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Growth factor functionalized biomaterial for drug delivery and tissue regeneration

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Abstract

Elastin like polypeptides (ELPs) are a class of naturally derived and non-immunogenic biomaterials that are widely used in drug delivery and tissue engineering. ELPs undergo temperature-mediated inverse phase transitioning, which allows them to be purified in a relatively simple manner from bacterial expression hosts. Being able to genetically encode ELPs allows for the incorporation of bioactive peptides thereby functionalizing them.

Here we report the synthesis of a biologically active epidermal growth factor-ELP (EGF-ELP) fusion protein that could aid in wound healing. EGF plays a crucial role in wound healing by inducing cell proliferation and migration. The use of exogenous EGF has seen success in the treatment of acute wounds, but has seen relatively minimal success in chronic wounds because the method of delivery does not prevent it from diffusing away from the application site.

Our data shows that EGF-ELP retained the biological activity of EGF and the phase transitioning property of ELP. Furthermore, the ability of the EGF-ELP to self-assemble near physiological temperatures could allow for the formation of drug depots at the wound site and minimize diffusion, increasing the bioavailability of EGF and enhancing tissue regeneration.

Keywords

Fusion protein; Elastin-like-polypeptides; EGF; Wound healing

Introduction

Epidermal growth factor (EGF) is a 53 amino acid single-chain polypeptide discovered by Dr. Stanley Cohen in 1962 while studying the effects of mice submaxillary gland extract on newborn mice^{1, 2}. EGF is a member of a family of growth factors, the EGF family, that are able to bind to the epidermal growth factor receptor (EGFR), a tyrosine kinase transmembrane protein found throughout the epidermis. Upon binding EGF, the EGFR undergoes dimerization and autophosphorylation, activating the mitogen-activated protein kinase pathway (MAPK). Ultimately, activation of the MAPK pathway influences the phosphorylation of various transcription factors and calcium release of protein kinase C. Furthermore, activation and dimerization of the EGFR due to EGF binding has been shown

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to play a crucial role in epidermal regeneration and corneal epithelial regeneration through enhancing epithelial cell proliferation and migration, stimulating the production of extracellular matrix proteins, and increasing fibroblasts proliferation ³.

The use of exogenous EGF in acute wound healing was originally examined in 1973 by Savage and Cohen, when they demonstrated corneal epithelial hyperplasia can be induced using EGF ⁴. Since then, the use of exogenous EGF has shown promise in the treatment of acute surgical wounds in rabbits ⁵, corneal epithelial wounds ⁶⁻⁸, and partial thickness burn wounds ⁹. However, the use of exogenous EGF to aid in the treatment in chronic wounds, such as diabetic ulcers or venous ulcers, has not been as successful. Exudative fluid withdrawn from chronic wounds has shown, compared to acute wounds, an increased level in protease activity and diminished EGF activity ^{10, 11}. This hints at the possibility EGF is degraded by excessive protease activity within the microenvironment of chronic wounds. Furthermore, Falenga et al. demonstrated the topical administration of aqueous EGF solutions to venous ulcers does not cause a statistically significant decrease in wound area after a period of 10 weeks ¹². Due to these studies, alternative delivery systems have been investigated.

As aforementioned, the microenvironment in chronic wounds leads to the excessive degradation of growth factors that enter the wound. The high rate of degradation means there is not a high enough concentration of EGF present at the wound site to make a significant difference in healing, and the growth factor is not present in the wound long enough for its presence to be detected ¹³. Additionally, when EGF is administered using simple bolus delivery methods, such as topical application ¹² or intralesional injections ¹⁴, it is able to easily diffuse away from the wound decreasing the local concentration of EGF, thus minimizing its impact on wound healing. This can lead to using large quantities of EGF and having to reapply the treatment continuously, potentially leading to tumor development and making the therapy expensive ¹³.

