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Novel Modeling Approach to Generate a Polymeric Nanofiber Scaffold for Salivary Gland Cells

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Abstract

Background—Electrospun nanofibers have been utilized in many biomedical applications as biomimetics of extracellular matrix proteins that promote self-organization of cells into 3D tissue constructs. As progress towards an artificial salivary gland tissue construct, we prepared nanofiber scaffolds using PLGA, a biodegradable and biocompatible material.

Method of Approach—We used electrospinning to prepare nanofiber scaffolds using PLGA with both DMF and HFIP as solvents. Using a design of experiment (DOE) approach, system and process parameters were optimized concurrently and their effects on the diameter of the resulting fibers were computed into a single model. A transfer function was used to reproducibly produce nanofibers of a defined diameter, which was confirmed by SEM. The mouse salivary gland epithelial cell line, SIMS, was seeded on the nanofiber scaffolds, and morphology, cell proliferation, and viability were assayed.

Results—Varying two or more parameters simultaneously yielded trends diverging from the linear response predicted by previous studies. Comparison of two solvents revealed that the diameter of PLGA nanofibers generated using HFIP is less sensitive to changes in the system and process parameters than are fibers generated using DMF. Inclusion of NaCl reduced morphological inconsistencies and minimized process variability. The resulting nanofiber scaffolds supported attachment, survival and cell proliferation of a mouse salivary gland epithelial cell line. In comparison with glass and flat PLGA films, the nanofibers promoted self-organization of the salivary gland cells into 3D cell clusters, or aggregates.

Conclusions—These data indicate that nanofiber scaffolds promote salivary gland cell organization, and suggest that a nanofiber scaffold could provide a platform for engineering of an artificial salivary gland tissue construct. This study additionally provides a method for efficient production of nanofiber scaffolds for general application in tissue engineering.

Keywords

Electrospinning; nanofibers; DOE; salivary gland; tissue engineering

1. Introduction

There is a significant clinical need for therapeutic options for patients having xerostomia (dry mouth) due to salivary gland hypofunction, or insufficient saliva production by the

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salivary gland acinar cells. There are three primary causes of xerostomia: 1) Sjögren' Syndrome, an autoimmune disease affecting 1-4 million Americans that is typified by the loss of acinar cell tissue [1]; 2) radiation therapy, which is administered to a large percentage of over 60,000 head and neck cancer patients diagnosed annually in the United States and causes an irreversible loss of acinar cells [2]; and 3) side effects from thousands of medications that affect the salivary function of millions of patients [3]. Patients suffering from Xerostomia experience a decreased quality of life due to increased dental caries, oropharyngeal infections, difficulties with swallowing (dysphagia) and digestion (mucositis), loss of taste, and pain resulting from these conditions [4, 5]. The current treatments for salivary hypofunction include stimulation of salivary flow in residual acinar cells with drugs that have unwanted side effects and artificial saliva, which is an inadequate substitute [6]. One increasingly popular therapeutic strategies for patients suffering from salivary hypofunction is to replace irreversibly damaged or lost tissue with functional engineered tissue [7].

In tissue engineering, one widely used approach has been to prepare a three dimensional (3D) bio-compatible scaffold onto which cells derived from various sources are grown. The scaffold's 3D architecture creates an environment similar to the natural extracellular matrix found in human tissues, leveraging its high surface area and porosity to provide structural support and enhanced cellular adhesion [8-11]. Many previous studies have reported that the specific nanotopography of biomimetic scaffolds affects cellular organization [12-23]. Recently, Oh et al. reported the ability to direct specific differentiation of stem cells by simply changing the nanotopography of the scaffold [24]. Based on these data, we hypothesized that nanofiber scaffolds could be engineered to have optimal properties to support salivary gland cell growth and differentiation.

Processed biocompatible polymer fibers have become a favored scaffold for tissue engineering [25]. Although many materials have been used in electrospun nanofiber scaffolds, poly(lactic-co-glycolic acid) PLGA has been one of the most widely used polymers. Since its approval by the FDA as a constituent in implantable dental and orthopedic devices [15], PLGA has quickly become a preferred synthetic polymer used in biological applications. While it is a hydrophobic material consisting of varying ratio of lactic and glycolic acid monomers, PLGA is classified as a biocompatible polymer in part because it is not known to have any native toxicity, and also because it undergoes degradation by hydrolysis *in vivo* to produce lactic acid and glycolic acid [26-28], which are products of cellular metabolism. The degradation rate can be controlled by altering the ratio of lactic acid to glycolic acid in the polymer [29]. We selected PLGA as a starting material for creating an electrospun scaffold to support salivary gland growth. Owing to the aforementioned advantages and its wide application, it is particularly important to be able to reliably and consistently reproduce PLGA nanofibers of defined dimensions, especially for the study of biological applications.

