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Bactericidal activity of elastin-like polypeptide biopolymer with polyhistidine domain and silver

Kishore K. Krishnani[a,1], Wilfred Chen[a,b], Ashok Mulchandani[a,*]

[a] Department of Chemical and Environmental Engineering, University of California, Riverside, CA, USA
[b] Department of Chemical and Biomolecular Engineering, University of Delaware, DE, USA

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ABSTRACT

In the present study, elastin-like biopolymer (ELP) composed of a polyhistidine domain has been investigated as a silver binding agent for antibacterial activity against *Escherichia coli*, a model test strain for Gram-negative bacteria for antibacterial assays of nanoparticles, and *Vibrio harveyi*, an opportunistic pathogen which cause mass mortality in shrimp *Penaeus monodon* reared in coastal aquaculture. The concentration dependent antimicrobial activity of ELPH-Ag on *E. coli* and *V. harveyi* was examined by agar well diffusion method and further confirmed through growth curves using spectrophotometer assisted absorption observations. The increased concentrations of ELP-Ag effectively checked the bacterial growth and increased the diameter of inhibition zone. The results showed a minimum inhibitory concentration of 37 μg/ml. This study has an application in formulating artificial protein based antibacterial in diverse fields of healthcare and management of disease in coastal aquaculture.

1. Introduction

Silver is a potent bactericidal agent due to its large surface area to volume ratios, physicochemical properties and a broad range of antimicrobial activities [1–3]. However, silver nanoparticles have the propensity to aggregate in solution, causing loss of their bactericidal activity over time [4]. Furthermore, over exposure to uncoated silver nanoparticles causes cellular toxicity [5]. Therefore, there is a need of stabilizing silver nanoparticles and reducing their toxicity [6,7]. Chemical approaches for producing stable silver-based materials require lengthy synthesis steps and the resulting products often have inadequate biocompatibility [8]. Biomimetic approaches based on the metals binding proteins and polymeric ligands have attracted attention in recent years [9–11]. Among promising candidates, one emerging technology is the use of tunable, metal-binding biopolymers based on elastin-like peptides (ELPs) [12–14], because of their mild and environmentally friendly synthesis conditions. ELPs are protein based biopolymers derived from natural biological building blocks consisting of a repeating pentapeptide Val-Pro-Gly-Val-Gly that undergo a reversible phase transition from water-soluble forms into aggregates upon increasing the temperature and under a broad range of pH and ionic strength [15,16]. The flexibility of tailoring the desired metal-binding domain in the ELP biopolymer is a unique property that can be easily exploited for improved affinity and specificity for the target metals [13,17].

The importance of histidine complexing with transition metal ions in biological systems has been known for a long time. The methodology of purifying proteins using the high affinity of histidine residues (His-tag) to nickel ions has long been exploited [18]. Elastin-like polypeptides composed of a polyhistidine domain has also been used as an environmentally benign chelating agent for metals removal [19,20]. We have previously [9,13] generated ELP biopolymers containing a polyhistidine tail (ELPH12) as an environmentally benign metal chelating domain, and demonstrated the feasibility of easy extraction of cadmium from contaminated water and soil. In the present study, elastin-like biopolymer (ELP) composed of a polyhistidine domain has been investigated as a silver binding agent for antibacterial activity against two model pathogens, *Escherichia coli* and *Vibrio harveyi*. *E. coli*, often used as a model test strain for Gram-negative bacteria for antibacterial assays of nanoparticles [21,22] is used as a model human pathogen. *V. harveyi* [23], an opportunistic Gram-negative pathogen that causes mortalities among *Penaeus monodon* larvae, post larvae and cultured shrimp and survive even the lime and chlorination treatment of ponds [24], is an emerging threat in aquaculture production world-wide [25].

* Corresponding author.
E-mail address: adani@engr.ucr.edu (A. Mulchandani).
[1] Present address: National Institute of Abiotic Stress Management, Baramati, Pune 413115, India.