To combat these issues, delivery systems utilizing hydrogels with EGF encapsulated within the polymer network have been investigated. While there has been moderate success using this method, there is little control over the release of EGF from the polymer network, resulting in a burst release profile of EGF ¹⁵. While there may be some instances where a burst release profile is preferred, most applications require the ability to control the release of EGF to ensure the growth factor is present throughout the healing process ⁶. To achieve this, growth factor delivery vehicles that allow for the covalent binding, or at least physical interactions, between growth factors and a scaffold are developed ¹³.

Elastin like peptides (ELPs) are a class of genetically encodable polymers that possess temperature dependent phase transition properties ¹⁶. The biocompatibility and ability to form nanostructures makes ELPs ideal for drug delivery and *in vivo* applications. Additionally, since ELPs are genetically encodable, it is relatively simple to fuse biologically active peptides or proteins to ELPs creating ELP fusion proteins ^{17, 18}. These ELP fusion proteins have been shown to retain not only the inverse phase transition property of traditional ELPs, but also the biological activity of the fused peptide ¹⁹. Hence, ELP

fusion proteins have the potential to serve as all-in-one drug delivery systems, containing the drug and delivery vehicle without the need for chemical conjugation.

Here we report the creation of a biologically active EGF-ELP fusion protein that could address the issues connected with the use of exogenous EGF in regenerative medicine. Currently, the administration of exogenous EGF is carried out by either a simple injection or topical application. Unfortunately with these techniques, EGF can be rapidly degraded and can diffuse away from the application site with relative ease, leading to a decreased local concentration of EGF and minimal improvements as far as tissue regeneration is concerned. We report that the biological activity of EGF was retained in the fusion as well as the physical properties of ELPs. We further show that the ELPs in the fusion result in aggregation of the protein at its transition temperature. Thus the fusion protein was rapidly and easily purified by inverse temperature cycling²⁰. This represents a seamless production of a functionalized biomaterial in one step eliminating the need of physically or chemically conjugating growth factors or other bioactive proteins. Due to the polymeric ELP backbone the fusion protein can also act as a scaffold for tissue engineering as well as a drug since it contains EGF. Finally, aggregates of EGF-ELP can also act as drug depots of EGF limiting the loss of the growth factor due to diffusion.

Materials and Methods

Materials

Restriction enzymes and other materials used for cloning were purchased from New England Biolabs (New England Biolabs, Ipswich, MA). EGF and V₄₀C₂ (ELP) genes were purchased from GenScript (Piscataway, NJ, peptide sequences are described in Table 1). DNA purification and gel extraction was conducted using kits produced by Qiagen (Germantown, MD). Top10f competent *Escherichia coli* used for DNA cloning and BLR(DE3) competent *E. coli* used for fusion protein expression were purchased by Invitrogen (Carlsbad, CA).

For cell culture purposes, fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Life Technologies (Carlsbad, CA), and antibiotic-antimycotic (AA) solution was purchased from Corning (Mediatech, Manassas, VA). Dr. Eric Haura from Moffitt Cancer Center generously donated A549 adenocarcinomic alveolar basal epithelial cells and A431 epidermoid carcinoma cells. Human skin fibroblasts were purchased from ATCC (Manassas, VA).

Fluorescent photographs for migration and cell counting experiments were taken using an EVOS® FL Digital Inverted Microscope (Life technologies, Carlsbad, CA). ImageJ (National Institutes of Health) was used to analyze fluorescent images. Fluorescent staining of cell cytoplasm was conducted using ethidium homodimer, purchased from Life Technologies. Cell nuclei were fluorescently stained using NucBlue from Life Technologies.

An Eon™ High Performance Microplate Spectrophotometer from Biotek was used for optical density readings at 350 nm (OD350), while a Synergy Multi-Mode Reader from Biotek (Winooski, VT) was used for fluorescence readings during A549 and fibroblasts

proliferation studies. Dynamic light scattering was conducted using a Zetasizer Nano S (Malvern Instruments, Malvern, UK).

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, 12% acrylamide gels were used. Antibodies were purchased from Cell Signaling Technologies (Beverly, MA) and LumiGLO® Reagent and peroxide were purchased from Cell Signaling Technologies.