Due to its simplicity and reproducibility, electrospinning is one of the most commonly used methods for the preparation of fibrous scaffolds that are able to mimic the structure of extracellular matrix scaffold proteins found in human tissues [30]. Electrospinning is a powerful technique for the processing of polymeric biomaterials into nanofibers, which offers control over the diameter and composition of the nanofibers themselves as well as the porosity and thickness of the nanofiber matrices using a relatively simple experimental setup (**Fig. 1**) [31]. Using this technique, fibers ranging from 50 nm to well over 1000 nm can be produced by applying an electric potential to a polymeric solution [30, 32]. When the electrical potential reaches a threshold sufficient to overcome the surface tension of the solution, the solution leaving the needle tip forms a conical shape known as the Taylor Cone (**Fig. 1b**) [33, 34]. Subsequently, overcoming the minimum threshold potential leads to the

elongation and thinning of the polymer as it leaves the cone, resulting in randomly oriented nano or micro fibers collected on a grounded metallic plate.

The physical properties of electrospun fibers are known to be highly dependent upon both the properties of the polymer solution and the experimental conditions. Vasita *et al.* divided the key parameters affecting the process of electrospinning in two categories: system parameters and process parameters [35]. The system parameters identified were solution viscosity, surface tension, and conductivity, which were shown to influence both the diameter of the nanofibers and their homogeneity. The inner diameter of the needle, the feeding rate, and the electric potential were identified as process parameters, which also affect the nanofiber diameter. Other process parameters, such as the distance between the needle and the collector, primarily affect the evaporation of the solvent, while movement of the collector affects the pattern of deposited nanofibers [36, 37].

Although there are reports suggesting that there is a simple linear relationship between system/process parameters and fiber diameter [28, 36, 38-40], many studies have implied that the relationship between different process and system parameters is complex. For example, it was reported that increasing the solution concentration leads to an increase in the nanofiber diameter according to a power law relationship; however, high concentrations often resulted in a bimodal distribution of fiber sizes [38]. These discrepancies in reported findings have prompted a need for a systematic and integrated approach to understand how the system and process parameters simultaneously influence fiber architecture. Few studies have investigated how one factor (i.e. solution concentration) varies fiber diameter and morphology when another factor (i.e. potential) is changed. This is evident in the fact that numerous recent publications report significant variability in fiber diameter, as well as production of fibers with diameters in the micron scale when the nano scale was desired [41].

In this study, we report an integrated and systematic approach to produce PLGA nanofibers of a defined diameter for use as cell scaffold as a first step in the design of an engineered salivary gland. Advanced statistical analysis software, Minitab 15™ was used to optimize multiple variables simultaneously. A Design of Experiment (DOE) was prepared to explore the relationship between fiber diameter (Y), and the systems and process parameters (X). Based on results from experimental runs generated by the DOE, Minitab was used to develop a transfer function equation to mathematically relate the parameters, and a series of validation experiments were performed to demonstrate the reliability of the transfer function's predicted values. A salivary gland cell line was seeded on the PLGA nanofiber scaffolds, and the cells were investigated for their ability to attach, survive, and proliferate. We here demonstrate that nanofiber scaffolds can provide a viable scaffold on which an artificial salivary gland can be based.

2. Experimental Methods

2.1. Materials

Poly(D-lactide-*co*-glycolide) (PLGA), with a lactic to glycolic acid ratio of 85:15 and a molecular weight of 95,000 Da, was purchased from Birmingham Polymers (Pelham, AL). Hexafluoroisopropanol (HFIP), dimethylformamide (DMF), sodium chloride (NaCl), and magnesium sulfate (MgSO₄) were purchased from Sigma-Aldrich USA (St. Louis, MO). An automatic syringe pump was purchased from New Era Pump Systems Inc. (Wantagh, NY). 3 ml syringes were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Needles with an inner diameter of 0.25, 0.41 and 0.51 mm were purchased from EFD Inc. (East Providence, RI). Polytetrafluoroethylene (PTFE) tubing was purchased from VWR

International (West Chester, Pennsylvania). All chemicals were used as supplied without further purification.

