Growth Factor Release from Microporous Annealed Particle (MAP) Hydrogel to Improve Wound Healing

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On our honor as University Students, we have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments.

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Abstract

Chronic wound healing from diabetic foot ulcers (DFUs) is an unmet clinical need, often resulting in severe consequences including lower-leg amputation. Microporous Annealed Particle (MAP) gel loaded with Epidermal Growth Factor (EGF) may provide an environment that is suitable for tissue integration and improved wound closure in these chronic wounds. An optimized formulation of MAP gel with 5% of the particles containing heparin and loaded with EGF at 1.0 ug/mL elicits a significant cellular response in a functional cell migration assay. The loading and release of MAP loaded with EGF was characterized using enzyme linked immunosorbent assays (ELISA). Heparin was used to load EGF into the microparticles due to its known electrostatic interactions with growth factors, and the loading efficiency was 87%. Finally, lyophilization of MAP gel with EGF maintains equivalent properties to non-lyophilized MAP gel with EGF as the release profiles from ELISAs and the functionality in cell migration studies did not significantly differ. The progress made on this MAP-EGF formulation has the potential to substantially improve the treatment options for those facing chronic diabetic wounds. The potential for lyophilization of this product would also increase its ability to translate into a clinical setting by extending its shelf life.

Introduction

Significance

Over 16 million people in the United States suffer from diabetes, a number that expands to over 200 million across the globe. Between 10% and 15% of those affected by diabetes will develop or currently suffer from diabetic foot ulcers (DFUs)[1]. DFUs can result from acute or chronic, cutaneous disruptions to the skin, arterial complications, peripheral neuropathy, or a blend of these factors[1]. Patients with DFUs experience numerous risks including decreased mobility, sleep deprivation, depression, and anxiety. DFUs precede 85% of lower leg amputations[2,3]. Further, DFUs and resulting amputations are associated with a 5-year mortality rate[1]. The diabetic population is growing with an anticipated global population of 366 million by 2030[1]. This disease is a major health concern of the 21st century, embodying a large, unmet clinical need that is only expected to grow. Yet, the options for clinical treatment of DFUs are sparse and inadequate. Because of the range of factors that contribute to DFUs, these wounds are difficult to treat. Current treatment options for DFUs include tissue removal, negative pressure therapy, and topical applications[1,2]. However, these therapies are still ineffective as 50% of DFUs do not heal because they do not promote a healthy environment for tissue regeneration, but rather only attempt to alleviate the side effects of diabetes and DFUs[2,4].

Traditional treatments lack the biological cues and stimulatory agents necessary to provide an ideal wound healing environment[2]. Hindered blood flow and impaired local neovascularization often delay the healing of DFUs, increasing the chances of infection and further complications[5]. With chronic wounds, such as DFUs, integration that allows for the development of the extracellular matrix and cell migration and proliferation is critical[5]. Specifically, the promotion of angiogenesis at the wound site could be crucial to a healthy diabetic wound closure[5]. With a

growing patient population, providing a treatment option that promotes wound healing in chronic wound sites is significant and can improve the treatment of life-threatening wounds such as DFUs, preventing amputations and lowering morbidity rates.

There are many causes of DFUs, including joint and bone deformation that increases plantar foot pressure, a decrease in the regulation and promotion of blood flow to the extremities and dermal layer of the skin, and a progression of peripheral arterial occlusive disease in the lower extremities[5]. Further, increased discomfort is associated with diabetic wounds or DFUs due to deterioration of the sensory, vasomotor and autonomic nerves[5]. Each wound site varies in its composition, requirements for tissue regeneration, and causative factors, calling for a robust and tunable treatment[2,6]. Recently, researchers in the chronic and diabetic wound healing field have begun to focus on the use of biomaterials and hydrogels to create an optimal wound healing and healthy tissue regeneration environment[2]. These microstructures enable bioactivity and stimulate natural tissue repair through their cell-binding properties, controllable degradation capabilities, and support for the native microenvironment through instructive cellular response cues[2].