EGF-ELP Gene Construction

The EGF gene was sent in a pUC57 plasmid with *Pfl*MI and *Bgl*II restriction enzyme sites flanking the gene. *Pfl*MI and *Bgl*II were used to remove the EGF gene from the pUC57 plasmid, and *Bgl*II was used to linearize the ELP pUC19 plasmid. The EGF gene was ligated into the linearized ELP pUC19 plasmid and transformed into Top10f competent cells. The ligation was carried out so that EGF was at the N-terminus of the fusion protein.

The EGF-ELP pUC19 plasmid was then cut using the restriction enzymes *Pfl*MI and *Bgl*II to excise the EGF-ELP gene. The EGF-ELP gene was then ligated into a linearized pET25b(+) expression vector and transformed into Top10f competent cells. The EGF-ELP pET25b(+) vector was sent for sequencing to confirm the EGF-ELP gene was successfully ligated and no mutations were present. The EGF-ELP pET25b(+) vector was then transformed into BLR(DE3) competent cells for fusion protein expression.

EGF-ELP Fusion Protein Expression and Purification

BLR(DE3) competent cells containing the EGF-ELP pET25(b+) plasmid were cultured in 50 mL of TB media (yeast extract: 24 g/L, tryptone: 12 g/L, potassium phosphate monobasic: 2.31 g/L, potassium phosphate monobasic: 12.54 g/L, proline: 11.5 g/L, glycerol: 4 ml/L, and carbenicillin: 100 µg/ml) overnight. The next day, the starter culture was added to 1 L of fresh TB media and cultured for 18h at 37°C and 180 RPM. *E. coli* was collected by centrifugation (3,000 G's, 4°, 20 min) and resuspended in 160 mL of 4°C phosphate buffered saline (PBS). Bacteria was then lysed using a Model 505 Sonic Dismembrator (Fisher Scientific, Waltham, MA) set to run at 50% amplitude for 12 min cycles (3 cycles total, pulse on 59s, pulse off 59s). After each cycle, the bacterial lysate was cooled at 4°C for 40 min. To remove cellular debris, the bacterial lysate was centrifuged (20,000 G's, 4°C, 20 min) and the supernatant containing EGF-ELP was collected.

After collecting the supernatant, EGF-ELP was purified using inverse temperature cycling (ITC). The supernatant was supplemented with 1 M NaCl and placed in a water bath at 45°C for 40 min. The EGF-ELP solution underwent a hot spin (20,000 G's, 40°C, 10 min), after which the supernatant was discarded and the pellet was resuspended in 100 mL PBS containing 10 mM dithiothreitol (DTT) at 4°C. DTT served as a reducing agent to prevent the formation of disulfide bonds, which would decrease the solubility of the EGF-ELP. Prior to cold spins (20,000 G's, 4°C, 20 min), the EGF-ELP solution was cooled at 4°C for 1h. The cycle of hot and cold spins was conducted 3 times, or until cellular debris was no longer visible after centrifugation.

After the last hot spin, the EGF-ELP pellet was resuspended in 50 mL deionized (DI) water at 4°C. The EGF-ELP solution was then dialyzed against DI H₂O for 18h at 4°C to remove any salts or DTT that may be present. After dialysis, the EGF-ELP was lyophilized.

Total Protein Stain and Western Blot Analysis

Total protein stains and western blot analysis were conducted using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were taken from various stages of ITC and were analyzed by SDS-PAGE. For western blots, anti-EGF antibody was used to label EGF-ELP. Horseradish peroxidase linked (HRP) anti-rabbit antibody was used to label anti-EGF antibody, and a chemiluminescent signal was produced using LumiGLO® Reagent and peroxide.

