted to β -turn structures upon heating. Through hydrophobic interaction of domains, the β -turn structures assembled into particles and interdigitated to achieve the most thermodynamically stable β -sheets. These structures subsequently assembled together to

form fibers. Images (Fig. 8) showed that fibers had lengths of more than 10 μm and were about 20 – 70 nm in diameters. [25].

Figure 7: Proposed model of the assembly of GPG nanofibers. [25]

Figure 8: Structures of GPG fibers after 7 days at 45°C. (A) AFM image, (B) SEM image of a freeze-dried sample [25]

2.4. Elastins as biomaterials

Elastins have been widely explored in the field of drug delivery and tissue engineering. Elastins have also been used in combination with other natural extracellular matrix (ECM) molecules such as collagens to fabricate a variety of hybrid biomaterials [24]. In addition, elastins elicit a variety of cellular behavior such as enhanced chemotaxis, cell proliferation and up-regulation of collagenase in cultured fibroblasts [23]. As a result, elastins are also widely studied for their role in dermal remodeling.

The properties of elastin-like recombinant proteins can be varied by controlling the amino acid sequence and peptide length. They can also be designed to influence cellular behavior by incorporating bioactive sequences derived from extracellular matrix [23]. As a result, elastin-like recombinant proteins have been found interesting applications in drug delivery, scaffolds or surface coatings [24] .

Three-dimensional scaffolds derived from tropoelastin, soluble elastin and elastin peptides have received considerable attention since they mimic the physical and biological properties of native elastin. There are existing methods to fabricate scaffolds out of elastin-based recombinant proteins such as electrospinning [26] or chemical crosslinking [27].

2.5. DSS cross-linking

Currently, many homobifunctional crosslinkers are available commercially based to meet the demand for crosslinking applications. To crosslink proteins, bifunctional crosslinkers are often used. These compounds contain two reactive functional groups at the ends of a variable carbon chain spacer. These functional groups can react with the primary amines found on lysines and arginines, carboxyl groups found on glutamic and aspartic acids, or thiol groups found on cysteines. In this work, a bifunctional crosslinker, disuccinimidyl suberate (DSS) will be used to crosslink the GPG-AG3 protein. Specifically, the activated esters located on both ends of the DSS molecule will react with the amines located on the lysines present on our GPG-AG3 protein backbone via the mechanism shown in Figure 9. The hydrocarbon spacer is non-cleavable creating irreversible formed cross-links [28].

Figure 9: Formation of amide bond cross-links using DSS [28].

2.6. Silver as antibacterial agents

Silver is one of the most studied metal due to its excellent electrical conductivity [29], optical properties [30] and wide-spectrum antimicrobial effects [31]. For several decades, silver had been demonstrated to have antimicrobial properties against gram-negative and gram-positive bacteria, as well as viruses, protozoa, fungi and antibiotic resistant strains [14]. As a result, silver has been widely incorporated in a wide variety of applications, including biosensors, coatings for biomedical implants, wound dressings and coatings in food packaging [32]. However, the mechanisms by which silver interacts with bacteria are still being elucidated. However, it is widely accepted that disruption of bacteria cell membrane occurred when the cell cytoplasm interacts with silver ions. Others have shown that interaction of silver ions with the ribosome halts the enzymes and proteins expression essential to ATP production [33].

Silver nanoparticles (AgNPs) are widely explored as antibacterial agents due to their ability to penetrate the bacteria cell wall more effectively. Currently, AgNPs are synthesized via the chemical reduction of silver ion precursors in the presence of additives. These additives include polymers and surfactants, which are essential for the initial nucleation, stabilization and growth of AgNPs. Recently, peptide-based strategies have also been

explored for the synthesis of silver due to their intrinsic solubility in aqueous media and increased biocompatibility [34].

2.7. Biomimetic synthesis of silver

In nature, proteins often serve as intrinsic scaffolds for the nucleation and growth of functional inorganic materials. Stone and colleagues first discovered silver-binding peptides using combinatorial phage display and showed that these peptides have the ability to mineralize silver in aqueous solution [12]. Specifically, several silver-binding domains were reported (Table 1). The overall charge of AG5 peptide (9.47) is the highest compared to that of AG3 and AG4. It was also proposed, that presence of Arginine (R) and Methionine (M) contributed to the recognition and reduction Ag^+ to Ag^0 [35]. In contrast, single amino acids or peptides consisting only of hydroxyl-containing amino acid residues (e.g., Serine (S) and Tyrosines (Y)) as well as Arginine (R) or Proline (P) were unable to precipitate silver particles [12].

Table 1 Amino acid sequences and properties of the silver-selected peptides.

		Isoelectric pH(pl) ^a
AG3	A Y S S G A P P M P P F	5.57
AG4	N P S S L F R Y L P S D	6.09
AG5	S L A T Q P P R T P P V	9.47
	● ● ● ★ ●	

^a pl calculated using pl/Mass program at www.expasy.ch

■ Amino acids with functional side groups.

★ Amino acids conserved in all three sequences.

● Amino acids conserved in two of the sequences.

Table 1: Amino acid sequences of silver-binding peptides [12].

They further proposed a silver nucleation mechanism via interaction of silver-binding peptides with silver nanoclusters or silver nuclei which were preformed in aqueous silver nitrate solution (Fig.10). Subsequent silver nanoparticles synthesis was followed by formation of chemically reducing environment at the interface between metal and peptides [12].

Figure 10: Proposed mechanism for the formation of silver particles via interaction of silver-binding peptides with silver cluster in aqueous silver nitrate solution. [12]

Silver nanoparticles exhibit unique optical properties, which can be detected via surface plasmon resonance (SPR). Silver absorbs light, typically at the wavelengths of 400 – 420 nm. However, the intensity of the light absorption peak is strongly dependent on shape, size and aggregation of particles [36-38]. In addition, the absorption wavelengths of the silver nanoparticles can also be tuned over a broad range wavelengths in the visible spectrum [39].