MAP Gel as a Candidate for Chronic Wound Healing

Microporous Annealed Particle, or MAP, gel provides a microporous and tunable porosity structure as well as a degradable wound healing environment that promotes cellular network formation and vascularization[6]. The goal of MAP gel is to apply it topically to an open wound and improve wound healing time scales and overall health of the tissue for a one-time solution to a chronic wound[6]. MAP gel has proven to be a more successful alternative over other non-porous hydrogel alternatives. For example, MAP gel reduced the wound area to 60% of its initial coverage over a five-day period, while the wound area remained at 100% when using comparative hydrogels[6]. In addition to the success of shorter wound closure times of MAP, it has also

demonstrated two other key improvements over comparative hydrogels by decreasing inflammation and increasing integration with healthy tissue. Another study of MAP gel displayed extensive cellular network formation by day two compared to a six-day stretch before the wound treated with a non-porous hydrogel began to show cellular network formation[6].

Chronic wounds such as DFUs, though, present a complex wound environment in which MAP gel has not proven as successful (Unpublished data from Griffin lab). Therefore, the overall goal of this project is to continue to build upon the success that MAP gel has already proven in acute wounds by developing a MAP-EGF formulation for chronic wounds. This development would address the unmet clinical need of diabetic wound healing as well as providing a stimulatory agent of EGF for these specific chronic wound sites.

MAP gel has a number of advantages in design and impact over other comparable hydrogels. First, the porosity capability that is inherent to the MAP gel structure is critical for MAP gels' application in diabetic wound healing. A microfluidic device uses aqueous and oil channels to create fine-tuned beads that structure the gel's porosity[6]. A delay in the natural healing properties of the tissue in DFUs are due to a decrease in peripheral blood flow and decreased local neovascularization[5]. The porosity of MAP gel, which is not typical of the current hydrogel alternatives, permits increased vascularization by allowing tissue growth through the gel's pores. In addition, MAP gel provides structural support during the regrowth of tissue by degrading at the rate of tissue regrowth[6]. Other hydrogels encouraged inflammation and prohibited natural growth as their denser, non-porous scaffold created a barrier to growth when the scaffold would not degrade at the rate of tissue growth. Contrastingly, MAP gel creates a stable structure during cell migration and tissue regeneration and subsequent resorption of the biomaterial after the tissue has been properly integrated.

While MAP gel increases vascularization at the wound site, a chronic wound requires an increase in cellular activity and biological cues to stimulate regrowth[4,7]. Thus, a formulation of MAP gel loaded with growth factors to promote healing is needed. Growth factors are necessary to provide cues for the native cells of the wound to remodel and restore the extracellular matrix[2]. EGF, specifically, has been proven to enhance epidermal regeneration[6]. The rate at which this extracellular matrix is rebuilt by the native cells is controlled by growth factor cues. The gel degradation rate must match the tissue growth rate to provide structure during the entire wound healing process without hindering the full integration of the newly restored tissue with its surrounding environment[2]. Delivery of sensitive biologics, including growth factors like EGF and nucleic acids, addresses the problem of the biological deficiency of decreased cellular response in these damaged tissues[2].

An optimized EGF-MAP formulation is a promising alternative treatment to chronic wounds, specifically DFUs, meeting an unmet clinical need for millions of patients across the world. Chronic wound environments are highly complex and individual, requiring a treatment that is highly specific and tunable. Due to the immense control over MAP gel's properties, including factors such as pore size and different growth factor deliveries, this MAP-EGF formulation has the potential to dramatically improve chronic wound healing.

Approach and Specific Aims

The wound healing advantages of MAP gel compared to standard, non-porous hydrogels coupled with literature supporting the curative effects of growth factors in wound healing environments motivates the evaluation of the intersection of these two treatments: a growth factor-loaded MAP gel. To evaluate the impact of EGF-loaded MAP gel on wound healing, functional and characterization assays were run and analyzed. First, a cell migration study was conducted to

determine the most efficient heparin-EGF formulation for significant cell migration (Aim 1). The resulting formulation became the optimized concentration of MAP gel moving forward. A standard ELISA assay was then run to characterize the loading and release of the EGF from the MAP gel and to test for burst release (Aim 2).