EGF-ELP Transition Temperature

EGF-ELP was dissolved in cold PBS (~4°C) to create a 5 µM solution and was then filtered using a 0.45 µm filter. Dynamic light scattering (DLS) was used to analyze the sample as the temperature was increased from 10°C to 40°C in 5°C increments using the Zetasizer Nano S (Malvern instruments, Malvern, UK). The sample was allowed to equilibrate for 15 min and the derived count rate (kcps) was recorded twice at each temperature.

EGF-ELP Hysteresis

EGF-ELP was diluted in cold PBS (~4°C) to a concentration of 15 µM. 100 µl was then loaded in a round bottom 96 well plate, which was then placed in the spectrophotometer. The optical density at 350 nm (OD 350) was measured as the EGF-ELP solution was heated from 26°C to 42°C in increments of 2°C. The OD 350 was also measured as the solution was cooled from 42°C to 26°C in increments of 2°C. The EGF-ELP solution was shaken for 30s and allowed to equilibrate for 10 min at each temperature before the OD 350 was measured.

EGF-ELP Size Characterization

A 5 µM EGF-ELP solution was prepared using cold PBS (~4°C) and filtered using a 0.45 µm filter. DLS was used to analyze the EGF-ELP solution at 15°C (below the T_i) and 37°C (above the T_i) using the Zetasizer Nano S (Malvern instruments, Malvern, UK). This apparatus produces a speckle pattern by illuminating the particles with a laser. The scattering intensity at a specific angle fluctuates with time, which is detected using a sensitive avalanche photodiode detector (APD). A digital autocorrelator generates a correlation function by analyzing the intensity changes. Particle size distribution can then be deduced by the correlation function. The EGF-ELP sample equilibrated at each temperature for 15 min prior to being analyzed. After equilibration, the EGF-ELP solution was analyzed three times at each temperature. The particle size distribution (mean intensity) and polydispersity index (PDI) were calculated by the Zetasizer Nano S software.

A549 Proliferation and Migration

For the A549 proliferation study, A549 cells were seeded in a 48 well plate at 15,000 cells/well in DMEM + 10% FBS + 1% AA for 24h. Next, cells were washed with 1x PBS and

serum starved for 24h. Cells were then treated with serum free media, and various amounts of ELP, EGF-ELP, and recombinant EGF (rEGF) for 48h. Cellular proliferation was measured by quantifying the DNA content of cells in response to the treatment by the Bromodeoxyuridine (BrdU) assay using a kit as per the manufacturer's recommendations (BD Biosciences, San Jose, CA). Briefly, 24h into treatment, bromodeoxyuridine (BrdU) was added to the wells. After 48h, the incorporated BrdU which is a direct measure of DNA synthesis was labeled by an antibody and quantified by measuring the fluorescence at 450 nm.

For the migration experiment, cells were prepared by seeding 10,000 cells/well in DMEM + 10% FBS + 1% AA. After 24h cells were washed with 1x PBS and serum starved for an additional 24h. Following serum starvation, cells were treated with EGF-ELP or ELP (5 μ M). After 48h of treatment, cells were treated with calcein and incubated at 37°C for 30 min. After incubation, photographs were taken at 10x and 20x.

Inhibition of A431 Cellular Proliferation

A431 epidermoid carcinoma cells were seeded in a 48 well plate at 10,000 cells/well in DMEM + 10% FBS + 1% AA for 24h. Cells were then washed with 1x PBS and cultured in 2% FBS for 24h. After 24h, cells were treated with different concentrations of ELP and EGF-ELP in 2% FBS for 48h. Cell nuclei were then stained with NucBlue (2 drops/ml media) (Life Technologies, Carlsbad, MA) and dead cells were stained with ethidium homodimer (1:500 dilution). Cells were then incubated at 37°C for 30 min. 9 pictures/well were taken at 10x magnification. Images were then analyzed using ImageJ software and a macro created by Raul Iglesias²¹.

