Optimizing Transfection Efficiency and Determining Release Kinetics of Plasmid DNA from Polyurethane Scaffolds *in vitro*

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KEYWORDS. Skin, healing, transfection, scaffolds

BRIEF. The purpose of this study was to deliver DNA from biomaterial scaffolds to cells in vitro.

ABSTRACT. Skin wound healing is driven by growth factor signaling. When the healing process fails, one way to fix it is by delivering DNA containing genes for growth factors to cells. DNA must be complexed with compounds that can help protect it from being destroyed before entering the cell. In this study we found the best method for delivering such complexes. Once the method was determined, the complexes were put in synthetic scaffolds to determine the effectiveness of these scaffolds as delivery vehicles. Over the course of 9 days, the amount of the complexes released from the scaffolds was measured. The complexes were able to diffuse out of the delivery vehicle; however, the genes were not expressed by the cells. Further steps will need to be taken to protect the complexes and ensure that cells are expressing the delivered genes to achieve successful skin regeneration.

INTRODUCTION.

The skin is a primary defense against infection or disease. Burns and cuts can lead to very large and severe skin wounds, leaving the body exposed to infection or disease for an extended amount of time. In the body, the healing process of these wounds is generally driven by growth factor signaling that stimulates cellular processes such as proliferation or differentiation. Abnormal wound healing can be due to communication failures or a lack of growth factors. One way to approach this problem is to use delivery vehicles to distribute genes for growth factors into cells. Delivering genes for growth factors into the skin wounds could enhance healing and lead to skin regeneration.

However, to successfully deliver genes for growth factors or transfect cells, there are several criteria that have to be met. Firstly, the delivery vehicle must successfully transport biological molecules: in this specific experiment, a gene that produces luminescence (known as luciferase) was used in place of a growth factor gene. For this study scaffolds made of synthetic biomaterial called polyurethane (PUR) were used as delivery vehicles to distribute luciferase. This specific material was chosen because of its porosity, injectability, and biodegradability. Porosity is an important characteristic because it allows cells to quickly migrate into the scaffold. Injectability is advantageous because it allows the scaffold to fill irregularly shaped wounds. Finally, biodegradability of the scaffold ensures that all complexes are released and avoids the need for surgery to remove the delivery vehicle from the wound site.

Secondly, a positively charged compound must be used to condense the DNA into complexes that are small, stable and positive – three characteristics needed for complexes to enter a cell. In this study two compounds, hyaluronic acid (HA) and polyethyleneimine (PEI), were added and different ratios were investigated to determine the best conditions in which the complexes containing the luciferase gene would enter as many cells as possible.

Once the best ratio was established, complexes made with these ratios were lyophilized (freeze dried) and added to the PUR scaffolds. For 9 days, the complexes were observed to determine whether they would diffuse from the delivery device. Complexes that were successfully released were then tested for their ability to enter cells. This experiment was conducted to ensure that eventually genes for growth factors that enhance wound healing could be successfully delivered from PUR scaffolds to a wound.

METHODS.

Transfection Procedures/Synthesis of PEI-HA-pDNA.

Cells from IMDF and MC3T3 cell lines were plated in 96 well plates at 100000 cells per well 24 hours before transfection. To make PEI-HA-pDNA complexes, PEI, plasmid DNA (pDNA), and HA were first diluted in DPBS or OPTI-MEM. Equal amounts of each dilution were then mixed together to obtain final mixtures with a DNA concentration of 3 ug/mL and PEI-HA-pDNA charge ratios of 12:0:1, 12:6:1, 12:12:1, and 12:24:1 (for PEI-HA-pDNA charge ratio of 12:0:1, DPBS or OPTI-MEM was added in place of HA). Half of the complexes made were added immediately to the cells at 100 µL per well while the remaining half were incubated at 37°C for 24 hours. To make complexes with positive control, pDNA and Lipofectamine 2000 were diluted in OPTI-MEM, and the resulting solutions were then mixed together to obtain a final mixture with a DNA concentration of 8 µg/mL. After incubating mixture at room temperature for 20 minutes, these positive control complexes were added to the wells at DNA concentrations of 4.8 µg/mL, 3.2 µg/mL, 2.13 µg/mL, and 1.42 µg/mL per well. After adding all complexes, cells were incubated with 5% CO_2 at 37°C. One hour after transfection, 100 µL of αMEM were added to wells containing complexes made in DPBS. Four hours after transfection all media was replaced and finally, 24 hours after transfection luciferin was added to cells, which was broken down by luciferase in a reaction that produced luminescence. A SpectraMax plate reader (Sunnyvale, CA) was then used to measure the emitted luminescence signal. The same transfection procedure was repeated for the PEI-HA-pDNA complexes that were incubated for 24 hours at 37°C.