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

The plasmid pET22b-GPG encoding the elastin-like GPG sequence (pET22b-GPG) was a generous gift from Sugawara-Narutaki at the University of Tokyo, Japan. Forward and reverse primers containing the AG3 sequence (AYSSGAPPMPPF) strands with flanking XhoI and SalI restriction sites were purchased from 1st Base Asia, Singapore (see Appendix A). *Escherichia coli* (*E. coli*) bacteria DH5 α and BL21(DE3) strains were purchased from Invitrogen (Carlsbad, CA). Reagents used for cloning including restriction enzymes, T4 DNA ligase and DNA ladders were purchased from New England Biolabs Inc. (Ipswich, MA). Plasmid DNA purification kit was obtained from Qiagen (Valencia, CA). Gel DNA Recovery Kit was obtained from Zymo Research (Irvine, CA). Reagents used for cell culture were purchased from BD Biosciences (San Jose, CA). Nickel-nitrilotriacetate (Ni-NTA) was obtained from Qiagen. Urea and chemicals used in purify protein were from Merck (Germany). Chemicals used for mammalian cell experiments were purchased from Invitrogen (Gibco). Cross-linker DSS and organic solvent DMSO were purchased from Sigma (St Louis, MO). Round 12 mm glass cover slides were from Hampton Research (Aliso Viejo, CA).

3.2. Construction of plasmid containing GPG-AG3

Double-stranded AG3 DNA fragment was constructed by DNA annealing. Briefly, both forward and reverse AG3 oligonucleotides were dissolved in water containing 10 mM Tris, 100 mM NaCl and 100 mM MgCl₂ (pH 8). The solution was heated to 100°C for 5 min and cooled overnight at rate of 1°C/min to room temperature. The annealed oligonucleotides AG3 were digested with XhoI and SalI for 3 h at 37°C, ran on 1.5% DNA agarose gel and subsequently recovered using Zymo Gel DNA Recovery Kit. The digested AG3 DNA was then ligated into the plasmid pET22b-GPG using XhoI site. The ligation mixture was transformed into DH5 α *E. coli* strain, and positive colonies were screened and verified by

sequencing. Figure 11 illustrates the overall cloning scheme in the construction of the plasmid containing GPG-AG3.

The verified pET22b-GPG-AG3 plasmid was finally transformed into *E. coli* strain BL21(DE3) cells using heat shock.

Figure 11: Cloning scheme of pET22b-GPG-AG3 plasmid.

3.3. Expression and purification of GPG-AG3

Starter cultures of BL21(DE3) cells containing the pET22b-GPG-AG3 plasmids were inoculated in 1 L TB (Terrific broth) containing 50 µg/mL ampicillin and grown overnight. The next day, cells were harvested and centrifuged at 8,000 rpm for 15 min at 4°C. The cell pellet was resuspended in denaturing lysis buffer (8 M urea, 100 mM sodium phosphate monobasic, 20 mM Tris-Cl, 250 mM NaCl, 10 mM Imidazole, pH 8.0) at 10 mL/g wet cell mass and sonicated (40% output for 5 x 3 second pulses) for 10 min on ice. The solution was then centrifuged at 18,000 rpm for 1 h and the supernatant was mixed with Nickel-nitrilotriacetate (Ni-NTA) slurry for 1 h at room temperature. The mixture was then loaded onto a Ni-NTA column, and the Ni-NTA slurry was washed three times with Wash buffer 1 (8 M urea, 100 mM sodium phosphate monobasic, 20 mM Tris-Cl, 500 mM NaCl, 20 mM Imidazole, pH 7.5) and three times with Wash buffer 2 (8 M urea, 100 mM sodium phosphate monobasic, 20 mM Tris-Cl, 1M NaCl, 20 mM Imidazole, pH 7.0). Bound proteins were eluted 2 times using Elution buffer 1 (8M urea, 100 mM sodium phosphate

monobasic, 20 mM Tris-Cl, 150 mM NaCl, 250 mM Imidazole, pH 6.5) and Elution buffer 2 (8 M urea, 100 mM sodium phosphate monobasic, 20 mM Tris-Cl, 150 mM NaCl, 250 mM Imidazole, pH 4.5) consecutively. Eluted proteins were dialyzed using dialysis membrane with 8 kDa MWCO against deionized water (pH 5.5) to remove urea and subsequently lyophilized. 20 μ L sample of each step was stored for verifying on SDS-PAGE electrophoresis (6% for the stacking gel and 15% for the resolving gel).

MALDI-TOF-MS

2 μ L eluted GPG-AG3 proteins was mixed with matrix solution α -Cyano-4-hydroxycinnamic acid in 60% Acetonitrile at ratio 1:1. The mixture was spotted on 384-Well Insert MALDI target plate. The spectrum was acquired in MS linear Mid Mass Positive mode at 20 kV, with 2000 shots per spectrum on MALDI-TOF-MS 4800 Plus (AB Sciex, Framingham, MA).

3.4. Characterization of GPG-AG3 proteins

3.4.1. UV-VIS spectroscopy

Determination of lower critical transition temperature (LCST)

The lower critical transition temperature (LCST) of GPG-AG3 was determined using UV-VIS spectroscopy. First, lyophilized GPG-AG3 protein was left to entirely dissolve in deionized water at 4°C and 10X PBS (4°C) was added to final concentration of 20 μ M protein in 1X PBS. The protein solution was pipetted into 1-cm quartz cell and placed in Peltier ETCR-762 thermostatted single cell holder. Samples were heated at 0.1°C/min and their optical transmittances at 350 nm were monitored using a Jasco V-670 UV-VIS spectrophotometer, referenced against PBS. The LCST was determined as the temperature at which a 10% reduction in the original transmittance of the solution was observed [40].

3.4.2. Preparation of spin-coated GPG-AG3 films

Round 12 mm cover slides were soaked in mixture of NaOH and Ethanol overnight and cleaned in ultrasonic bath for 30 min. Cover slides were rinsed with water and dried in air. Lyophilized GPG-AG3 protein was dissolved in DMSO (5 wt%) for 1 h. Disuccinimidyl

suberate (DSS; 1 mg) was dissolved in 10 μL ddH₂O and added to 90 μL of protein solution. The stoichiometric ratio of activated esters to lysines (K) is roughly 1:1. A 10 μL volume of DSS-protein solution was then pipetted onto a glass cover slide and spun at 5,000 rpm for 30 sec at RT. Spin-coated films were stored overnight at RT to allow thorough crosslinking. The films were then rinsed in water for 30 min (5 washes) to remove the excess DMSO. Rinsed films were dried in air before use.