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2. Materials and methods

2.1. Production and purification of ELPH12 biopolymer

E. coli BLR(DE3) (Novagen) containing pET-Ela78H12 [9] coding for ELPH12 was grown in Luria–Bertani (LB) media supplemented with 100 μg/ml ampicillin in 3000 ml Erlenmeyer flasks filled with 1000 ml medium agitated at 250 rpm for 6 h and 30 °C on an incubator-shaker until OD of 0.6 when 1 mM IPTG was added and incubated for another 30 h at 250 rpm and 37 °C. At this time the cells were harvested by centrifugation (5000 rpm for 15 min and 4 °C), washed in 0.9% NaCl, resuspended in ice cold buffer (10 mM Tris–HCl, pH 8) containing lysozyme (1 mg/ml) and kept on ice for 1 h. Subsequently, the cells were lysed by sonication, the cell debris removed by centrifugation for 15 min at 30,000 × *g* and the biopolymers purified from the cell free extract by three cycles of inverse temperature transition [26] using NaCl to a final concentration of 1 M. For each cycle, the sample was heated to 37 °C and centrifuged at 30,000 × *g* at 30 °C, and the pellet containing the biopolymer was dissolved in ice-cold sterile deionized water (pH 7.4). NaCl was added for precipitation during temperature transition. Purified biopolymers were stored at −20 °C, and used within 24 h.

2.2. Determination of biopolymer concentration

The biopolymer concentration was determined by measuring the absorbance at 215 nm using a Beckman DU-800 UV-VIS spectrophotometer. Amount of biopolymer was determined using following equation: $Abs_{215} \times 69.9 \times 100 = \mu g/ml$, reported previously [9].

2.3. Silver-binding of ELPH12

Experiments to determine the silver-binding capacity of ELPH12 were performed in 1 ml of nanopure water (pH 7.4). Varying amounts of biopolymer were mixed with different concentrations of Ag^+ in the form of $AgNO_3$ and incubated for 24 h at room temperature with intermittent shaking. The silver–biopolymer complex was precipitated by heating the solution at 37 °C for 10 min and centrifuged for 5 min at 14,000 × *g* and 37 °C. To avoid formation of AgCl, sodium chloride was not used during precipitation of biopolymers. The resulting pellets were re-dissolved in deionized water and used for bactericidal assay. For silver analysis, each sample was diluted by adding 0.1% nitric acid to the appropriate dilution prior to measurement. The amount of bound Ag was analyzed by ICP – Varian Vista MPX Simultaneous Inductively Coupled Plasma Optical Emission Spectrometer.

2.4. Antibacterial activity

E. coli BLR(DE3) (Novagen) and *V. harveyi* ATCC 14126, used as indicator bacteria, were grown on LB broth/agar and photobacterium broth/agar (ATCC medium 101), respectively.

2.4.1. Microbial agar well diffusion method

The antibacterial properties of samples were measured by minimal inhibitory concentration (MIC). Solid agar media were used to evaluate growth inhibiting properties of Ag bound material. Solid media (25 ml in Petri dishes) were seeded with bacteria. Wells were made using sterile tip. Silver-ELP containing 9.3, 10.7, 12.4, 14.5, 18.4, 34.3, 37 and 43.7 μg silver was put in the wells. The plates were then stored for 30 min to allow for pre-diffusion of the sample into the agar medium. Subsequently, the plates were incubated at 37 °C and zones of inhibition were read after 24 h. MIC was determined according to such a standard that the lowest concentration of antibacterial solution needed to prevent visible growth of test microorganism was defined as the MIC against the microorganism.