Synthesis of Lyophilized PEI-HA-pDNA.

To make lyophilized complexes, PEI, HA, and pDNA were diluted in water and mixed together to obtain final mixtures with a DNA concentration of 6 µg/mL and PEI-HA-pDNA ratios of 12:0:1, 12:6:1, 12:12:1, and 12:24:1. To each complex, trehalose was added at a trehalose to DNA mass ratio of 1000:1. Complexes were then frozen at -80°C and freeze-dried overnight. After freeze-drying, all complexes were reconstituted in DPBS or OPTI-MEM and only half were added immediately to cells using the same transfection procedure as above. The remaining half were incubated at 37°C and added to cells 24 hours following incubation. For both Day 0 and Day 1 lyophilized complexes, luminescence was detected 24 hours after transfection using a SpectraMax plate reader (Sunnyvale, CA).

Size Measurement and Zeta-Potential.

Complex sizes and zeta potentials were measured with a Zetasizer (Worcesterchire, UK). Aqueous solutions prepared the day of complex formation were used to analyze size distribution of complexes and zeta potentials.

Synthesis of PUR Foams/Incorporation of Complexes.

A blank PUR foam (without lyophilized complexes) was made by mixing together a polyol mix comprising glycolide, lactide, and caprolactone; water; triethylene diamine catalyst; and calcium stearate pore opener with lysine triisocyanate (LTI), to make a final foam with a mass of 200 mg. The PUR mixture was incubated at room temperature until curing was achieved. A similar procedure was followed for the PUR foam containing complexes. Lyophilized (powdered) complexes were added to the polyol mix before mixing the polyol with the isocyanate. This plasmid foam was also incubated at room temperature until curing was achieved.

Measuring the Release Kinetics of pDNA from PUR Foams.

Both blank and plasmid foams were cut into three pieces, each piece being approximately 0.044g. Each piece was immersed in 1000 µL of phosphate-buffered saline (PBS) and incubated at 37°C for 24 hours at a time for 9 days. After each 24 hour time point, the releasate (PBS) was collected and each foam piece was reimmersed in fresh PBS and incubated again for 24 hours. Part of each releasate was used to analyze complex size with a Zetasizer (Worcesterchire, UK) and to test for presence of pDNA using a Nanodrop Spectrophotometer (Wilmington, DE) while the remaining was added to IMDF cells to determine whether or not the releasate was still able to transfect cells. When it was determined that complexes in the releasate were not transfecting cells, only pDNA release was monitored over the remaining 8 days.

Data Quantification and Analysis.

To determine transfection efficiency, positive control standard curves were formulated using relative luminescence data. However, due to significant inconsistencies among the standard curves produced transfection efficiencies were not calculated. Instead, optimal conditions were determined using the luminescence signal readings of each of the complexes. Furthermore, data provided by the zetasizer was used to compare complex sizes and determine whether or not HA had stabilized the complexes. Similar size measurements were repeated for complexes in foams. In addition, DNA concentration in the releasate determined by the nanodrop was used to measure cumulative release of the complexes from the scaffold over the span of 9 days.

RESULTS.

Optimizing Transfection Efficiency in vitro.

After thorough analysis of the relative luminescence data obtained from each experiment, the optimal condition for cell transfection was determined. Both fresh (data not shown) and lyophilized results showed that complexes made in DPBS with a PEI:pDNA charge ratio of 12:1 that were added to IMDF cells transfected the greatest number of cells (Figure 1 and S1). This was made evident through the positive relative luminescence signal. Complexes without HA for both days 0 and day 1 had relative luminescence units (RLU's) greater than 50 while all complexes containing HA had RLU values of 0.