Human Skin Fibroblast Proliferation

Human skin fibroblast were seeded in a 48 well plate with 20,000 cells/well in DMEM + 10% FBS + 1% AA. After 24h, cells were serum starved in DMEM + 1% AA for 48h. Following serum starvation, cells were treated with various amounts of EGF-ELP, ELP, and recombinant EGF (rEGF), for 48h in serum-free culture. Following treatment, cell proliferation was measured by quantifying the DNA content using a Hoechst 33258 assay (Life technologies, Carlsbad, CA). Fluorescence changes were recorded using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Statistical Analysis

Statistical significance for biological activity assays was determined using the p-value calculated from ANOVA.

Results

EGF-ELP is Purified Using Inverse Transition Cycling

The total protein stain at various stages of the purification procedure shows successful purification of the protein by inverse transition cycling (ITC) (Figure 1A). The sample in Lane 1 was taken from the bacterial lysate and contains high protein content, as shown by the intense signal and lack of distinct protein bands. The samples used in Lane 2 and Lane 3

were taken during the later stages of ITC, with the sample in Lane 3 being taken after Lane 2. The protein content in Lane 2 appears to be similar to the protein found in Lane 1, indicating high protein content. In contrast, Lane 3 contains less protein than the bacterial lysate and Lane 2, and a single band between 15 kDa and 35 kDa is apparent. This demonstrates that ITC does remove impurities as the cycle progresses. Lane 4 contains the lyophilized EGF-ELP. The protein sample at this stage has been through the entire ITC process, dialyzed for 18h, and lyophilized. Lane 4 comprises a single band at approximately 30 kDa, demonstrating it is possible to successfully remove impurities while purifying the EGF-ELP using ITC.

To confirm EGF-ELP was the protein isolated during ITC, samples from several stages of ITC were taken and underwent western blot analysis using anti-EGF antibody (Figure 1B). The protein bands near 30 kDa indicate the presence of EGF-ELP (Figure 1B, MW=26.7 kDa). The western blot shows the EGF-ELP did not solubilize completely as it is present in the cold pellet (Lane 2) and cold supernatant (Lane 3) in roughly equal amounts after the first cold centrifugation. Additionally, the protein bands present in the hot pellet (Lane 4) and hot supernatant (Lane 5) indicate the EGF-ELP is present in both samples after the first hot centrifugation. However, the signal in the hot pellet is more intense than the signal in the hot supernatant, indicating there is a larger quantity of the EGF-ELP in the hot pellet. Finally, the band seen in the lyophilized product demonstrates the final product contains the EGF-ELP (Lane 6), and the minimal background noise indicates there are relatively few impurities present in the lyophilized EGF-ELP product.

EGF-ELP Transitions Below Physiological Temperature

The transition temperature of the EGF-ELP was determined using dynamic light scattering (DLS). The mean count rate of a 25 μ M sample was recorded over a temperature range of 10°C–40°C in 5°C intervals (Figure 2). An increase in derived count rate corresponds to the transition of EGF-ELP from monomers to aggregates. From 10°C–25°C, the mean count rate stays fairly constant at approximately 200 kilocounts/s (kcps). Once the temperature reached 30°C, the mean count rate increased from 200 kcps to 850 kcps, indicating the aggregation of EGF-ELP's. The transition temperature likely lies between 30°C–35°C. As the temperature was increased further to 35°C and 40°C the mean count rate increased to 1250 kcps and 2600 kcps, respectively. The continued increase in mean count rate denotes a further increase in EGF-ELP aggregate size.

EGF-ELP demonstrates hysteresis behavior

To further determine whether the phase transition behavior of EGF-ELP is elastic similar to ELPs, we measured the optical density of a 15 μ M solution of EGF-ELP solution at increasing and decreasing temperatures (Figure 3). The OD 350 was measured as the EGF-ELP solution was heated from 26°C–42°C and then cooled from 42°C–26°C in increments of 2°C. As the sample was heated from 26°C–42°C, the OD350 value increased from 0.152 (+/- 0.006) to 1.322 (+/- 0.004), indicating the formation of EGF-ELP aggregates. Though, the OD 350 value did not increase significantly until the sample was heated to 38°C. When the solution was cooled from 42°C to 26°C, the OD 350 value decreased from 1.322 (+/- 0.004) to 0.241 (+/- 0.018), showing the EGF-ELP was able to be solubilized after

forming aggregates. Also, the EGF-ELP during cooling did not follow the same path as heating, thereby displaying a hysteresis behavior in phase transition.