3.5. Preparation of silver nanoparticles

3.5.1. Biomimetic synthesis of silver using GPG-AG3

Lyophilized GPG-AG3 protein was dissolved in cold ddH₂O and adjusted with 10x PBS to yield a final protein concentration of 100 μM in 1x PBS. Silver nitrate was subsequently added to the protein mixture to achieve a final concentration of 0.2 mM AgNO₃. The mixture was shaken at room temperature for up to 6 days. Samples were collected using centrifugation and washed twice with 10% SDS and then 5 times with ddH₂O. Since silver nanoparticles possess size-dependent characteristic surface plasmon, the presence of silver precipitation in silver nitrate solution was confirmed using UV-VIS Spectrometer 2501PC (at wavelengths between 300 nm – 900 nm) [37].

3.5.2. Biomimetic synthesis of silver nanoparticles on GPG-AG3 films

GPG-AG3 films were incubated in 1x PBS containing 1 mM AgNO₃ and in 1x Phosphate buffer (pH 7.2) containing 1 mM AgNO₃ and 2 mM NaCl at room temperature in 3 days. Films were then washed vigorously in ddH₂O to remove contaminants such as NaCl and AgCl.

3.6. Silver particles synthesis in various conditions

Varying NaCl concentration

GPG-AG3 thin films were incubated in PBS containing 1 mM AgNO₃, Phosphate buffer pH 7 and varying amounts of NaCl (0, 0.1, 0.5, 1 and 5 mM).

Varying pH solution

Spin-coated GPG-AG3 thin films were incubated in Phosphate buffer containing 1 mM AgNO₃, 1 mM NaCl and adjusted to various pH values (5, 6, 7, 8 and 9).

3.7. Electron microscopy

Field emission scanning electron microscopy (FESEM)

Samples were dropped onto carbon tape and left to dry at room temperature. Samples were visualized using a Field Emission Scanning Microscopy (JOEL, FESEM, 6340F), using 5 kV accelerating voltage and 12 mA emission current. The particle size was quantified by manually tracing the diameters of each particle using ImageJ (v1.47, NIH).

Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM, JEM-2010), 5 μ L of 20 mg synthesized AgNPs/mL were dropped onto copper grids with carbon film and dried at room temperature. TEM images and diffraction pattern were visualized using an emission current 105 mA and an accelerating voltage of 200 kV. No stain method was used in TEM sample preparation.

3.8. Antibacterial assays

*Construction of *E. coli* growth curve*

Bacteria *E. coli* BL21(DE3) was grown in 2xYT medium. The growth of *E. coli* was monitored over time by measuring the optical density (OD_{600nm}) of the culture at 30 min intervals. At each time point, 100 μ L of the bacteria culture was diluted (10^{-3} , 10^{-4} or 10^{-5}), plated on 2xYT agar plates and followed by overnight incubation at 37°C. The number of colonies formed was counted on the next day. The logarithm of the number of colonies formed was plotted as a function of OD_{600nm}, and the data was subjected to a linear fit.

2xYT agar plates

Approximately 10^7 colony forming unit (CFU) of gram-negative *E. coli* bacteria were subsequently cultured on 2xYT agar plates pre-coated with the synthesized silver nanoparticles (0 – 100 μ g/mL), and incubated overnight at 37°C. The antibacterial properties

of the silver nanoparticles were examined by counting the amount of colonies formed on each plate.

Separately, the silver-coated GPG-AG3 films were placed in direct contact with 2xYT agar plate surfaces that were pre-coated with 10^7 *E. coli*.

Liquid 2xYT media

E. coli bacterial culture was grown to log phase (i.e., optical density OD_{600nm} 0.6 – 0.8) and subsequently re-inoculated at 1:100 dilution into 2xYT media supplemented with varying concentrations of synthesized silver nanoparticles.

Likewise, 3 mL *E. coli* bacterial culture in 2xYT was prepared as described above. Silver-coated GPG-AG3 films were added to the media with shaking at 37°C. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm at regular time intervals for up to 24 h.

3.9. MTS assay

The cytotoxicity of silver coated GPG-AG3 thin films were evaluated *in vitro* using mammalian cell line, L929 mouse fibroblast cells. The CellTiter96® Aqueous Non-Radioactive Cell proliferation Assay MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was performed [41]. The fibroblasts (passage 13) were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-Glutamine, 1% sodium pyruvate and 1% non-essential amino acids without the presence of antibiotics at 37°C, 5.0% CO₂ in a humidified incubator. After 2 to 3 days, 10^3 cells (cells were subjected to Trypan blue staining and counted using a hemacytometer) were seeded in 24-well plate containing GPG-AG3 thin films and silver-coated GPG-AG3 films respectively. Both films were incubated at 37°C, 5.0% CO₂ for up to 7 days. At various time points, cells were rinsed in PBS before replacing medium with a mixture of media and MTS solution (at ratio 5:1) and further incubated for 2 hours. The absorbance at 490 nm was then recorded using a microplate reader

(TECAN Infinite M200). The absorbance data for each sample was normalized to control (tissue culture polystyrene) and reported as percent viable cells.

3.10. Statistical analysis

For all experimental data, results were expressed as means $\pm$ SD of three independent experiments. The statistical significance of differences was determined using student's t-test. Differences were taken to be statistically significant at $P \leq 0.05$.

4. RESULTS AND DISCUSSION

4.1. Construction of pET22b-GPG-AG3

The plasmid encoding the GPG-AG3 fusion protein (i.e., pET22b-GPG-AG3) was successfully constructed using the cloning scheme outlined in Figure 11. To confirm the presence of the GPG-AG3 gene, double digestion of the plasmid using XhoI and NdeI restriction enzymes was performed. A digested product with molecular weight of ~ 600 bp was obtained, corresponding to the expected molecular weight of the GPG-AG3 gene (Fig. 12; red boxed). The amino acid sequence of GPG-AG3 can be found in Appendix B.

Figure 12: DNA gel showing digested products of pET22b-GPG-AG3. Double digestion of the pET22b-GPG-AG3 plasmid with XhoI and NdeI yielded a band of ~ 600 bp (boxed; lane 3).

