Incorporation of DNA Particles into Chitosan Nanofibers for Tissue Regeneration

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Abstract: Controlled DNA delivery to a localized target is an important tool for tissue regeneration. A problem associated with controlled DNA delivery is that naked DNA may degrade, causing a loss of function and decreased control of the release rate. Encapsulation may protect DNA from such degradation. In addition, a sufficient delivery vehicle and incorporation method is needed for targeted delivery. In this study, encapsulation was performed by mixing DNA with a cationic polymer, polyethylene imine (PEI), which surrounded the negatively charged phosphate DNA backbone via charge neutralization. A DNA delivery vehicle of a chitosan-based nanofiber scaffold was investigated. Chitosan nanofibers have been found to possess high surface area and porosity, which maximizes cell interaction and tissue regeneration, and it is biodegradable and biocompatible. Chitosan was mixed with polyethylene oxide and the scaffolds were prepared with an electrospinning process. DNA-PEI nanoparticles were incorporated into the nanofibrous scaffolds before electrospinning by addition to the polymer solution. By this method, encapsulated DNA was successfully incorporated into chitosan-based nanofibers. Further investigation of the degradation behavior of the nanofibrous scaffolds and stability of the DNA function will determine the system potential for controlled DNA delivery.

1. INTRODUCTION

The ability to control DNA release at a localized site is an important tool for the advancement of gene therapy and tissue regeneration. For controlled release, maintained stability and DNA function are required. Naked DNA is susceptible to degradation due to environmental factors inside the tissue causing a decrease in function and control of the release rate. Encapsulation of DNA has been found to increase DNA stability and transfection efficiency. DNA can be encapsulated through the formation of DNA complexes with a cationic polymer, such as polyethylene imine (PEI), which has been successfully used to condense DNA into positively charged nanoparticles. The negatively charged nitrogen of the PEI surrounds the negatively charged phosphate backbone of the DNA, shielding the DNA from degradation [2].

For localized DNA delivery, a sufficient delivery vehicle is required. To mimic the extracellular matrix (ECM), nanoscale polymer scaffolds have been investigated with high surface area and porosity to promote tissue regeneration. Nanofibrous mats can be produced by electrospinning polymers and have shown to promote cell adhesion and viability. The electrospinning method uses a highly charged (10 – 25kV) polymer, which charges the ions that are attracted to a grounded collector. Nanofibers can be formed and deposited using a specific polymer concentration and solution viscosity. Biodegradable polymers such as polycaprolactone (PCL) and poly-lactic-co-glycolic acid (PLGA) have been electrospun to form nanofibrous scaffolds [3]. Dissolution of PCL solutions requires an acidic environment which would alter the negatively charged DNA and is therefore not investigated as a DNA delivery vehicle in this study. Chitosan nanofibers have been successfully produced via electrospinning and are widely used as a biomaterial due to biocompatibility, biodegradability and low cytotoxicity. Chitosan is soluble in 2% acetic acid which is a desirable solvent for DNA-PEI complexes due to the mild pH. For these reasons, chitosan was chosen as the primary polymer to create nanofibrous scaffolds for DNA delivery. Chitosan polymer solutions are often too viscous for the electrospinning process, however introduction of other polymers, such as polyethylene oxide (PEO) decreases the viscosity of the chitosan solution and allows for successful electrospinning of nanofibers [1]. PEO will be investigated with chitosan to produce nanofibrous scaffolds to serve as a DNA delivery vehicle for tissue regeneration.

For DNA delivery, the DNA must be incorporated into the nanofibrous mats. Covalent
linking of DNA to polymer surfaces has been reported to decrease in functionality and release rate control. An alternative method to incorporate DNA is prior creation of the nanofibrous mats. Incorporating DNA-PEI complexes into the polymer solution before electrospinning allows for controlled DNA concentrations in the nanofibrous mats.

2. MATERIALS AND METHODS

2.1 Materials
Chitosan, medium molecular weight, and PEO were obtained from Aldrich. Dimethylsulfoxide (DMSO) and Triton X-100 were obtained from J.T Baker and VWR International, respectively. Oregon Green 488-X succinimidyl ester and Sybr Green were purchased from Invitrogen. Genepin was obtained from Challenge Bioproducts.

2.2 DNA encapsulation
DNA and PEI solutions were prepared separately prior to DNA encapsulation. Plasmid DNA was prepared at a concentration of 870 μg/ml. PEI was dissolved in HEPES buffer solution (HBS) with a final concentration of 10 mg/ml. DNA and PEI solutions volumes were calculated using the molar weights at a desired ratio of 19:1 nitrogen:phosphate (N/P) such that the final concentration of DNA was 100 μg/ml in HBS. Different N/P ratios were tried and a 19:1 N/P ratio gave the best results for DNA encapsulation to create DNA nanoparticles. First, DNA was mixed with HBS in a microcentrifuge tube followed by PEI. The solution was vortexed for a few seconds and incubated for 10 min. The nanoparticle size was verified with dynamic light scattering, passing a beam of light through a solution causing the light to scatter when it comes in contact with particles. To incorporate into the chitosan/PEO, the DNA-PEI solution was diluted to 50 μg/ml with 2% acetic acid. To ensure stability of the DNA-PEI complexes, the nanoparticle size was verified with DTS.