EGF-ELP Partially Solubilizes below the transition temperature

Dynamic light scattering (DLS) was conducted below the transition temperature (4°C, Figure 4A) and above the transition temperature (37°C, Figure 4B) to determine the size of EGF-ELP monomers and aggregates, and determine the particle size distribution. 3 measurements were recorded at each temperature. At 4°C, the Z-average value of the EGF-ELP was 61.00 nm with an average PDI value of 0.377 (data not shown).

Below the transition temperature we observed that the particle size distribution is multimodal, with an approximate range of 5.61 nm–5560 nm (Figure 4A). Relative maxima for mean intensity are found at 11.7 nm (4.7%) and 5560 nm (3.8%) with an absolute maximum at 142 nm (4.9%).

When DLS was conducted at 37°C, the Z-average of the EGF-ELP aggregates was determined to be 684.15 nm with an average PDI of 0.055 (data not shown). The relatively low PDI value indicates the Z-average value is reliable and that the data is monodispersed. Also, above the transition temperature the particle size distribution is monomodal and monodispersed, with a range of approximately 459 nm–1100 nm (Figure 4B). The maximum mean intensity value was found to occur at 712 nm with a mean intensity value of 25.7%.

EGF-ELP retained the biological activity of the fused EGF

To test the biological activity of the fused EGF in the EGF-ELP we performed several cellular assays involving multiple cells lines. Each assay confirmed that the EGF in the fused EGF-ELP was active. These assays are described below.

EGF-ELP Induces Proliferation of A549 Cells—Recombinant EGF (rEGF) has been shown to induce proliferation of non-small cell lung cancer (NSCLC) cell lines, such as A549 adenocarcinomic epithelial cells. NSCLC cell lines overexpress the EGFR, making them ideal to test the biological activity of the EGF-ELP ²². Cell proliferation was measured using a bromodeoxyuridine (BrdU) assay to quantify the synthesis of new DNA after 48h of treatment. The results show that EGF-ELP retained the biological activity of rEGF by increasing cellular proliferation nearly 20% when compared to the control after 48h of treatment for all 3 concentrations used (Figure 5A).

EGF-ELP Induces Migration of A549 Cells—EGF has also been shown to induce the migration of A549 cells ²³. To further investigate the biological activity of the EGF-ELP, A549 cells were treated with serum free media as a control, ELP or EGF-ELP (5 µM) for 48h, after which they were stained with calcein and photographed to observe differences in migration patterns. Treatment with ELP did not produce any change in cell or cell colony morphology when compared to the control group, with both forming tightly packed colonies. Treatment with EGF-ELP induced a change in cell morphology compared to the control and ELP treatment. A549 cells treated with EGF-ELP appear to have an elongated

morphology and do not form distinct colonies as the control and ELP treated cells do (Figure 5B).

EGF-ELP Inhibits Proliferation of A431 Cells—It has been demonstrated that EGF is able to inhibit cell proliferation of certain cells overexpressing the EGFR, such as A431 epidermoid carcinoma cells²⁴. To determine whether the EGF-ELP was biologically active, A431 cells were treated with various concentrations of ELP or EGF-ELP for 48h, after which the number of live cells was counted. When A431 cells were treated with EGF-ELP (5 μ M, 10 μ M, and 15 μ M), the number of live cells counted decreased by nearly 50% when compared to the untreated control and the ELP controls (Figure 5C). ELP controls did not experience a statistically significant increase or decrease in cell count after 48 h of treatment when compared to the untreated control.