4.2. Expression and purification of GPG-AG3 protein

The purity and molecular weight of purified GPG-AG3 fusion protein was confirmed by SDS-PAGE electrophoresis and MALDI-TOF/TOF MS respectively (Figs. 13A – B). The typical lyophilized protein yield was 40 mg/L.

Figure 13: Verification of GPG-AG3 purity and molecular weight using SDS-PAGE and MALDI-TOF. (A) 15% SDS-PAGE gel confirming the expression and purification of GPG-AG3 proteins (red box); Lane M: Molecular weight marker; Lane 1: cell lysate after 24 hours autoinduction; Lane 2: Flow through; Lane 3: First wash; Lane 4: Second wash; Lane 5 – 8: elution fractions. (B) MALDI spectra of 100 μ M purified GPG-AG3 protein dissolved in water. The theoretical molecular weight of GPG-AG3 was calculated to be 18.2 kDa.

The presence of a band between 15 kDa and 25 kDa (Fig. 13A; enclosed by red box) indicated that GPG-AG3. The His-tagged GPG-AG3 proteins were mostly bound to the column since a large population of non-specific proteins was eliminated in the flow through (lane 2). Washing the sample with wash buffers at pH 7.5 (1st wash, lane 3) and at pH 7.0 (2nd wash, lane 4) were able to successfully remove most of the background proteins. Most of the GPG-AG3 proteins were eluted in the first elution buffer at pH 6.5 (Lanes 5 – 6), while the remainder of GPG-AG3 proteins were recovered from the second elution at pH 4.5 (Lanes 7 – 8).

A sample solution of GPG-AG3 proteins, combining all the eluates, were analyzed using MALDI-TOF/TOF-MS. Figure 13B shows the MALDI-TOF spectra containing a peak with the highest intensity at the molecular weight of about 18.2 kDa, corresponding to theoretical molecular weight of GPG-AG3. From the peak intensity, it was calculated that the sample was about 95% pure. The protein purity was sufficient for subsequent experiments.

4.3. Characterization of GPG-AG3

To understand the thermoresponsive behavior of GPG-AG3 in solution, we first determined the lower critical solubility temperature (LCST) of the protein using UV-VIS spectroscopy. Figure 14A shows the light absorption ($\lambda = 350$ nm) of GPG-AG3 in PBS (pH 7.4) with increasing temperature. The LCST was found to be $\sim 15^{\circ}\text{C}$, suggesting that self-assembly of GPG-AG3 proteins is likely to occur at room temperature.

The presence of GPG-AG3 protein aggregates was verified using FESEM. Figures 14B – C are FESEM images of GPG-AG3 protein aggregates self-assembled in ddH₂O and PBS (pH 7.4) at room temperature respectively. The average diameter of the protein aggregates in both cases was found to be about 100 nm.

Figure 14: Characterization of GPG-AG3 using UV-VIS and FESEM. (A) Light absorbance ($\lambda = 350$ nm) of GPG-AG3 in PBS (pH 7.4) with increasing temperature. (B – C) FESEM images of GPG-AG3 protein aggregates in (B) ddH₂O and (C) PBS (pH 7.4) at room temperature.

4.4. Preparation of GPG-AG3 thin films

To see if silver particles could be synthesized on a 2-D substrate, the GPG-AG3 thin films were prepared. The GPG-AG3 proteins were dissolved in DMSO and crosslinked with DSS as previously described. The protein solution was spin-coated onto glass cover slides. Figure 15 shows an image of a crosslinked GPG-AG3 thin film obtained by spin-coating. The GPG-AG3 thin films were insoluble in ddH₂O, PBS or DMEM solution for up to 3 days with shaking.

Figure 15: Image of crosslinked GPG-AG3 thin film spun-coated on round 12mm glass cover slides.

4.5. Silver precipitation on GPG-AG3 in solution

Figure 16: UV spectroscopy at day 3 for various samples. A characteristic surface plasmon resonance peak at 420 nm indicates the presence of silver particles.

The ability of GPG-AG3 to nucleate silver biomimetically was examined using UV-VIS spectroscopy. Figure 16 shows the UV-VIS spectra of samples prepared using various conditions. A characteristic surface plasmon resonance peak at 420 nm was observed in the presence of 100 μ M GPG-AG3 in PBS (pH 7.4, containing 137 mM NaCl) after 3 days (blue curve, Fig. 16). In comparison, no such peaks were observed in our controls. We, however,

noted a peak at 420 nm for the control protein GPG, but the peak intensity was 3-folds lower (red curve, Fig. 16). This is likely due to the non-specific binding of silver ions to the Histidine (H) residues at the C-terminus of GPG [42]. Taken together, our data suggests that the AG3 silver-binding domain is responsible for the increased nucleation of silver.

We also noted that silver nucleation on GPG-AG3 only occurred in the presence of NaCl (no absorbance peaks were observed for green and pink curves; Fig. 16). This observation was consistent with reported literature, where chloride ions have been thought to play an important role to provide electrostatic stability to silver nanocrystallites nucleated on the AG3 peptides [43].

Figure 17: Intensities of the absorption peak at 420 nm over time for 100 μ M GPG-AG3 in 0.2 mM AgNO_3 and PBS. Error bars represent means $\pm$ SD. *, significant difference than Day 1.

In the presence of 100 μ M GPG-AG3, 0.2 mM AgNO_3 and PBS, the characteristic peak of silver at 420 nm could be detected as early as Day 1 using UV-VIS spectroscopy. The amount of silver formed was the highest after Day 3, and the peak intensities reached a plateau after 3 days (Fig. 17).

While the main mechanism of silver nanoparticles synthesis using protein templates has not been elucidated, it is possible that silver ions were reduced by the exposed AG3

domains present on the surface of GPG-AG3 proteins [12]. According to the self-assembly model proposed by Sugawara-Narutaki and coworkers, the GPG domains within the GPG-AG3 proteins undergo self-assembly as a result of their hydrophobicity. As a result, the hydrophilic AG3 sequence and His-tag were displayed on the surface of the self-assembled GPG-AG3 aggregates [25]. Hence, the AG3 domains interact with the silver ions, silver crystals were able to nucleate and grow over time. The growth of silver crystals over time contributes to the steady increase in the intensity of the silver surface plasmon peak (at 420 nm) from Day 1 to 3 (see Fig. 17).