2.2. Electrospinning Setup

To electrospin a nonwoven mat of nano- and micro-scale fibers, PLGA was dissolved in HFIP or DMF and loaded into a 3 ml syringe. The syringe was loaded into an automatic syringe pump, and PTFE tubing was used to shuttle the polymeric solution from the syringe to a metal needle tip. The needle was suspended vertically over a grounded aluminum collector plate at a fixed distance, and a voltage supply was wired to the metal needle with an alligator clip. The distance between the needle tip and the collector is labeled as the “spinneret-to-collector” distance in all of the figures and tables therein.

2.3. PLGA-DMF Fibers

PLGA was dissolved in *N,N*-dimethylformamide (DMF) solvent at concentrations ranging from 30-35% (w/v) and stirred in a closed container for 18 hours at room temperature to create a homogenous solution, as reported previously [36]. The pumping rate for PLGA-DMF experiments was between 6 and 8.5 $\mu\text{l}/\text{min}$ and the needle inner diameter was 0.41 mm. A positive DC potential ranging from 15 to 20 kV was applied at a spinneret-to-collector distance ranging from 15 to 20 cm.

2.4. PLGA-HFIP Fibers

PLGA was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solvent at concentrations ranging from 5 to 18% (w/v), and stirred in a closed container for 18 hours at room temperature. The pumping rate for PLGA-HFIP experiments was between 3 and 25 $\mu\text{l}/\text{min}$ and the needle inner diameter ranged from 0.25 to 0.51 mm. The electric potential difference applied ranged from 8 to 25 kV and the distances between the needle tip and the collector ranged from 5 to 15 cm. To eliminate HFIP-induced damage to the tube and connectors, metal luer connectors and polytetrafluoroethylene (PTFE) tubing were used.

2.5. Scanning Electron Microscopy (SEM) Characterization of Nanofibers

Fiber diameter and general morphology were characterized using a 1550 field emission scanning electron microscope (SEM) (Leo Electron Microscopy Ltd, Cambridge, UK; Carl Zeiss, Jena, Germany). Samples were mounted on 1cm² stubs using carbon tape and were sputter-coated with ~5nm gold-palladium to avoid charging the sample. Images were collected at a working distance of 3 mm and with an acceleration gun voltage of 2 kV. Using the incorporated digital annotation software, diameter measurements (15 per sample) were acquired, and arithmetic means and standard deviations were calculated for all fibers.

2.6. Statistical Analyses and Surface Plots

All statistical analyses were performed using the statistical software package MinitabTM. Design of Experiment (DOE) analyses were performed using a response surface design with non-coded values. The data was analyzed by mean of regression analysis. The R-squared values were greater than 95%. Within this dataset, none of the system and process parameters evaluated were found to be confounded. Factors with a p-value greater than 0.05 were excluded. The exclusion of factors that failed the null hypothesis yielded the transfer functions which were further optimized using Minitab's optimizer tool and Microsoft Excel's solver tool.

2.7. Cell Culture, Viability Assays and SEM Characterization of Cells on Nanofibers

SIMS, an immortalized adult mouse submandibular salivary gland ductal epithelial cell line [20, 24], was cultured in complete cell media, Dulbecco's Modified Eagle Medium

(DMEM), 10% fetal bovine serum (FBS), and 100 U/ml penicillin and 100 µg/ml streptomycin, as previously described (all Invitrogen, Carlsbad, CA)

PLGA fibers were electrospun onto 12 mm glass coverslips coated with Vectabond (Vector Laboratories) as per manufacturer's protocols and placed into individual wells in a 24-well tissue culture plate. Vectabond-coated glass coverslips without fibers and flat PLGA polymer-coated glass coverslips were used as flat surface and material controls, respectively. Approximately 50 µl of 3% (w/v) PLGA in HFIP was added to each coverslip and air-dried for 24 hrs at room temperature to create the flat PLGA coatings. All surfaces were sterilized by UV irradiation for at least 1 hr, pre-soaked in sterile phosphate buffered saline (PBS) for 48 hrs, followed by 1 hr in complete cell media before cell culture.