Next, an evaluation of the effects of lyophilization on the functionality and release of the growth factor was conducted by running additional cell migration assays and ELISAs for the loaded MAP gel post-lyophilization (Aim 3). This culmination of studies builds upon previous work in the Griffin lab and in the larger scientific community, addressing the need for a biomaterial that promotes wound healing and tissue integration in chronic wound healing environments, especially in diabetic wounds.

Aim 1: Optimize the concentration of EGF loaded into MAP gel using a cell migration assay to achieve the most efficient concentration of EGF that promotes a cell response. Within limits, the concentration of EGF was optimized. This is not a full optimization study as a model for concentration of EGF will not be created. Therefore, the potential concentrations of EGF evaluated in this study varied by orders of magnitude. This method of optimization was adapted from similar studies, such as an evaluation of Regranex, an approved treatment of delivery of growth factors. During clinical trials on Regranex, chosen concentrations were based upon orders of magnitude and predetermined levels, rather than the creation of a full optimization study with a model[8]. In this study, the potential EGF concentrations were 0.1 ug/mL or 1 ug/mL. The percentage of particles containing heparin were either 0.5% or 5%.

Aim 2: Characterize the loading and release of EGF from MAP gel using an ELISA to better understand the quantity of EGF retained by the gel. With the most efficient EGF loading concentration determined by functional cell migration assays, a characterization of the

growth factor release associated with the known loading concentration provided a profile of the active free EGF and protein that remains contained by the gel. This characterization, provided by running an ELISA over predetermined time points, detailed the release kinetics of the gel, determining whether there is a burst release of the growth factor. Prior studies indicate that the addition of Heparin slows the release of its attached factors, therefore a burst release was not anticipated[9]. Following the results and analysis of an ELISA, a complete, quantitative measure of the released, active EGF in the MAP gel is known for the associated amount loaded into the gel.

Aim 3: Determine the effects of lyophilization of MAP on protein loading. Lyophilization promotes stability of samples and can prolong the shelf life of loaded proteins in storage[10]. Assuming lyophilization has minimal effects on the efficacy and characterization of the EGF-loaded gel, the stabilization of the product is more commercializable and uniform for users. A cell migration assay and ELISA was run with lyophilized and non-lyophilized samples of the MAP-EGF formulation to evaluate the effects of lyophilization on protein release and efficacy on cell spreading.

Results

Aim 1: Optimization of MAP-EGF Formulation

First, this project aimed to find an ideal formulation of EGF loaded into MAP gel. This formulation would be used in the two subsequent aims of the project. A full optimization was not conducted due to time and resource constraints as this would have required a modeling component for optimization. Therefore, orders of magnitude were employed in order to decide upon an ideal formulation. The original variables for test conditions were percent of gel particles containing heparin and concentration of EGF in the MAP gel. Heparin, a strongly negative GAG, was

incorporated into the gel to promote strong interactions between the growth factor and attract it to the gel. The percentages of heparin in the gel were either 0.5% or 5%. The concentrations of EGF were either 0.1 ug/mL or 1.0 ug/mL of EGF. The control condition for the first phase of Aim 1 contained no heparin or EGF, a standard MAP gel previously proven to elicit cell spreading.

The fold changes of the area of the cell spreading from the initial time point to each of the successive time points, including 24, 48, 72 and 96 hours, were analyzed and compared to each other. The fold changes at 24 and 48 hours demonstrated the most conclusive results and thus were considered in the decision of the condition with which to move forward. The cell spreading at time points following 48 hours (72 and 96 hours) was extensive, producing much variability in the data. In addition, the sample containing 0.5% heparin and 0.1 ug/mL of EGF was excluded from analysis and eliminated moving forward as it qualitatively demonstrated less cell spreading early on.