Induction of Human Skin Fibroblast Proliferation Using EGF-ELP—EGF has been shown to promote cellular proliferation in human fibroblast^{25, 26}. So to further test the biological activity of the EGF-ELP, human skin fibroblasts were treated with EGF-ELP for 48h and a Hoechst assay was performed to determine cell proliferation. The data show that the EGF-ELP does induce proliferation of human skin fibroblasts when compared to serum-free controls and ELP controls. Both EGF-ELP treatments, 5 μ M and 15 μ M, caused an approximately 45% increase in cell proliferation. ELP treatments did not cause any statistically significant increase in fibroblast proliferation (Figure 5D).

Discussion

The aim of this study was to create a recombinant biologically active biomaterial that could be used for drug delivery and tissue engineering applications. Exogenous EGF has been used in regenerative medicine with moderate success to treat acute wounds, but minimal success has been seen in chronic wounds. This has been attributed to the harsh microenvironment in chronic wounds, which leads to enhanced degradation of exogenous growth factors^{10, 11}. Additionally, EGF is typically administered in a bolus solution, which allows EGF to easily diffuse away from the injection site. Both increased degradation and diffusion lead to poor bioavailability of the growth factor; hence, minimal improvements are seen with EGF administration in chronic wounds¹².

The EGF-ELP fusion protein we have fabricated may assist in overcoming these hurdles in EGF administration. To start, the ability to undergo self-assembly at physiological temperature may allow for the formation of drug depots at the injection site, increasing the local concentration of EGF and possibly limiting diffusion of EGF. In addition, the EGF-ELP fusion protein can be used to create EGF-ELP infused hydrogels, similar to fibrin hydrogels infused with KGF-ELP fusion protein¹⁹. The ELP domain of the EGF-ELP could also be crosslinked with a scaffold to immobilize EGF, thereby creating a concentration gradient promoting cell migration and proliferation into the scaffold which could further aid tissue regeneration²⁷. Another benefit of the EGF-ELP fusion protein is the drug and drug carrier are one and produced as one by bacteria, removing the need to physically or chemically conjugate EGF with a delivery vehicle.

The biological activity assays using human skin fibroblasts, A549 cancer cells, and A431 cancer cells all show the EGF-ELP retains the biological activity of EGF. In particular, the proliferation of fibroblast and induced migration of A549 cells demonstrates the potential for the EGF-ELP to be used for wound regeneration as fibroblast and epithelial cell proliferation and migration are important components of the wound healing process, such as granulation tissue formation and epithelialization^{28–30}. Although, the treatments did not appear to show any dose dependence, which is likely due to a dose threshold being reached using EGF-ELP. Future studies will use lower concentrations of EGF-ELP to determine the most effective concentration. Additionally, further experiments will be necessary to compare the regenerative capabilities of the EGF-ELP and rEGF.

The enhanced migration of A549 cells treated with EGF is indicated by the apparent epithelial-to-mesenchymal transition (EMT) of the cells, and the increased distance between cells. The elongated nature of the A549 cells treated with EGF is indicative of EMT, which is an important step in tissue repair, as it coincides with a decrease in cell-to-cell adhesion and increase in cell migratory capability^{31, 32}. Additionally, EGF has been shown to induce EMT in epithelial cells³³.

The ability to create ELP fusion proteins that retain the biological activity of peptides and the inverse phase transition property of ELPs makes them ideal candidates for localized drug delivery. There are a number of ways ELP fusion proteins can be utilized for local drug delivery. One method has seen the creation of coacervate drug depots via aggregation of ELP fusion proteins for treatment of osteoarthritis³⁴ and neuroinflammation³⁵.

Furthermore, keratinocyte growth factor-ELP fusion protein coacervates have been used in conjunction with fibrin hydrogels for delivery of keratinocyte growth factor-ELP fusions to wounds in animal models¹⁹. Another method utilized for the local delivery of ELP fusion proteins is chemically crosslinking ELP domains to create a three-dimensional scaffold²⁷.