For trypan blue viability and cell proliferation assays, SIMS cells (10,000 cells/well) were seeded in complete media. At the indicated intervals, coverslips were removed, transferred to fresh plates, prior to cells being detached by mild trypsinization, treated with trypan blue, and manually counted using a hemocytometer. For fluorescent live-dead assays (Invitrogen), SIMS cells were seeded (10,000 cells/well) on different surfaces as indicated and cultured for 24hrs in complete media. Cells were washed in PBS to remove media and incubated with a mixture of calcein (1µM), to detect live cells and ethidium homodimer-1 (2µM) to label dead cells for 20 mins. Images were obtained using an inverted fluorescent microscope (CellObserver, Zeiss, Jena, Germany) with a 20X objective at excitation wavelengths of 488 nm (to detect the fluorescent biproduct of calcein metabolism) and 543 nm (to detect ethidium).

For SEM, approximately 3×10^4 cells/well were seeded and allowed to adhere overnight to different surfaces before fixing and processing for SEM. Briefly, cells were fixed with 3% glutaraldehyde solution in 0.1 M phosphate buffer containing 0.1 M sucrose for 2 hrs at room temperature. The samples were then rinsed three times in PBS, dehydrated in a graded ethanol series and slowly infiltrated with hexamethyldisilazane (HMDS) for drying. SEM characterization of the samples was done using an FEI Nova NanoSEM 600 field emission scanning electron microscope (FEI Company, Hillsboro, Oregon). Uncoated samples were mounted on 1 cm² stubs using carbon tape and imaged in low vacuum mode with water vapor introduced into the specimen chamber to minimize sample charging. An extraction voltage of 4 kV and a working distance of 5 mm was used for imaging.

3. Results and discussion

3.1. Development of a transfer function for PLGA nanofiber synthesis using DMF as a solvent

To examine the suitability of a nanofiber scaffold for salivary gland cell growth, it was necessary to reproducibly produce fibers of a defined diameter. Since previous work and our own preliminary studies indicated that there is not a linear relationship between all the process and systems parameters, we used the statistical analysis software package Minitab 15™ to define conditions for representative experimental runs. For this analysis, the independent variables were defined as PLGA concentration (X_1), potential (X_2), feeding rate (X_3) and the spinneret-to-collector distance (X_4). These parameters were selected using non-coded low and high values for each. Parameters and run orders were randomized using a custom response surface design of experiment (DOE). For the first set of runs, the polar, aprotic dimethylformamide (DMF) was used as the solvent. DMF has a high boiling point (153°C) and a high dielectric constant of 38.3 at 20°C, which makes it a suitable solvent for electrospinning. Using the DOE-specified parameters, multiple nanofibers were produced, and the diameter of the resulting nanofibers were measured using SEM (**Appendix A**) (**Fig. 2**).

From the dataset, we generated surface plots to examine the complex relationship between the individual parameters. Four of the possible six plots are presented (**Fig. 3**). Contrary to previous reports that increasing percentage of PLGA is directly proportional to the diameter of the resulting fiber [36], the diameter does not increase linearly when the potential and the PLGA are evaluated concurrently (**Fig. 3a**). Similarly, the PLGA concentration and feeding rate (**Fig. 3b**), the spinneret-to-collector distance and % PLGA (**Fig. 3c**) and feeding rate and potential (**Fig. 3d**) all demonstrate complex relationships under these conditions. While the potential and the feeding rate are each found to individually promote an increase in the diameter of the nanofibers; when evaluated in conjunction, they adversely affect the diameter of the fibers (**Fig. 3d**). More importantly, fibers generated under these conditions were found to contain non-uniformities referred to as “beads”[36], which are undesirable due to complications in cell imaging and potential non-uniform effects on cell behavior (**Appendix B**), Table 1. For these reasons, an alternative solvent was investigated.

3.2. Evaluation of hexafluoroisopropanol as a solvent for PLGA nanofiber synthesis

Hexafluoroisopropanol (HFIP) was selected as an alternate solvent due to its low boiling point of 58°C, and dielectric constant of 16.70 at 20°C, which is nearly half of that of DMF. Due to the drastic difference in boiling point, a much lower potential can be used to electrospin the nanofibers using HFIP as a solvent as compared to fibers produced when using DMF as a solvent[42]. When fibers were electrospun from a PLGA-HFIP solution, a positive linear relationship was observed, as reported in previous studies [40]. Although beading was reduced with increased PLGA concentration, beading was still prevalent in all samples regardless of solution concentration (**Fig. 4a, 4b, 4c**). To generate a reliable transfer function for electrospinning PLGA nanofibers of uniform morphology, additional changes to the properties of the solution were required.