Analysis of the fold changes at 24 hours allowed for further elimination of potential MAP-EGF candidates. As seen in Figure 1, the fold changes for the 5% heparin gel groups were greater than both the control and the 0.5% heparin conditions. The no heparin and no EGF control gel resulted in a fold change of 1.11. The 0.5% heparin, 1 ug/mL of EGF gel had a fold change of 1.16. The 5% heparin conditions of 0.1 ug/mL of

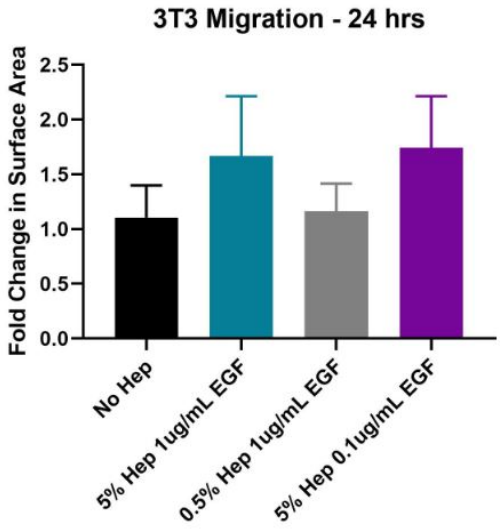

Figure 1: 3T3 Cell Migration 24 Hour Fold Changes. The surface area fold change at the 24 hour time point depicts a nearly equivalent surface area for both 5% heparin gel samples. Through a one way ANOVA, there was no significant difference amongst the groups as the number of samples of 4 was low. Through qualitative analysis, the 0.5% heparin gel was removed from further study as it produced visibly lower cell spreading.

EGF and 1.0 ug/mL of EGF demonstrated greater fold changes at 1.74 and 1.67, respectively. This phase of Aim 1 allowed for the elimination of the 0.5% heparin gels from future assays due to their lower fold changes.

Phase 2 of Aim 1 focused on the 5% heparin gels to determine whether 0.1 ug/mL or 1.0 ug/mL of EGF was an ideal concentration for the MAP-EGF formulation. A comparable assay to phase 1 was carried out, except for the addition of Human Dermal Fibroblasts (HDFs) instead of 3T3s. HDFs were used in this study because as a primary cell line, rather than the immortal cell line of 3T3s, they are more relevant for wound healing. In addition, a second control of 5% heparin and no EGF was included as a control to analyze the sole effect of heparin on cell spreading. This cell migration study extended over 48 hours with time points also taken at the initial 0 hours and 24 hours.

Analysis of the fold changes at 24 hours yielded a statistically significant difference in cell spreading (Figure 2). The no heparin and no EGF control gel, the second control of 5% heparin and no EGF, 5% heparin and 1 ug/mL of EGF, and 5% heparin and 0.1 ug/mL of EGF resulted in fold changes of 1.12, 1.27, 1.73, and 1.26, respectively. A one-way ANOVA was carried out to determine significance. As Figure 2 displays, the 5% heparin and 1 ug/mL of EGF has a significantly higher fold change than the other conditions. This phase guided the

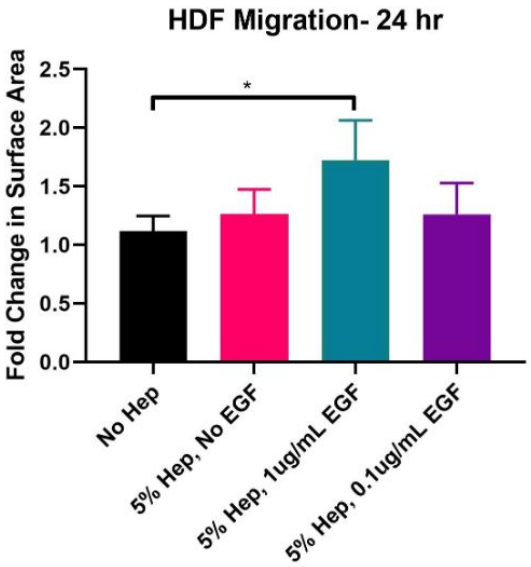

Figure 2: HDF Cell Migration 24 Hour Fold Changes. A one way ANOVA determined that the surface area fold changes at the 24 hour time point were significantly different amongst the groups, except for 5% Heparin, 0.1 ug/mL of EGF. Therefore, the project proceeded with 5% Heparin and 1 ug/mL of EGF.

decision of the desired formulation of 5% heparin and 1 ug/mL of EGF due to its higher fold change.