2.3 Chitosan/PEO solution preparation
Chitosan and PEO solutions were prepared at 3 wt% in 2% acetic acid. A solution with a 50/50 chitosan to PEO ratio was prepared and 1% Triton X-100 and 10% DMSO was added and mixed overnight. For DNA incorporation, a 4 wt% PEO solution in 2% acetic acid was diluted to 3 wt% PEO by addition of the DNA-PEI nanoparticle solution.

2.4 Electrospinning
A 3 ml syringe was filled with the polymer solution and fitted with a pipet tip. The syringe was placed in a holder and the electrode was secured in the syringe. A thin piece of aluminum foil was taped to the circular collector drum. The solution was electrospun at 22 kV with a rotating drum for varying amounts of time.

2.5 Nanofiber/DNA characterization
The morphology of the nanofibers was observed using scanning electron microscopy (SEM, JEOL 7000). The DNA was characterized using SEM and fluorescent labeling of the DNA-PEI nanoparticles. PEI was fluorescently labeled using Oregon Green by the following method. PEI was dissolved in HBS with a final concentration of 10 mg/ml. Oregon Green was dissolved in DMSO at a concentration of 5 mg/ml and 50 μl of the solution was added to the PEI solution and mixed for 1 hr in the dark. The solution was then incubated overnight at 4°C in the dark. The labeled DNA-PEI solution was purified with a PD-10 column using HBS. The labeled PEI was used for DNA encapsulation at a final concentration of 9.52 mg/ml.

DNA was fluorescently labeled using Sybr Green which was diluted to a 1x solution by making a 1:10,000 dilution of Sybr Green to TAE buffer. A solution of DNA was then made with a ratio of 16:1 dye/DNA. This DNA was then used to make the DNA-PEI particles with a final concentration of DNA (~50μg/ml). All solutions were kept in the dark and the electrospinning of fibers with the fluorescently labeled DNA particles was done in the dark.

A degradation test was done on a chitosan/PEO nanofibers mat with incorporated DNA. The mat was neutralized with 1M sodium hydroxide in ethanol for 15 min. It was then rinsed with ethanol and then soaked in 1% Genepin to crosslink the nanofibers and incubated in water overnight.
3. RESULTS AND DISCUSSION

3.1 DNA encapsulation

The encapsulated DNA nanoparticles with a N/P ratio of 19 had an average size of 152nm. After dilution of the 100 μg/ml DNA solution to 50μg/ml with 2% acetic acid, the average size of the nanoparticles did not change immediately after dilution or after solution was kept overnight. To this end, the encapsulated DNA nanoparticles were stable in the acetic acid solution which was the solvent for electrospinning of the chitosan/PEO nanofibers. Both PEI and DNA were fluorescently labeled for DNA characterization in the nanofibers. The method of PEI labeling caused a dramatic increase in particle size for unknown reasons. An alternative labeling method, directly labeling double stranded DNA with Sybr Green, resulted in 174 nm particles. It is desirable for the nanoparticles to maintain a smaller size to minimize interference with nanofiber formation.

3.2 Chitosan/PEO nanofibers

Nanofibers were produced from chitosan polymer solutions both with and without DNA-PEI complexes (Figure 1). In each image in Figure 1, small (~600 nm) regions can be seen dispersed throughout the fibers. These may be due to spray or polymer aggregates formed during the electrospinning process as they are seen in the fibers with and without the DNA nanoparticles. Comparing Figure 1A and 1B, the incorporation of DNA did not affect the size or general morphology of the chitosan nanofibers. In both cases, the fibers ranged from 100-300 nm in diameter. Figure 1C shows chitosan/PEO nanofibers with incorporated DNA after degradation, indicating the general morphology of the fibers was maintained with no significant swelling or degradation, suggesting that the scaffolds were stable in an aqueous environment.

3.3 DNA characterization

Labeling the DNA instead of the PEI produced smaller DNA-PEI particles which affected the nanofiber diameter (Figure 2). Figure 2A shows chitosan/PEO nanofibers with incorporated DNA-PEI particles with fluorescently labeled PEI. The fibers ranged from 200-300 nm in size and the fluorescently labeled DNA fibers (Figure 1B) were smaller, ranging from 50-150 nm. This suggests that the size of the incorporated DNA particles affects the size of the nanofibers.

Figure 3 shows the fluorescence microscope images of chitosan/PEO nanofibers with incorporated DNA-PEI particles with labeled PEI. Figure 3A is a bright field image in which small black areas can be seen inside the fibers, which are believed to be DNA-PEI complexes. Figure 3B is the fluorescent image of Figure 3A, however the signal is weak. Small fluorescing aligned along fibers can be seen at higher magnification (Figure 3C). Chitosan/PEO fibers with no DNA were also examined and some fluorescence was seen in them as well. For this reason, these results were not conclusive and DNA characterization methods are still being investigated, such as the use of Sybr Green to fluorescently label double stranded DNA.
4. CONCLUSIONS

From this study, it has been found that encapsulated DNA can be incorporated into electrospun nanofibers with no effect on the morphology of the nanofibers. It has also been concluded that fluorescent labeling techniques affect the size of DNA nanoparticles. Fluorescent labeling of PEI with Oregon Green requires a purification process which greatly increases the DNA-PEI particle size. Fluorescent labeling of DNA with Sybr Green does not dramatically affect the particle size and is still being investigated for DNA characterization inside the nanofibers. The size of the DNA particles incorporated into nanofibers affects the size of the nanofibers and smaller sized DNA particles are desired. The chitosan based nanofibrous mats with DNA were stable in water. Future studies including
additional DNA characterization with Sybr Green and in vitro studies are currently being investigated.

REFERENCES