3.3. Evaluation of the effect of salts on nanofiber diameter uniformity

Previous reports have indicated that the presence of salts can eliminate beading from electrospun nanofibers [36]. We evaluated sodium chloride (NaCl) and magnesium sulfate (MgSO₄), at 1 wt% in each solution, for their effect on nanofiber uniformity when HFIP was used as a solvent. Both salts reduced the total number of beads observed; however NaCl eliminated beads much more effectively than MgSO₄. (**Fig. 4d, e**). The addition of NaCl to an 8.5 wt% solution yielded fiber diameters which ranged from an average of 110 nm to 278 nm, whereas addition of MgSO₄ yielded fibers whose average diameters ranged from 315 nm to 379 nm (**Table 2**). Additionally, the diameter distribution is significantly less with NaCl than it is with MgSO₄ under specific conditions. These effects can also be observed in **Fig. 4d and e**, since addition of NaCl results in fibers of a more consistent diameter than those generated in the presence of MgSO₄. Since NaCl expanded the range over which bead-free nanofibers were produced, all subsequent fibers were prepared using 1 wt% NaCl in HFIP solvent, and the subsequent transfer function generated by Minitab for HFIP used DOE experimental runs with 1% NaCl in solution.

3.4. Design of Experiment (DOE) to produce PLGA Nanofibers using HFIP as a solvent

Using HFIP and 1 wt% NaCl, a DOE-based approach was again used to design a series of experiments to assess the effects of varying both system and process parameters on nanofiber diameter (**Table 3**). A response surface regression was performed to compare the effects of PLGA concentration, the potential, the feeding rate and the spinneret-to-collector distance on the average diameter of the fibers. Another process parameter, the diameter of the needle tip, was controlled and kept constant at 0.51 mm. The analysis was performed using non-coded units and the regression coefficients were evaluated (**Table 3**). As previously observed with DMF, the concentration has the strongest positive correlation with the average diameter of the fibers. An increase in the potential and the spinneret-to-collector

distance negatively affected the diameter of the fibers, while the feeding rate showed the weakest correlation to the average diameter of the fibers (**Table 3**).

To visually map the effect of select factors on the average diameter of the fibers, 3D surface plots were generated. Four of the possible six unique surface maps are displayed (**Fig. 5**). Since the PLGA concentration had the strongest positive effect on the average diameter of the fibers, its effect in concert with the other parameters (potential, spinneret-to-collector distance, feeding rate) was evaluated (**Fig. 5a-c**). In general, as the concentration of PLGA increased along with the other parameters, the diameter of the fibers appears increased. However, at low PLGA concentrations, the other process parameters have a stronger influence than at high PLGA concentrations. As indicated in **Fig. 5a-c** when the value of the process parameters are increased above a certain threshold, the concentration of the PLGA must be increased significantly to overcome the irregularity in the diameter of the fibers. This understanding allows for the modulation of both parameters in parallel in order to change the diameter of the fibers without the need for PLGA concentrations as high as those used with DMF as the solvent. Table 4 Fig. 6

To facilitate synthesis of nanofibers of a defined diameter, we performed a regression analysis of these data to produce a transfer function for the average diameter of the nanofibers electrospun in HFIP:

$$d_{ave}=161X_1 - 308X_2+0.1X_3 - 71.2X_4 - 3078$$

Equation 1. Transfer function of the average diameter (d_{ave}) of fibers electrospun from a solution of PLGA in HFIP and 1% NaCl. The Independent variables are PLGA concentration (X_1), the potential (X_2), the feeding rate (X_3) and the spinneret-to-collector distance (X_4).

3.5. Validation of transfer function for PLGA-HFIP solution with 1% NaCl

To validate the transfer function, two target fiber diameters were selected: 250 nm (nanofibers) and 1200 nm (microfibers), and the equation was solved for these two values to provide settings for each of the process and systems parameters. We set standard deviations of 50 nm and 200 nm as acceptable for each fiber diameter, respectively. Using the parameters specified by the transfer function (**Table 3**), we generated nanofibers by electrospinning, and measured the diameters of the resulting fibers using SEM. As indicated in **Table 3** and visualized in **Fig. 5**, we obtained beadless nanofibers of an average diameter of 247.2 and 1276.5, under the two conditions, which fell within the acceptable limits we set for thin and thick fibers, respectively. These data validate the applicability and effectiveness of the transfer function for producing uniform PLGA nanofibers of a defined diameter.