4.6. TEM characterization of silver nanoparticles

To characterize the silver nanoparticles synthesized on GPG-AG3, we subjected samples to TEM analysis. Figure 18 shows a TEM image of AgNPs nucleated on the GPG-AG3 aggregates after Day 3. The dark spots are the silver nanoparticles.

Figure 18: TEM image of silver nanoparticles nucleated on GPG-AG3 after 3 days.

To visualize the silver nanoparticles alone, we removed the GPG-AG3 protein templates by thorough washing with 10% SDS, followed by ddH₂O. Figure 19A shows TEM image of AgNPs obtained after the removal of GPG-AG3. Multiply twinned particles were

observed (white arrows, Fig. 19A) confirmed the presence of metal crystal particles [44]. The diameters of the silver nanoparticles ranged from 20 – 25 nm (Fig. 19B).

Figure 19: TEM analysis of silver nucleated using GPG-AG3. (A) Image of silver nanoparticles obtained after the removal of GPG-AG3 proteins, white arrows: multiple twin particles. (B) Distribution of diameters of the silver nanoparticles. (C) TEM image of single particle. (D) High

resolution TEM image of red area in (C). (E) Selected area electron diffraction (SAED) patterns of silver nanoparticles. (F) EDX spectrum confirming the presence of elemental silver.

Figure 19D is an enlarged image of an area of the particle found in Fig. 19C. The selected area electron diffraction (SAED) patterns (Fig. 19E) were obtained by directing an incident electron beam perpendicular to the flat face of single particle. The hexagonal symmetry of the spot array characteristics correspond to the [111] beam orientation of silver particle. The high-resolution TEM image in Fig. 19D clearly shows the resolved lattice fringes of a crystal structure. The lattice spacing was determined to be about 2.30 Å, which corresponds to the [111] plane of face-centered cubic lattice on silver crystal [45]. Finally, the presence of elemental silver in silver nanoparticles samples was confirmed by energy-dispersive X-ray (EDX) spectrum (Fig. 19E). The copper, carbon, oxygen, silicon signals are from the copper sample grid used for TEM analysis.

Taken together, we confirmed that silver crystalline nanoparticles were indeed synthesized using GPG-AG3 nanoaggregates as templates.

4.7. Silver precipitation on crosslinked GPG-AG3 thin films

Crosslinked GPG-AG3 thin films were incubated in PBS solutions containing different NaCl concentrations to examine the effect of NaCl on silver nucleation. Films were incubated for 3 days and subsequently visualized using field emission scanning electron microscopy (FESEM) (Fig. 20). Figure 20A shows GPG-AG3 films incubated in PBS (pH 7.2) containing 1 mM AgNO₃ and 137 mM NaCl. Figure 20B shows GPG-AG3 films incubated in PBS (pH 7.2) containing 1 mM AgNO₃ and 2 mM NaCl.

Under both conditions, a brown coloration was observed in the GPG-AG3 films, which suggests that silver was present on the surfaces of the films. Unfortunately, we were unable to obtain reliable UV or visual light data due to the opacity of the films. From FESEM images shown in Figures 20A – B, we observed that the silver particles nucleated on the surfaces of GPG-AG3 thin films were mostly spherical.

Figure 20: Field emission scanning electron microscopy analysis of silver nucleated on GPG-AG3 film. (A) Image of silver particles formed on GPG-AG3 film incubated in PBS (pH 7.2) containing 1 mM AgNO_3 and 137 mM NaCl after 3 days. (B) Silver particles formed on GPG-AG3 film incubated in PBS (pH 7.2) containing 1 mM AgNO_3 and 2 mM NaCl after 3 days. (C – D) EDX spectra of silver particles in (A) and (B) respectively.

Different sizes of AgNPs ($869 \text{ nm} \pm 353 \text{ nm}$) and ($471 \text{ nm} \pm 178 \text{ nm}$) were obtained from solutions containing 137 mM and 2 mM of NaCl respectively. The presence of silver in both cases was confirmed via energy-dispersive x-ray spectroscopy analysis (Figs. 20C – D). The silicon and oxygen peaks were from glass coverslip and protein film, respectively. The GPG-AG3 film samples were washed vigorously in water to solubilize any AgCl that may be precipitated on the films since the differentiation of AgCl from silver particles would not be possible using microscopy. EDX spectra data indicated the presence of Cl^- in both samples.

Trace amount of Chloride contamination was also observed on the surface of silk-silver-binding protein films reported by Currie *et al.* [14].

The size of the silver nanoparticles increases with increasing concentrations of Cl^- ions present in the solution. This observation suggests that Cl^- ions were involved in the redox reaction of silver reduction as well as influencing the shape of the resulting silver nanoparticles. These observations are consistent with previous reports on role of chloride ions in silver particles synthesis [43, 46, 47]. For example, Xia *et al.* showed that the presence of chloride ions in the conventional polyol synthesis of AgNPs facilitated the etching and dissolution of Ag seeds, and instead formed monodispersed silver nanocubes. In contrast, in the absence of Cl^- , larger irregularly-shaped silver particles were obtained [43]. Similarly, Wiley *et al.* found that the oxidation and preferential etching process of twinned Ag nucleates, were accelerated by addition of NaCl amount and leaving the growth of single silver particles [46]. In addition, Xu *et al.* verified that Cl^- was able to act as an etchant to selectively sculpt the [110] facets of silver nanoprisms into planar disk-like shape [47].

Taken together the outcomes of experiments, a possible mechanism for the synthesis of silver nanoparticles in the presence of GPG-AG3 proteins is proposed (Figure 21).

Figure 21: Proposed schematic of silver nanoparticles growth mechanism on protein GPG-AG3 template.

The proposed mechanism where silver ions are reduced by peptides/proteins has yet to be validated to date. Gruen *et al.* suggested that at neutral pH, Methionine (M) which contains sulfur can interact with Ag^+ via silver/sulfur bond [35]. In addition, Blanc *et. al.* demonstrated that the peptides containing methionine was effective for reducing silver ions [48]. Hence, it is likely that the Met presents on our AG3 sequence was responsible for the recognition and reduction of silver ions observed in our work and that of Naik and coworkers [12].