Aim 2: Load and Release of EGF from MAP Gel

The goal of the second aim was to characterize and understand how much EGF was loaded into MAP gel and released by the gel. The ideal formulation decided upon in Aim 1 of 5% heparin and 1 ug/mL EGF was used. The characterization was accomplished through ELISAs run over specific time points over 72 hours. The remaining EGF in the supernatant solution after the gel was passively loaded was compared to the known total amount of EGF loaded into the gel (calculated from 1 ug/mL) to yield a loading efficiency of 87%. In addition, the release profile was obtained in conjunction with the same assay for Aim 3.

Aim 3: Lyophilization of MAP-EGF Formulation

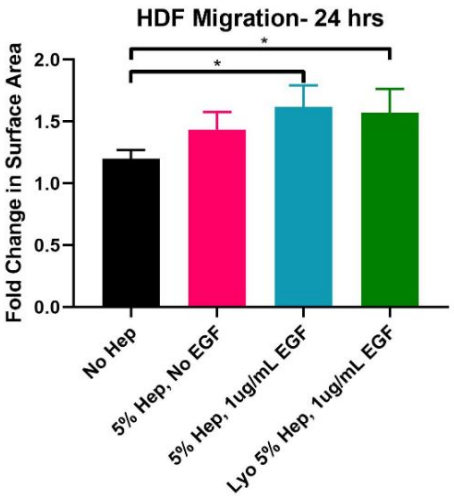

Figure 3: Lyophilization vs. No Lyophilization Cell Migration 24 Hour Fold Changes. In order to determine whether lyophilization impacts the effects of the gel on cell migration, the otherwise identical lyophilized and non-lyophilized samples were compared to each other and two controls: no heparin gel and 5% heparin gel. Two one-way ANOVAs determined that there was a significant difference between each of the experimental conditions, non-lyophilized vs. lyophilized 5% heparin and 1 ug/mL of EGF, and the control conditions. There was no significant difference in effect between non-lyophilized and lyophilized.

The final phase of this project aimed to evaluate the impact of lyophilization on the efficacy of the MAP-EGF scaffold. A lyophilized product is advantageous as it improves stability and shelf life. To assess the effects of lyophilization, a functional cell migration assay and an ELISA, to characterize the release profiles, were run on lyophilized and non-lyophilized samples of the optimized MAP-EGF formulation.

In the cell migration assay, four conditions of MAP gel were plated with spheroids of HDFs: the two samples of the lyophilized and non-lyophilized 5% heparin, 1 ug/mL MAP gel and two controls, one

without heparin and one with heparin but without EGF. The no heparin gel represented the original, unmanipulated MAP gel. The 5% heparin, no EGF gel served as a comparison to evaluate the effects of additional EGF as seen in the optimized gel samples. The fold change, or the ratio between the amount of cell spreading since plating over the initial spheroid area, 24 hours following gel and cell plating were 1.20, 1.44, 1.62, and 1.57 for no heparin, 5% heparin no EGF, non-lyophilized MAP-EGF, and lyophilized MAP-EGF, respectively (Figure 3). Following a one-way ANOVA with a p-value of less than 0.05, both optimized conditions, lyophilized and non-lyophilized, had fold changes that significantly varied from the no heparin, no EGF gel. However, the fold change of the lyophilized gel did not statistically differ from the non-lyophilized gel.

To characterize and compare the release profiles of lyophilized and non-lyophilized gel, an ELISA was run on supernatant samples of the loaded lyophilized and non-lyophilized gels. As seen in Figure 4, the difference between the release profiles of the gels is not statistically significant, as each pair of data points falls within each other's standard deviations. Both gels were loaded with approximately 7500 picograms (pg) of EGF. One hour following the loading of the gels, the lyophilized and non-lyophilized gels had released 2077 and 2777 pg, respectively. Following the last release sample taken 72 hours after loading, the lyophilized and non-lyophilized gels had released 3060 and 3511 pg, respectively.