3.6. Utility of the transfer function in the preparation of specified PLGA nanofiber diameter for use as salivary gland epithelial cell scaffold

To assess the suitability of the 250 nm nanofibers as a substrate for salivary gland cells, we used the SIMS mouse submandibular gland cell line as a model cell. To determine if the salivary gland cells could attach to the nanofibers produced during this study, we compared the overall viability and morphology of SIMS salivary gland epithelial cells seeded on glass (a typical substrate for in vitro cell culture), a PLGA film (a material control) to those seeded on the PLGA nanofibers. To examine cell proliferative ability, SIMS cells were cultured on flat glass, flat PLGA, or PLGA nanofibers (247.2 nm mean diameter) for up to 96 hrs, and cells were counted at 24 hr intervals. Cells proliferated comparably on all three substrates, and perhaps slightly better on either PLGA flat films or nanofibers than on glass (**Fig. 7**), indicating that the cells could at least proliferate as well on nanofibers as on flat

controls. To account for possible differences in cell viability on the different surfaces, trypan blue staining was used in cell counting experiments to exclude non-viable cells, but significant differences in cell viability on the different substrates was not apparent (**Fig. 7**). As an additional confirmation of cell viability, the cells were exposed to calcein, which is metabolized to produce a product that fluoresces green in live cells, and ethidium, which is non-specifically absorbed only by dead cells and fluoresces red. Few ethidium-positive cells were detected under any condition, and yet almost all cells fluoresced green, indicative of a viable cell population (**Fig. 7**).

While the cells seeded upon glass or flat PLGA surfaces generally grew as single dispersed cells, SIMS cells seeded upon the PLGA nanofibers self-organized into cellular aggregates suggestive of an acinar cell cluster (**Fig. 7**). To characterize cell aggregate morphology further, SIMS cells were again seeded on both flat surfaces and on the nanofibers, grown for 18 hrs, fixed, and imaged using scanning electron microscopy SEM (**Fig. 7**). The cells seeded on the flat surfaces flattened and spread significantly, characteristic of cells in a 2D monolayer, while the SIMS cells grown on nanofibers attached to the fibers and assumed a more three-dimensional, rounded morphology, more typical of salivary gland cells *in vivo*. The cells did not migrate between the nanofibers, but instead remained on top of the nanofiber scaffold. These data indicate the nanofibers facilitate self-organization of salivary gland cells into a more desirable 3D morphology than is observed on flat substrates.

4. Conclusions

Here we report the first use of a nanofiber scaffold as a substrate for growth of salivary gland cells. The PLGA nanofibers supported the attachment, proliferation, and survival of the salivary gland epithelial cells. While the PLGA material itself supported cell attachment and cell growth, the arrangement of PLGA into nanofiber structures promoted the self-organization of cells on this material into cellular aggregates. Since the SIMS cells are an epithelial salivary gland cell line, we anticipated that the cells would not migrate into the nanofiber scaffold, but would instead interpret the scaffold as a mesenchymal-derived extracellular matrix. While this study demonstrates the ability of the nanofibers to facilitate the self-organization of cells into epithelial-like cell clusters or aggregates, further investigation will be required to determine if the cells can achieve apical-basal polarity, formation of apically-restricted tight junctions, and expression of proteinaceous components of saliva, such as alpha-amylase. Further research will also be necessary to investigate the effect of nanofiber diameter and topology on salivary gland cell differentiation. Although the PLGA nanofibers do not require surface modification to support cell attachment, surface modification and attachment of growth regulatory molecules may be required to achieve the desired acinar cell organization and cell function. While we have used the SIMS salivary gland cell as a model salivary gland cell line in this study, this study indicates that primary salivary gland cells may also respond favorably to these nanofibers.