In addition to possessing the biological activity of EGF, the EGF-ELP retained the ability to undergo reversible inverse phase transition, though the phase transition displayed some hysteresis during cooling. This is not unusual for some ELPs as the kinetics for transitioning and re-solubilization are different. Indeed, DLS data and the western blot show that some aggregates were still present when the transitioned protein solution was cooled to below the transition temperature. This could be attributed to the hydrophobicity of EGF or the secondary structure of EGF in the fusion may also play a role. Further studies are needed to identify the cause of this hysteresis behavior.

Even though the fusion protein displayed hysteresis, the EGF-ELP was purified via ITC though some protein was lost during the purification steps. This could provide a relatively simple and inexpensive purification technique for recombinant EGF when compared to affinity chromatography methods. We believe that the hysteresis of EGF-ELP can be addressed by fusing a more hydrophilic ELP to EGF. This may help in increasing the yield of EGF-ELP, however care must be taken in choosing the ELP sequence as a higher hydrophilic ELP may result in an increase in the transition temperature making the transition of EGF-ELP difficult.

In summary we were successfully able to create a fusion protein that retained the biological activity of EGF as well as the physical phase transition property of ELPs. The ELP portion of the fusion property functions as a polymeric backbone imparting not only temperature responsive behavior but also as scaffold for tissue engineering and drug delivery. Thus, it can serve as a biologically active biomaterial with potential application in tissue engineering (as a scaffold) or wound healing (as drug delivery vehicle). Finally, the ELP aggregating behavior enables the delivery of combination therapy involving multiple growth factors and bioactive peptides. Thus, this novel biomaterial will have far reaching impact in tissue engineering and drug delivery.

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Figure 1. Expression and purification of EGF-ELP. **(A)** Total protein stain from different steps during inverse transition cycling (ITC). **(B)** Western blot analysis conducted on samples taken during various steps of ITC. Anti-EGF was used to label EGF-ELP.

Figure 2. EGF-ELP transition temperature

Dynamic light scattering (DLS) was used to measure the mean count rate at temperatures between 10°C–40°C in 5°C intervals.

Figure 3. Thermoresponsive behavior of EGF-ELP

The OD 350 was measured while increasing the temperature from 26°C–42°C and while decreasing the temperature from 42°C–26°C.

Figure 4. EGF-ELP particle size distribution

EGF-ELP was diluted in PBS to a concentration of 5 μ M and analyzed using DLS. Particle size distribution is represented as mean intensity (%). (A) was conducted at 4°C and (B) at 37°C.

A **B**

Figure 5. Biological activity of EGF-ELP fusion protein

(A) A549 adenocarcinomic epithelial cells were treated for 48h with serum free (SF) control media, recombinant EGF (rEGF), and EGF-ELP. Cell proliferation was quantified by measuring the DNA content as described in materials and methods (B) Cell migration was observed by treating A549 cells with SF control media, ELP (5 μ M), or EGF-ELP (5 μ M) for 48h. Cells were then stained with calcein to aid in visualization of cell morphology. Clearly the colonies are more scattered and there are longer distances between the cells that show migratory phenotype in the EGF-ELP sample. (C) A431 cells were treated with control media (2% serum), ELP, or EGF-ELP for 48h, after which cell nuclei were stained with NucBlue and dead cell nuclei were stained with ethidium homodimer. (D) Human skin fibroblasts were treated for 48h with SF control media, ELP, or EGF-ELP, after which DNA

was quantified to evaluate cell proliferation as described in materials and methods. For all cell proliferation assays, * denotes a p-value ≤ 0.05 and ** denotes a p-value ≤ 0.10 .

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Table 1

Amino acid and molecular weight information for EGF, ELP, and EGF-ELP fusion protein.

Protein	Sequence	Molecular Weight
EGF	<i>NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVG YIGERCQYRDLKWWELR</i>	6216 Da
ELP	<i>(VPGVG)₄₀[(VPGVG)₂(VPGCG)(VPGVG)₂]₂</i>	20496 Da
EGF-ELP	<i>NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVG YIGERCQYRDLKWWELR (VPGVG)₄₀[(VPGVG)₂(VPGCG)(VPGVG)₂]₂</i>	26712 Da