However, according to the size distribution graphs, only those complexes containing HA remained stable over time (Figure 2). Between day 0 and day 1 of reconstituting lyophilized complexes, all complexes with HA maintained a size of roughly 200 nm, whereas, the complex condition not containing HA aggregated over time from 521.6 nm to 997.6 nm (Table S1 and S2). Zeta potential values also show that complexes containing HA had a net negative charge while the complex without HA had a net positive charge.

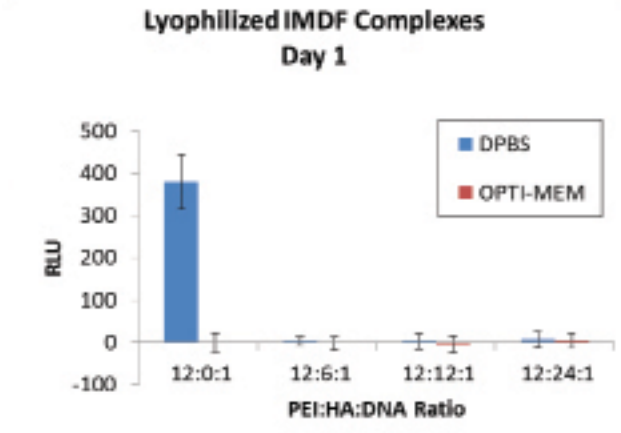

Figure 1. Luminescence signal of PEI:HA:pDNA complexes at day 1 (24h after being reconstituted). Only complexes without HA in DPBS produced positive luminescence readings.

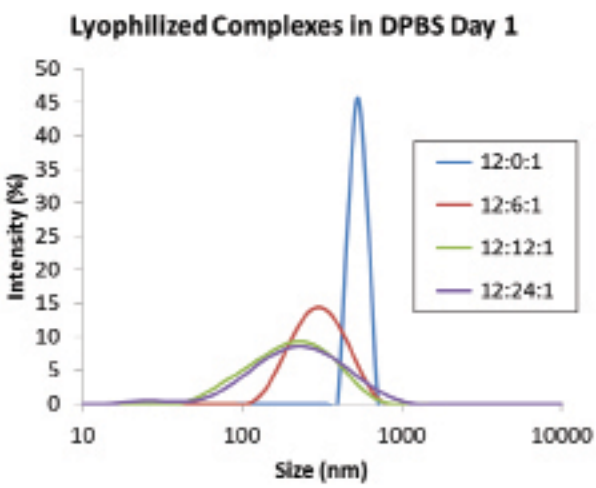

Figure 2. Size distribution of lyophilized complexes at day 1. Complexes containing HA remained stable maintaining relatively small sizes while complexes without HA appeared to be greater in size.

Measuring the Release Kinetics in vitro.

Pore size, release kinetics, and cellular transfection ability were determined to further characterize the complexes and PUR foams. A scanning electron microscope image of the foam showed 200-500 um sized pores with an aggregated amount of complexes in the center (Figure S2). Aggregated complexes are less likely to transfect cells because of their large sizes, therefore, an even distribution of complexes throughout the scaffold is more favorable.

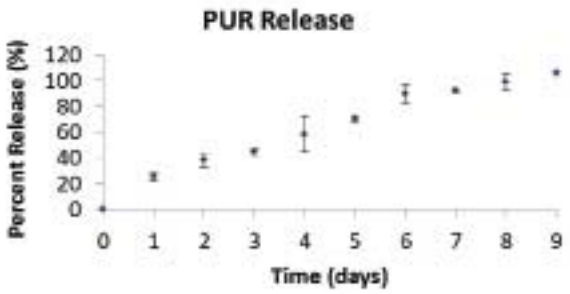

Figure 3. Cumulative release of complexes from PUR foam over nine days. No intial burst was observed, but instead a sustained release over time.