2.4.2. Culture growth suppression spectrophotometric method

E. coli and *V. harveyi* strains were grown in their respective broth for 18 h and then on respective solid (agar) medium. From agar plates, fresh colonies were inoculated into 5 ml of broth medium and growth monitored by UV–vis spectrophotometer till the optical density reached 0.2 at 600 nm. Subsequently, 0.1 ml of the suspension cultures were further added to 5 ml of freshly prepared broth media supplemented with ELPH12-Ag with the final concentrations of 9.3, 18.4 and 37 μg/ml silver. Control broth solution containing only ELP was also used. All the culture tubes were incubated in a rotary shaker at 200 rpm and 30 °C. The growth was monitored at an interval of 12 h for 48 h by measuring absorbance at 600 nm by a UV–vis spectrophotometer (Beckman DU800), for which 0.1 ml of culture from the control and treated sample were taken out and diluted in 10-fold increments.

2.4.3. Determination of residual amount of bacteria

The residual amount and reduction ratio of bacteria were estimated by the direct plate counting method. In brief, after 48 h 100 μl of appropriately diluted *E. coli* and *V. harveyi* broth were plated on LB and photobacterium agar, respectively, and incubated at 37 °C for 24 h. At this time the plates having an ideal number of colonies between 30 and 300 were counted and the residual amount of viable cells per milliliter in the original sample was calculated based on the dilution used in the plating. The percentage reduction ratio of the bacteria was evaluated by the following equation:

$$R\% = \left[\frac{(\text{Number of bacterial colonies from control sample} - \text{Number of bacterial colonies from the treated sample})}{\text{Number of bacterial colonies from control sample}}\right] \times 100$$

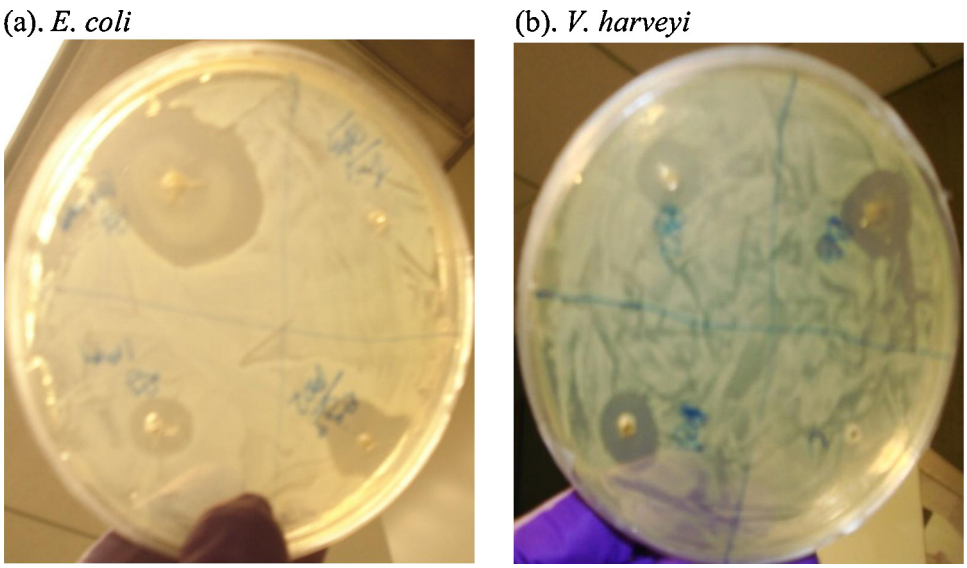
3. Results

3.1. Silver-binding characteristics of ELPH12

In order to avoid the formation of silver salt precipitate, experiments to determine the silver-binding capacity of ELPH12 were performed in nanopure water (pH 7.4) instead of saline buffer, and the silver–biopolymer complex was precipitated by heating at 37 °C without addition of sodium chloride. Incubation of varying amounts of biopolymer with different concentrations of Ag^+ in the form of $AgNO_3$ resulted in the formation of ELP-Ag complexes containing 372, 428, 497, 580, 737, 1372, 1480, and 1748 mM Ag ion, which were subsequently used as stock solutions.