Silver ions are assembled on the surface of the proteins, and were stabilized by the chloride ions through electrostatic interactions [46]. The AG3 domains further reduce silver ions to Ag^0 , forming silver nuclei seeds for subsequent growth of silver crystals over time. Silver chloride (AgCl) initially forms from the reaction of free Ag^+ and Cl^- ions in the solution. However, depletion of Ag^+ from the solution due to silver nucleation is able to result in the dissolution of the existing AgCl [49]. This process provides a constant reservoir of Ag^+ ions for subsequent growth of the silver nuclei to form multiply twin particles, which are typically formed in the early stage of silver synthesis [50].

4.8. Antibacterial assays

A standard growth curve for *E. coli* was constructed to allow us to calculate colony-forming unit (CFU) based on the $\text{OD}_{600\text{nm}}$ readings (Fig. 22).

Figure 22: Standard *E. coli* growth curve in 2xYT media. Data was subjected to linear fit.

We tested the bactericidal properties of the silver nanoparticles towards gram-negative *E. coli* bacteria. We cultured 10^7 CFU of *E. coli* bacteria on 2xYT agar plates pre-coated with varying concentrations of synthesized silver nanoparticles, and incubated overnight at 37°C. Figure 23A shows a comparative image of 2xYT agar plates after 24 h. No colonies could be observed on agar plates pre-coated with silver nanoparticles.

Next, *E. coli* bacteria were grown in 2xYT media containing varying concentrations of synthesized silver nanoparticles. Figure 23B shows the growth curves obtained for each concentration of silver tested. Bacterial growth was completely inhibited by 20 µg/ml of silver nanoparticles, compared to the control (i.e., regular bacterial culture). Likewise, when only GPG-AG3 proteins were presented, bacterial growth could be observed after 8 h. Consistent with our agar plate experiments, all cultures containing silver nanoparticles did not promote the growth of bacteria, confirming the antibacterial properties of the silver nanoparticles.

Figure 23: Bacterial growth in the presence of different AgNPs concentrations. Bacteria growth on (A) 2xYT agar plates and (B) 2xYT liquid media; both supplemented with varying concentrations of silver nanoparticles.

The overall antibacterial properties of the silver nanoparticles synthesized in our work were also found to be higher than that observed by others. For instance, silver nanoparticles synthesized chemically by Sondi and coworkers, with a modal diameter of 12 nm, were only able to completely inhibit bacterial growth at a concentration of 50 – 60 $\mu\text{g/mL}$ [51].

Similar antibacterial properties were also noted for the silver-coated GPG-AG3 thin films (Fig. 24). A bacterial inhibition zone could be clearly observed along the periphery of silver-coated GPG-AG3 thin film (black arrow, Fig. 24A). By contrast, bacterial growth could be found along the edges of the controls (i.e., glass coverslip and uncoated GPG-AG3 protein film). Similarly, *E. coli* bacterial growth was also inhibited in the presence of silver-coated GPG-AG3 thin films in 2xYT liquid media (Fig. 24B).

Figure 24: Bacterial growth in the presence of silver-coated GPG-AG3 thin films on 2xYT agar plates (A) and in 2xYT liquid media (B).

4.9. MTS assay

Mouse L929 fibroblasts were cultured on silver-coated GPG-AG3 thin films and controls of uncoated GPG-AG3 films. MTS assay was performed to evaluate viability of cells on these surfaces after 3 and 7 days. Figure 25 shows that silver-coated GPG-AG3 thin films supported cell growth up to 3 days, comparable to our controls. However, the

percentage of viable cells was dramatically reduced on silver-coated GPG-AG3 surfaces (AgNPs film) by day 7, suggesting that there might be silver-associated cytotoxicity effects.

Figure 25: L929 mouse fibroblast viability on GPG-AG3 thin films and silver-coated GPG-AG3 thin films over time. Data reported was normalized to control. A total of three independent experiments were performed.

Although there were some studies confirming the biocompatibility of nanosilver-based dressing [52, 53], their studies were limited to cytotoxicity data obtained in a short period of time (e.g., 1 day). On the other hand, researchers also have found that silver-based materials were toxic when silver ions contacted directly to mammalian cells [54-56]. Our cell culture data implies that further work is needed to reduce the toxicity of our silver-coated GPG-AG3 films while maintaining their antibacterial effects.

4.10. Evaluation of silver particles synthesis in different conditions

4.10.1. Effect of varying NaCl concentrations

Consistent with literature, chloride ions (Cl^-) were suggested to play a role in controlling the size and the shape of AgNPs nucleated by silver-binding peptides. To

investigate the role of Cl^- , protein films were incubated in Phosphate buffer (pH 7) containing 1 mM AgNO_3 with varying NaCl concentrations. Figure 26 shows the respective FESEM images of silver particles obtained in the presence of increasing NaCl concentrations.

Figure 26: FESEM images of AgNPs synthesized in medium containing PBS pH 7, 1 mM AgNO_3 and varying NaCl concentrations. (A) 0 mM NaCl, (B) 0.1mM NaCl, (C) 0.5mM NaCl, (D) 1mM NaCl and (E) 5mM NaCl; (F) EDX spectra of AgNPs in samples (B, C, D and E).

Square, long, irregular structures were found on GPG-AG3 thin film when film was incubated in medium without NaCl (Fig. 26A). EDX spectra confirmed that these irregular structures were indeed silver. In comparison, smaller particles (100 – 200 nm, Fig. 27) were observed in the presence of 0.1, 0.5 and 1 mM NaCl (Fig. 26B, C and D). With 5 mM NaCl, large spherical particles were found (Fig. 26E) with diameters of about 1 μm (Fig. 27, sample E). Figure 26F shows a representative EDX spectrum of particles in samples B, C, D and E, confirming the presence of elemental silver.

Figure 27: Average diameters of AgNPs nucleated on GPG-AG3 thin films with various NaCl concentrations. N = average size of silver nanoparticles. Error bars represent means $\pm$ SD. *, significant difference than E sample at $P < 0.05$.