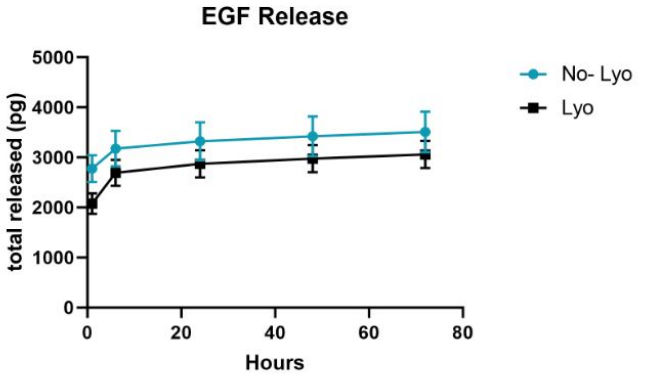

Figure 4: Total EGF Release of Lyophilized and Non-Lyophilized MAP Gel. The cumulative release curves of lyophilized and non-lyophilized gels are comparable with no qualitative difference. The gels were loaded with approximately 7500 pg of EGF. Both gels released less than 3000 pg in the first hour and have a sustained release across the 72 hour study.

Discussion

Aim 1: Optimization of EGF-MAP Formulation

Through an orders of magnitude optimization study of the MAP-EGF formulation, the decided upon formulation was 5% heparin and 1 ug/mL of EGF. The first phase of 3T3 cell migration studies allowed for the elimination of 0.5% heparin gels from the conditions as they displayed lower fold changes. In the second phase, through HDF cell migration studies, it was determined that 5% heparin and 1.0 ug/mL of EGF is optimal as it had a significantly higher fold change at 24 hours. In addition, this formulation provided higher confidence due to its higher order of magnitude of 1.0 ug/mL of EGF, presenting the possibility of less variation when preparing the gels loaded with EGF. This formulation of 5% heparin and 1.0 ug/mL of EGF was subsequently used in the studies for Aims 2 and 3.

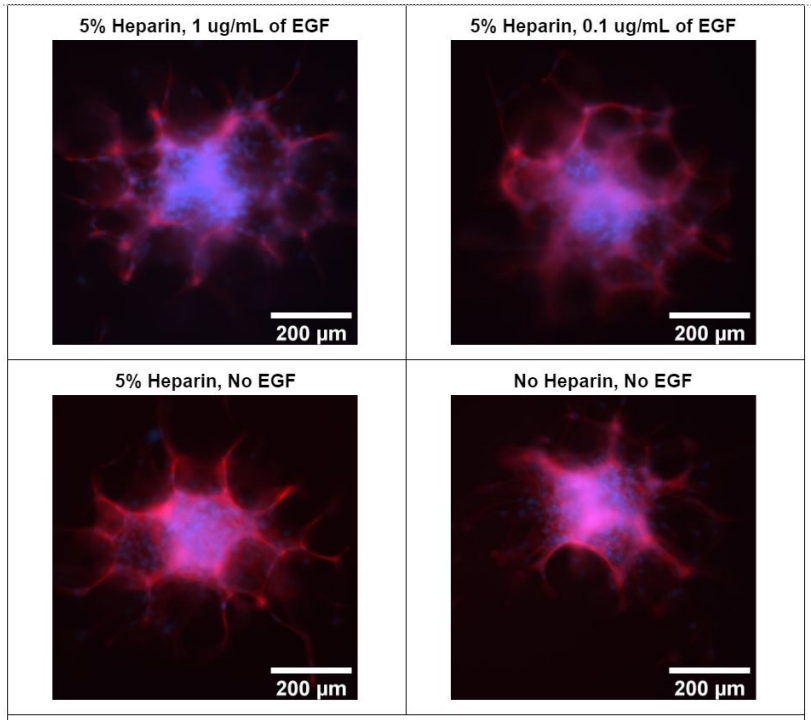

Figure 5: Cell Spreading of HDFs on 5% Heparin, 1.0 ug/mL EGF MAP Gel at 48 Hours. Qualitatively, the gels containing EGF show more cell spreading compared to the MAP gel that was not loaded with EGF. Blue stained DAPI. Red stained for actin.