3.2. Inhibitory effect of ELP-Ag on growth and viability of bacterial cells

The bacteriological tests of ELPH12-Ag against *E. coli* and *V. harveyi* at different concentrations of silver (9.3, 18.4 and 37 μg/ml) were performed and growth curves were obtained using spectrophotometer assisted absorption observations. In addition, the concentration dependent antibacterial activity of the ELPH12-Ag on *E. coli* and the shrimp pathogenic bacteria *V. harveyi* was determined by the agar well diffusion method using ELPH12-Ag



Picture 1. Inhibition zones of ELPH12-Ag containing different concentrations of silver against (a) *E. coli* (43.7, 14.5, 9.3 μg and control) and (b). *V. harveyi* (18.4, 12.4, 10.7 μg and control). Control test with only biopolymer had no inhibition effect on bacterial growth.

containing different concentrations of Ag. All the blanks and control tests, including supplementing media with only biopolymer, had no inhibition effect on bacterial growth. In contrast, the bactericidal activity of ELPH12-Ag was confirmed as evidenced from the bacterial growth curves and the increased diameter of inhibition zones (Picture 1).

3.2.1. Effect on E. coli

The *E. coli* strain used in the present study was *E. coli* BLR(DE3) (Novagen). The width of the inhibition zones of *E. coli* BLR(DE3) using antibacterial ELPH12-Ag is given in Fig. 1. The width of inhibition zones increased from 1 to 2.1 cm with increased doses of the ELPH12-Ag containing 9.3–43.7 μg silver. The growth curve (Fig. 2a) revealed that the ELPH12-Ag caused substantial decrease in cell growth and viability of *E. coli* BLR(DE3). From Table 1, it is evident that 93.03% and 99.92% reduction ratio of *E. coli* were achieved at 9.3 and 18.4 μg/ml. Fig. 3a shows the changes in the residual amount of viable *E. coli*. On the ELPH12-Ag containing 37 μg Ag/ml concentration for 48 h contact, no residual *E. coli* was detected, which indicates that 100% bacteria were killed due to the inhibitory effect of Ag^+ ions. Hence, the minimum inhibitory concentration (MIC) for ELPH12-Ag against *E. coli* BLR(DE3) was 37 μg/ml.

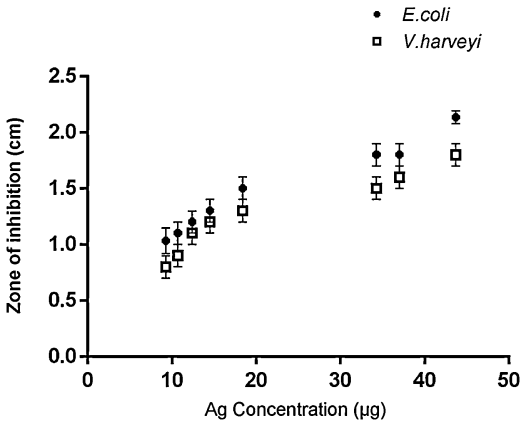


Fig. 1. Inhibition zones of samples as a function of different amounts of ELPH12-Ag against *E. coli* and *V. harveyi*. Each point represents an average of triplicate measurements.