Furthermore, Figure 3 shows that for complexes made in DPBS with a PEI:HA:pDNA charge ratio of 12:0:1, there was a sustained release over the course of roughly a week. No initial burst was observed which ensures that the complexes will be released at a constant rate over time.

To ensure that complexes being released from the foam were not only able to escape from the scaffold but also able to transfect cells following release, part of the releasate was added to IMDF cells and a luminescence reading was taken (Figure S3). The three replicates of the same PUR scaffold incubated in PBS were unable to transfect any cells as indicated by the values near 0 for RLU's (Figure S3). This was compared with the lyophilized complexes (while still a powder, before being incorporated into PUR foams) which successfully transfected a greater number of cells and therefore had RLU's surpassing 40.

Finally, size distribution of the complexes in the releasate and lyophilized complexes was measured (Figure S4). Although complexes in the releasate were unable to transfect cells while lyophilized complexes were, it was determined that all complexes were roughly the same size ranging from 740 nm to 990 nm (Table S3).

DISCUSSION.

Complexes made in DPBS media with a PEI:HA:pDNA charge ratio of 12:0:1, were determined to be the optimal complexes for cell transfection *in vitro*. These unique complexes demonstrated the most reasonable characteristics compared with other observed conditions. Complexes made in OPTI-MEM aggregated a large amount over a period of 24 hours and were therefore unable to penetrate cell membranes (data not shown). This aggregation could have been due to the interactions between the PEI in the complexes and the serum proteins present in OPTI-MEM media [3]. On the other hand, complexes made in DPBS were smaller in size primarily because of the lack of serum proteins in this media. It is important to consider this aggregation issue when complexes will be incorporated *in vivo*. Here, serum protein-based blood will be one of the main body fluids with which complexes will come into contact, and aggregation between PEI and this abundance of serum proteins must be prevented to allow for complexes to penetrate cell membranes.

Furthermore, complexes with HA demonstrated qualities not suitable for cell transfection. As established by the *American Society of Gene and Cell Therapy*, HA did stabilize all complexes over time by preventing drastic aggregation [3]. However, HA also made all complexes have a net negative charge which prevented them from being engulfed by cells. As a result all complexes containing HA were unable to transfect any cells even though their size distribution range was a lot smaller and they were more stable than complexes without HA. A smaller ratio could possibly be used to decrease the influence of HA on the net charge of complexes while still maintaining the stabilizing characteristics of HA.

Despite slight size instability and a small positive zeta potential value, complexes with the PEI:HA:pDNA ratio of 12:0:1 were chosen for incorporating into PUR foams. Several challenges arose during this process as well. First of all, SEM images show that complexes remained in clumps once inside the foam. Unfortunately, none of the complexes were spread evenly throughout the scaffolds in any of the three replicates produced. Secondly, although a sustained cumulative release was observed for the complexes from the scaffold, none of the released complexes transfected any cells *in vitro*. This led to the hypothesis that the lyophilization process had altered the characteristics of the complexes, making them incapable of transfection. However, lyophilized complexes were tested under the same conditions of the complexes from the releasate and relative luminescence readings show that the lyophilized complexes were still active and capable of transfection.

As a result, it was proposed that the size of the aggregated complexes in the scaffold was the characteristic intervening with the transfection process. Yet, looking at size distribution data, increased aggregation was not observed for any of the complexes in the releasate compared to lyophilized complexes at Day 1. Therefore, if neither freeze-drying nor size were the issue, an alternative explanation for the lack of transfection could be that a chemical reaction in the formation of the PUR scaffold adversely affected the complexes. Further steps could be taken to encapsulate complexes in microspheres before incorporation into the PUR scaffold to prevent degradation by any reaction. This perhaps could ensure efficient gene delivery and lead to successful skin regeneration.

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SUPPORTING INFORMATION.

Figure S1. Lyophilized Complexes in DPBS Day 0
Figure S2. Scanning Electron Microscope Image
Figure S3. Luminescence signal of PUR releasate
Figure S4. PUR Delivery Complex Sizes
Table S1. Complex Size Day 0
Table S2. Complex Size Day 1
Table S3. Average size of complexes in releasate

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