Figure 5, stained HDF cell migration images of the second phase of Aim 1, displays qualitatively better cell spreading in the 5% heparin gels after 48 hours. As images such as these were analyzed to calculate fold change, it must be noted that the initial spheroid size for each condition may have been variable. These images still display that there was qualitatively more cell

spreading in the 5% heparin and 1 ug/mL of EGF than the other groups. The actin can be seen extending through the pores in MAP gel in response to the released EGF. In the future, this ideal formulation will be carried forth in designing a treatment option for diabetic wound healing. Paired with similar controls, an identified next step of this project is testing this formulation in murine models. In addition, if brought through the regulatory process of the FDA in anticipation of clinical use, this formulation would be tested in human clinical trials in order to understand its functionality in wound healing.

Aim 2: Load and Release of EGF from MAP Gel

Heparin, as a glycosaminoglycan, is incorporated into the MAP scaffold due to its strong negative electrostatic interactions with EGF[9]. The 87% loading efficiency indicates that heparin is an effective mechanism of attraction of EGF for MAP. Further, sustained release of EGF was observed over the entire 72-hour study, presumably due to the loading efficiency of heparin. Therefore, no burst release of EGF occurred. Through the release profile of EGF from MAP (expanded upon in Aim 3), it was determined that heparin successfully fosters an interaction between MAP and EGF and promotes a sustained release over the entire 72 hours.

Aim 2 ELISAs provided important preliminary data for the Griffin lab if ever this formulation of MAP-EGF were brought to market. The FDA would inquire into how much EGF is actually administered to a patient and thus loading efficiency of EGF into the gel would be a factor in this equation. In addition, it would be vital to know the release profile of EGF by MAP in a patient to know at what times the wound is receiving the released EGF.

Aim 3: Lyophilization of MAP-EGF Formulation

Qualitatively, the fold changes of the 5% heparin, 1 ug/mL of EGF lyophilized and non-lyophilized gels were higher than both controls (no heparin, no EGF and heparin, no EGF) but do

not appear different from each other. The quantitative statistical difference between the optimized samples and the controls confirms that the MAP-EGF formulation has a significant impact on cell spreading (Figure 3). Further, the lack of a statistically significant difference in fold change between the lyophilized and non-lyophilized gel indicates that lyophilization does not affect the MAP-EGF's impact on cell spreading.

The release profiles of the lyophilized and non-lyophilized gels qualitatively follow a similar pattern (Figure 4). Quantitatively, the lack of statistically significant difference in the release profiles between the two gels suggests that lyophilization does not impact the release of EGF from the gel. Because their release profiles do not significantly differ, it can also be assumed that they have a comparable loading capacity. Further, less than ⅓ of the approximate amount loaded into the gels was released in the first hour. This confirms that the gel does not have a burst release as it retained the majority of the EGF. Over the 72 hours of the study, the gel retained over half of the EGF loaded, successfully implementing a sustained release. This is promising data for a future murine wound healing model, as it will last over the same time period of 72 hours.

With both the functional and characterization assays yielding no statistically significant difference between the lyophilized and non-lyophilized gels, it is concluded that lyophilization does not significantly impact the efficacy of the MAP-EGF formulation.

Materials and Methods

MAP Gel Formulation

MAP gel is composed of two solutions combined in a one-to-one mixture using microfluidic water-in-oil emulsion. The first solution contains a four-arm poly(ethylene glycol) maleimide (PEG-MAL) and RGD cell adhesive peptide (Ac-RGDSPGGC-NH2) dissolved in 10X

phosphate buffer saline (PBS) at pH = 1.5. The second solution consists of a four-arm poly(ethylene glycol) thiol (PEG-SH) and heparin dissolved in 1X PBS at a pH of 7.4. A 3.2 wt% gel is used for the no heparin gel and a 2.2 wt%, 6 mg/mL heparin gel when heparin is incorporated. The final heparin concentration, therefore, is 3 mg/mL, which matches mouse skin heparin concentration. These gels are mechanically matched at 18kPA, though. These two solutions combine to form the aqueous pre-gel solution. Following preparation of the microfluidic device, the solutions were combined one-to-one.