Silver nanoparticles obtained using AG3 silver binding peptides in solution were hexagonal, spherical and triangular shape [12]. As discussed previously in Section 4.7, Cl^- ions behave as etchants to sculpt the shape of the silver particles. In a separate work by An *et al.*, a mechanism for the interaction of chloride ions with the silver particles [47] was proposed. Figure 28 illustrates the manner in which Cl^- ions interact with silver particles. The Cl^- ions surround the silver particles, dissociating the silver to release silver atoms. The dissociation process continues until the corners of silver were completely dissolved, resulting in a spherical morphology. The dissociated silver atoms aggregate in solution and subsequently formed nanoclusters, which are stabilized by chloride ions. The stabilized silver

clusters are then re-deposited on or around the [111] facets of the original silver particles. As a result, the thickness of the original silver particle increased. This mechanism in part, allowed us to account for spherical AgNPs observed on GPG-AG3 proteins.

Figure 28: A schematic process illustrating how chloride ions affect the shape of nucleated silver particles in solution. [47]

At higher Cl^- concentrations, a higher number of silver nanoclusters formed from dissociated silver atoms can be stabilized by the higher Cl^- concentration. This in turn will lead to a higher number of stabilized silver clusters. More of these silver clusters can re-deposit onto the surface of silver nanoparticles, leading to an overall increase in silver particle size. This explanation would also account for the larger mean particle size of the silver particles obtained in the case of 5 mM NaCl (~ 901 nm) compared to the rest of the samples (Fig. 26). The silver particle size at 5 mM NaCl was comparable to that at 137 mM NaCl (around 1 μm in diameter see Fig. 20A). This suggests that the effect of Cl^- ions on particle size could have saturated at 5 mM NaCl, where subsequent increase in NaCl concentrations had no further effect on particle size.

Since Cl^- ions were involved in shaping the nucleated silver nanoparticles, it is reasonable to observe randomly shaped silver particles in the absence of Cl^- (Fig. 26A).

4.10.2. Effect of varying pH

Figure 29: FESEM images of AgNPs synthesized in medium containing 1 mM AgNO_3 , 1 mM NaCl and Phosphate buffer with varying pH. (A) pH = 5, (B) pH = 6, (C) pH = 7, (D) pH = 8 and (E) pH = 9; (F) EDX spectra of AgNPs in samples (A, B, C, D and E)

The pH of the incubation media was also varied while keeping other parameters unchanged (i.e., 1 mM AgNO₃, 1 mM NaCl). In all cases, silver nanoparticles were spherical in shape (Figs. 29A – E) and were similarly in size (Fig. 30). EDX spectra confirmed the presence of silver particles in samples (Fig. 29F). The O and C peaks were from protein film while Si, Pt, Ca, Na and Mg peaks were from glass cover-slide and SEM coating material, respectively. Interestingly, the agglomeration of AgNPs was reduced at higher pH (Figs. 29A – E). At higher pH values, the deprotonation of film acts as a barrier for agglomeration of AgNPs. As a result, the distance between each AgNP increased [57].

Figure 30: Size of synthesized AgNPs in incubation media at various pH. N = average size of silver nanoparticles. Error bars represent means $\pm$ SD.

4.10.3. Antibacterial effect of AgNPs formed on GPG-AG3 thin films under various conditions

Next, silver-coated GPG-AG3 films prepared under various conditions were evaluated for their antibacterial effects on 2xYT agar plates coated with 10⁷ bacteria *E. coli*. Figure 31 shows that all silver films have antibacterial capabilities.

Figure 31: Antimicrobial activity of silver-coated GPG-AG3 thin films. (A) Silver-coated GPG-AG3 thin film synthesized from different NaCl concentration. (B) Silver-coated GPG-AG3 thin film synthesized from different pH of phosphate buffer.

To study the effect of NaCl and pH on the resulting antibacterial properties, the width of the inhibition zone was measured for each sample (Table 2) using ImageJ and the diameters of AgNPs synthesized under different conditions were compared (Fig. 32).

Effect of varying NaCl concentration				Effect of varying pH			
NaCl concentration	Sample No.	Particle size (nm)	Inhibition zone distance (mm)	pH	Sample No.	Particle size (nm)	Inhibition zone distance (mm)
0 mM	1	N.A	2	pH 5	1	131 ± 57	3.1 ± 0.30
0.1 mM	2	208 ± 87	2.1 ± 0.41	pH 6	2	141 ± 60	2.9 ± 0.49
0.5 mM	3	149 ± 59	2.3 ± 0.28	pH 7	3	127 ± 72	2.7 ± 0.21
1 mM	4	153 ± 56	1.9 ± 0.20	pH 8	4	141 ± 40	2.9 ± 0.49
5 mM	5	901 ± 342	1.0 ± 0.28	pH 9	5	166 ± 66	3.0 ± 0.21

Table 2: Quantification of the bacterial inhibition zones for silver-coated GPG-AG3 thin films for various conditions.

Figure 32: Distance of the bacterial inhibition zones for silver-coated GPG-AG3 thin films. (A) silver-coated GPG-AG3 thin films were from different NaCl concentration. **(B)** silver-coated GPG-AG3 thin films were from different pH solution. Data are means $\pm$ SD from two independent experiments.

The bacterial inhibition zone for GPG-AG3 containing silver particles prepared with 5 mM NaCl was the smallest, suggesting that micron-sized AgNPs were less effective in inhibiting the growth of bacteria. This observation is consistent with literature reports [58]. We also noted some variations in the inhibition zones on films incubated at varying pH. In these cases however, the shape and size of the AgNPs were not significantly different. Hence, differences in widths of the inhibition zone could have attributed to variations in the densities of AgNPs on the films. Unfortunately, the surface densities of the AgNPs could not be determined due to the opacity of the films.

5. CONCLUSIONS

Elastin-like proteins containing the AG3 silver-binding domains (GPG-AG3) were successfully constructed and synthesized. We showed that GPG-AG3 proteins can be prepared as nanoaggregates as well as crosslinked thin films. Both forms of GPG-AG3 templates mineralized silver in the presence of silver nitrate at room temperature. We studied the effect of templating and synthesis parameters on the size, shape and antibacterial properties of the nucleated silver. The key findings of this work are summarized:

(1) Silver particles formed on GPG-AG3 nanoaggregates had an average diameter of 20 – 25 nm while silver particles formed on GPG-AG3 thin films were significantly larger ($869 \text{ nm} \pm 353 \text{ nm}$). The striking differences in particle size could be attributed to higher concentrations of AgNO_3 used (0.2 mM in nanoaggregates solution and 1 mM in solution for thin films) as well as differences in the presentation of AG3 domains on the template surface.