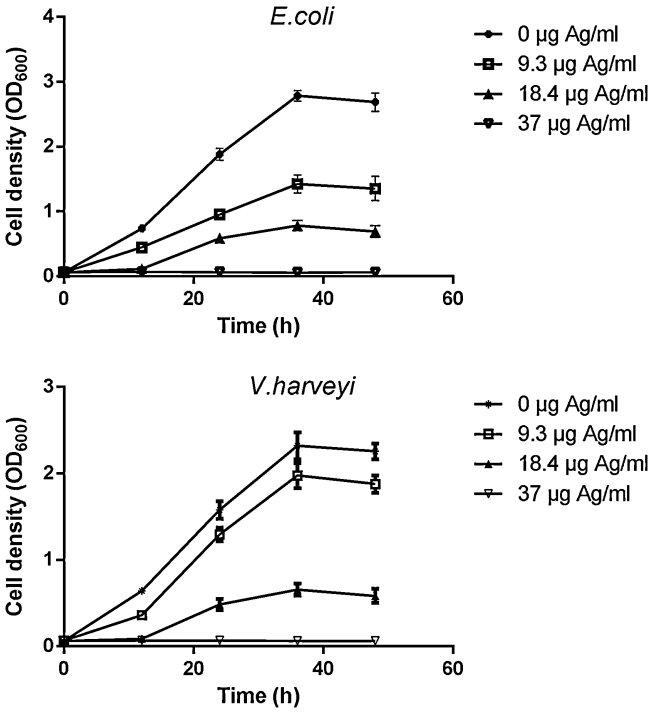


Fig. 2. Bacterial growth curves with increasing concentration of ELPH12-Ag. Each point represents an average of triplicate measurements.

Table 1
Bacterial log reduction after treatment with ELPH12-Ag containing different concentrations of silver for 48 h.

Concentration (μg Ag/ml)	*E. coli*	*V. harveyi*
9.3	1.1514	1.0463
18.4	3.0956	3.0861
37	9.0393	8.9890

Log reduction was calculated by log (Number of bacterial colonies from control sample/Number of bacterial colonies from treated sample).

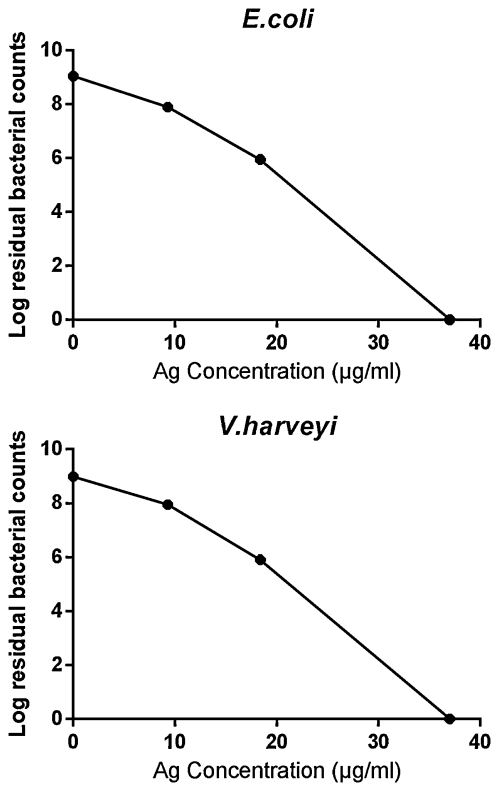


Fig. 3. The changes in residual amount of (a) *E. coli* and (b) *V. harveyi* with different amounts of ELPH12-Ag. Each point represents an average of triplicate measurements (error bars are smaller than symbols). The detection limit of bacteria for direct plate counting method was >10 CFU/ml.

3.2.2. Effect on V. harveyi

The *V. harveyi* strain used in the present study has been isolated from dead, luminescing amphipod (*Talorchestia* sp.), Woods Hole, MA. This organism produces restriction endonuclease VhaI. The width of the inhibition zone of the antibacterial ELPH12-Ag measured for this strain increased from 0.8 to 1.8 cm (Fig. 1) with increased doses of the ELPH12-Ag containing 9.3–43.7 μg silver. The growth curves of *V. harveyi*, shown in Fig. 2b, revealed that the ELPH12-Ag caused substantial decrease in cell growth and viability of *V. harveyi*. A 99.91% reduction ratio of *V. harveyi* was achieved at 18.4 μg Ag/ml (Table 1). Fig. 3b shows changes in the residual amount of *V. harveyi*. No residual *V. harveyi* was detected at 37 μg Ag/ml concentration of ELPH12-Ag after 48 h contact, which indicates that the MIC for ELPH12-Ag against *V. harveyi* was 37 μg/ml.