The microfluidic device has two inlet channels and one outlet that are connected to different sets of tubing (Figure 6). The inlet channels were fit with tygon tubing fixed with peek tubing on both ends, one to serve as the aqueous tubing and one to serve as surfactant tubing. The outlet tubing consists of tygon tubing. With the tubings connected to their corresponding ports, the device was flushed with oil. Then, the surfactant tubing was primed with surfactant solution (1% Picosurf).

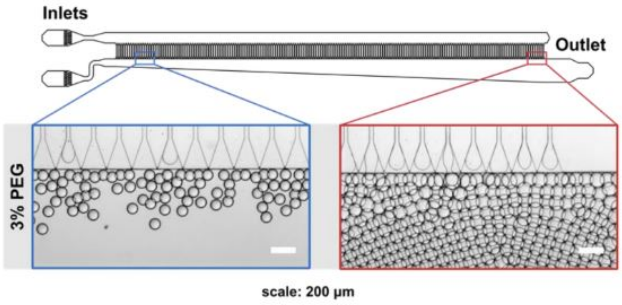

Figure 6: Microfluidic Production of MAP Gel. The microfluidic oil-in-water system creates consistently monodisperse gel particles quickly and efficiently. J. M. de Rutte, J. Koh, D. D. Carlo, *Advanced Functional Materials*.**0**, 1900071.

The pre-gel solution was loaded into a syringe following the loading of 0.05 mL of oil to prevent the gel from sticking to the walls of the syringe, tubing, or device. The gel was combined at a speed of 5 mL/hour using a syringe pump. After allowing the gel to settle, the gel was purified using a series of oil washes followed by PBS washes and hexane-PBS washes. The gel was then quenched and sterilized with a number of 70% IPA washes and PBS washes.

To load the growth factor into the gel, 200 uL of MAP was resuspended at a one-to-one ratio in either 0.1 or 1 ug/mL protein solutions and incubated for 48 hours while rotating at 4

degrees Celsius. The solutions were then lightly centrifuged and filtered through a 0.22 um filter to separate the gel from the supernatant solution. The gel was then placed in PBS until use.

Cell Migration

MAP gel loaded with EGF of varying weight percentages and concentrations are evaluated using a cell migration assay. Gels of 5% and 0.5% heparin weight percentages and 1 ug/mL and 0.1 ug/mL EGF loading concentrations were made for a total of four different combinations of EGF-loaded gels. The migration assay quantitatively measures migration in a 3D environment. First, cell spheroids were created by a hanging drop method over a 3-day incubation period. A cylindrical puck of MAP (infused with 2mM LAP in media) 1 mm in thickness will be annealed and transferred to a 24-well non-tissue culture treated plate well. The spheroid was then transferred to the surface of the puck, incubated, and imaged at desired time points. The gel with the most significant migratory response indicates the optimal EGF weight percentage and concentration moving forward.

Two cell lines, 3T3s and HDFs, were used in the cell migration assays. Both cell lines were cultured and maintained following manufacturers' guidelines. 3T3s were initially used before switching to HDFs as a primary, more sensitive cell line was needed in order to conduct more meaningful analysis of the 5% heparin and 1 ug/mL and 0.1 ug/mL of EGF conditions. Using an EVOS microscope, images were taken every 24 hour period over 72 hours, starting with the first image one hour after plating.

Loading and Release Characterization

Using the 5% heparin, 1 ug/mL of EGF MAP formulation determined in Aim 1, an ELISA was run to characterize the release associated with the known loaded concentration using an ELISA kit (R&D Biosystems, Inc.). A standard ELISA was run with samples taken over multiple time

points in the first day (1 hour, 6 hour) and 24 hour periods following the release from the gel over 72 hours.

Gel Lyophilization

The gel was placed in 90% IPA at a one-to-one ratio and mixed with a positive displacement pipette. The gel-IPA mixture was then flash frozen using liquid nitrogen. Following flash freezing, the sample was lyophilized for 48 hours. Once the gel was lyophilized and ready for use, the desired amount was rehydrated with a PBS-EGF mixture and sonicated for one hour.

Image Analysis

Cell migration images were analyzed using ImageJ's tracer tool. The perimeter of the cell spreading was traced and the resulting area quantified. Fold changes were then calculated by comparing the data across the time points for each condition.

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