(2) We studied the effect of NaCl concentration and pH on silver nucleation on GPG-AG3 thin films. When the concentration of NaCl in the solution was varied, we found that silver particle size increases with increasing NaCl concentration, achieving up to 1 μm at the highest NaCl concentration tested (137 mM). In the presence of NaCl, spherical silver particles were obtained, while randomly shaped silver particles were obtained when NaCl was absent. This suggested that Cl^- was necessary to produce spherical silver particles. We also examined the effect of varying pH on the silver nucleation on GPG-AG3 thin films. However, varying pH had no effect on the shape and size of the silver nanoparticles.

(3) Silver nanoparticles nucleated on GPG-AG3 protein nanoaggregates and on cross-linked thin films had antibacterial properties. Gram negative *E. coli* growths in both 2xYT media and on solid agarose were inhibited in the presence of AgNPs and silver-coated GPG-AG3 films. The antibacterial properties of silver-coated GPG-AG3 thin films were also found to be dependent on the size of the nucleated silver particles. The bacterial inhibition

zone was found to be the smallest for GPG-AG3 films containing micron-sized silver particles.

In summary, elastin-based silver-binding fusion proteins have the ability to mineralize silver particles. Both GPG-AG3 nanoaggregates and thin films were able to nucleate AgNPs in aqueous silver nitrate solution. In this work, the synthesized silver particles were shown to possess antibacterial properties against Gram-negative bacteria. Taken together, this work demonstrated the potential for silver-coated elastin-based materials to be used in tissue engineering and regenerative medicine to impart antibacterial functions.

6. FUTURE WORK

In our current work, we have successfully demonstrated that GPG-AG3 proteins were able to self-assemble into 3D structures and be crosslinked to yield stable thin films. Both 3D GPG-AG3 structures and 2D thin films nucleate silver under physiological conditions. The silver particles obtained in our work exhibit potent antibacterial properties against gram-negative *E. coli* bacteria.

We found, however, that silver-coated GPG-AG3 thin films have long-term toxicity effects on mammalian cells. We recognized that the toxicity of silver presented on the GPG-AG3 thin films might limit their applications in many biomedical applications. In our future work, we propose to optimize the density of AgNPs on the surface of the films to increase the overall biocompatibility of silver-coated GPG-AG3 thin films.

The elastin GPG framework has recently been shown to have the ability to self-assemble into nanofibers in aqueous solutions. The fibers were 20 - 70 nm in diameter and more than 10 μm in length [25]. Our future goal is to utilize protein GPG-AG3 nanofibers as templates for the fabrication of silver nanowires. Silver nanowires are attractive due to their intriguing electrical, thermal and optical properties [59]. More importantly, they also have exceptional antibacterial properties due to their large surface areas and nano-scale features, with potential applications as wound dressings.

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APPENDIX A

Oligonucleotide sequences of primers used in construction of AG3 DNA fragment:

Forward:

5' – gccg^{**SalI**}gtcgacgcttattcttctggtgctcctcctatgcctcctttt^{**XhoI**}ctcgagcgcg–3'

Reverse:

5' – ccgc^{**XhoI**}ctcgagaaaaggaggcataggaggagcaccagaagaataagc^{**SalI**}gtcgaccggc–3'

APPENDIX B

Nucleotide and amino acid sequence of GPG-AG3:

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ATG AAG CTT GTG GGC GGT GTG GGC GTT GGT GGC GTG GGT GTA GGC GGT GTA GGC GTG
M   K   L   V   G   G   V   G   V   G   G   V   G   V   G   G   V   G   V
GGT GGC GTG GGT GTT GGC GGT GTG GGC CTG TGG CTC GGA TCC GGG GTG CCG GGT GTG GGC
G   G   V   G   V   G   G   V   G   L   W   L   G   S   G   V   P   G   V   G
GTT CCG GGC GTG GGT GTA CCG GGC TTC GGT GTC CCG GGC GTA GGT GTT CCG GGT GTC GGG
V   P   G   V   G   V   P   G   F   G   V   P   G   V   G   V   P   G   V   G
GTG CCG GGT GTG GGC GTT CCG GGC GTG GGT GTA CCG GGC TTC GGT GTC CCG GGC GTA GGT
V   P   G   V   G   V   P   G   V   G   V   P   G   F   G   V   P   G   V   G
GTT CCG GGT GTC GGG GTG CCG GGT GTG GGC GTT CCG GGC GTG GGT GTA CCG GGC TTC GGT
V   P   G   V   G   V   P   G   V   G   V   P   G   V   G   V   P   G   F   G
GTC CCG GGC GTA GGT GTT CCG GGT GTC GGG GTG CCG GGT GTG GGC GTT CCG GGC GTG GGT
V   P   G   V   G   V   P   G   V   G   V   P   G   V   G   V   P   G   V   G
GTA CCG GGC TTC GGT GTC CCG GGC GTA GGT GTT CCG GGT GTC GGG GTG CCG GGT GTG GGC
V   P   G   F   G   V   P   G   V   G   V   P   G   V   G   V   P   G   V   G
GTT CCG GGC GTG GGT GTA CCG GGC TTC GGT GTC CCG GGC GTA GGT GTT CCG GGT GTC GGG
V   P   G   V   G   V   P   G   F   G   V   P   G   V   G   V   P   G   V   G
AAG CTT GTG GGC GGT GTG GGC GTT GGT GGC GTG GGT GTA GGC GGT GTA GGC GTG GGT GGC
K   L   V   G   G   V   G   V   G   G   V   G   V   G   G   G   V   G   V   G   G
GTG GGT GTT GGC GGT GTG GGC CTG TGG CTC GAG GCT TAT TCT TCT GGT GCT CCT CCT ATG
V   G   V   G   G   V   G   L   W   L   E   A   Y   S   S   G   A   P   P   M
C C T   C C T   T T T   G T C   G A G   C A C   C A C   C A C   C A C   C A C   C A C
P       P       F       V       E       H       H       H       H       H       H       H

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