4. Discussion

Genetic and protein engineering have emerged as the latest tools for the construction of nanoscale materials that can be controlled precisely at the molecular level [17]. Artificial proteins produced through recombinant DNA techniques can be engineered to have high affinities for metals [27,28]. The most significant feature of these nanoscale biopolymers is that they are specifically pre-programmed within a synthetic gene template and can be controlled precisely in terms of sizes, compositions and functions at the molecular level. Biopolymers composed of repeating elastin units have been shown to undergo phase transitions within a wide range of conditions [15]. Compared to chemical polymers or chelators, these biopolymers are environmental friendly as no toxic chemicals are required for their synthesis and they can be easily produced in mass quantity and regenerated [17]. Selected metal-binding proteins can be fused to the tunable biopolymers to provide metal-binding functionalities [29–32]. The use of these metal-binding domains has significant advantages over existing chemical chelators, including higher specificity and affinity. Kostal et al. [9] and Prabhukumar et al. [13] exploited elastin-like polypeptide composed of a polyhistidine tail (ELPH12) as a tunable metal-binding biopolymer with high affinity toward cadmium. The addition of six extra histidines increased the Cd^{2+} binding capacity to a ratio of 1.5:1, suggesting that the metal-binding capability of the biopolymers can be easily modified. ELPH12 has the advantage of undergoing a reversible thermal precipitation for easy purification. Kostal et al. [17] has highlighted the tunable biopolymer technologies and their potential applications and future uses of particular relevance. Tunable biopolymers can be easily regenerated and reused for many repeating cycles. Production and purification of biopolymers are based on the same phase transition principle and could be easily scaled-up to large quantity; therefore, providing a low-cost and environmentally benign technology for heavy metal removal [17].

Diverse studies have demonstrated the antimicrobial effect of silver nanoparticles on a broad spectrum of microorganisms [33–36] including *E. coli*, *Pseudomonas aeruginosa*, *Vibrio cholera* and *Bacillus subtilis*. Anh et al. [8] demonstrated that both GPG-AG3 protein aggregates and crosslinked thin films were able to cause the biomimetic nucleation of silver. Their antibacterial assay results showed that silver coated elastin-like material GPG-AG3 thin films and silver nanoparticles (20 μg/ml) completely inhibited *E. coli* (10^7 CFU) growth on agar plates and in liquid medium. Hernandez-Sierra et al. [37] demonstrated the antibacterial activity of silver nanoparticles against *Streptococcus mutans* with an average MIC of 4.86 ± 2.71 μg/ml. In the present paper, we have extended the application of ELP biopolymer through successful demonstration of its silver ion binding property for possible utilization as an antibacterial material against *E. coli* and *V. harveyi*. The results showed minimum inhibitory concentration of 37 μg/ml.

For the antibacterial activity of silver nanoparticles, protein inactivation and loss of replication ability of DNA are suggested [21]. This effect is dependent on superficial contact, in that silver can inhibit enzymatic systems of the respiratory chain and alter DNA synthesis [36,38]. The cellular permeability in the case of *E. coli* is largely controlled by the presence of a lipopolysaccharide (LPS) layer on the outer surface of the cellular membrane [39]. The heavily saturated fatty acids on LPS links it to the membrane backbone, which itself contains many negative ions and the binding of even simple cations to LPS weakens the membrane backbone, which leads to the disintegration of the membrane [40]. Silver nanoparticles cause irreparable damage to the cellular membrane which enables the accumulation of nanoparticles in the cytoplasm, hence, action of silver nanoparticle arises due to this damage and not its toxicity [41].

5. Conclusions

In conclusion, this work successfully demonstrated the feasibility of tailoring ELP biopolymer with enhanced silver binding affinity by employing a polyhistidine binding moiety. Silver-ELP complexes were shown to have promising bactericidal activity against *V. harveyi* and *E. coli*. Silver-ELP complexes can be proposed as a low cost material for bactericidal activity for aquaculture use. Successful further studies on the use of Ag-ELP could be beneficial for disease management in aquaculture systems.

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