To our knowledge, this study represents the first use of a systematic design of experiment (DOE) approach towards electrospinning of PLGA nanofibers having a predictable and defined diameter for use as cellular scaffolds. This approach is more efficient than evaluating parameters individually because it allows factors to be evaluated in concert, but still allows the effect of each parameter to be individually quantified. Our results demonstrate HFIP proved to be a more suitable solvent than DMF for the synthesis of nanofibers of defined diameters due to the extreme sensitivity of the diameter to minor changes in process parameters when DMF is used as a solvent. The addition of salt, particularly NaCl, expanded the range of fiber diameters that could be produced without inducing undesired beads and with minimal changes to the process parameters. The transfer function generated for a PLGA-HFIP solution with 1% NaCl was validated, as uniform

fibers of predicted diameter were able to be electrospun using parameters suggested by the transfer function. Similar methods can be used to reproducibly generate nanofibers composed of different materials for other biomedical applications.

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Nomenclature

PLGA	poly(lactic- <i>co</i> -glycolic acid
FDA	U.S. Food and Drug Association
DOE	design of experiment
HFIP	Hexafluoroisopropanol HFIP
DMF	dimethylformamide
NaCl	sodium chloride
MgSO₄	magnesium sulfate
SEM	scanning electron microscopy
SIMS	submandibular gland epithelial cell line

References

1. Delaleu N, Jonsson R, Koller MM. Sjogren's syndrome. *European journal of oral sciences*. 2005; 113(2):101–113. [PubMed: 15819815]
2. Bhide SA, Miah AB, Harrington KJ, Newbold KL, Nutting CM. Radiation-induced xerostomia: pathophysiology, prevention and treatment. *Clinical oncology (Royal College of Radiologists (Great Britain))*. 2009; 21(10):737–744. [PubMed: 19833490]
3. Pedersen AM, Nauntofte B. Primary Sjogren's syndrome: oral aspects on pathogenesis, diagnostic criteria, clinical features and approaches for therapy. *Expert opinion on pharmacotherapy*. 2001; 2(9):1415–1436. [PubMed: 11585021]
4. Napenas JJ, Brennan MT, Fox PC. Diagnosis and treatment of xerostomia (dry mouth). *Odontology / the Society of the Nippon Dental University*. 2009; 97(2):76–83. [PubMed: 19639449]
5. Mese H, Matsuo R. Salivary secretion, taste and hyposalivation. *Journal of oral rehabilitation*. 2007; 34(10):711–723. [PubMed: 17824883]
6. Sreebny LM, Schwartz SS. A reference guide to drugs and dry mouth--2nd edition. *Gerodontology*. 1997; 14(1):33–47. [PubMed: 9610301]
7. Kagami H, Wang S, Hai B. Restoring the function of salivary glands. *Oral diseases*. 2008; 14(1):15–24. [PubMed: 18173444]
8. Smith LA, Ma PX. Nano-fibrous scaffolds for tissue engineering. *Colloids and surfaces*. 2004; 39(3):125–131. [PubMed: 15556341]
9. Li WJ, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. *Journal of biomedical materials research*. 2002; 60(4):613–621. [PubMed: 11948520]
10. Ma PX, Zhang R. Synthetic nano-scale fibrous extracellular matrix. *Journal of biomedical materials research*. 1999; 46(1):60–72. [PubMed: 10357136]
11. Ma Z, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue engineering*. 2005; 11(1-2):101–109. [PubMed: 15738665]
12. Andrews KD, Feugier P, Black RA, Hunt JA. Vascular prostheses: performance related to cell-shear responses. *The Journal of surgical research*. 2008; 149(1):39–46. [PubMed: 18395748]

13. Andrews KD, Hunt JA. Upregulation of matrix and adhesion molecules induced by controlled topography. *J Mater Sci Mater Med*. 2008; 19(4):1601–1608. [PubMed: 18214646]
14. Curtis A, Wilkinson C. New depths in cell behaviour: reactions of cells to nanotopography. *Biochemical Society symposium*. 1999; 65:15–26. [PubMed: 10320930]
15. Gogoleski S, Mainil-Varlet P. Effect of Thermal Treatment on Sterility, Molecular and Mechanical Properties of Various Polylactides. *Biomaterials*. 1997; 18:251–255. [PubMed: 9031727]
16. Curtis AS, Casey B, Gallagher JO, Pasqui D, Wood MA, Wilkinson CD. Substratum nanotopography and the adhesion of biological cells. Are symmetry or regularity of nanotopography important? *Biophysical chemistry*. 2001; 94(3):275–283. [PubMed: 11804737]
17. Dalby MJ, Riehle MO, Yarwood SJ, Wilkinson CD, Curtis AS. Nucleus alignment and cell signaling in fibroblasts: response to a micro-grooved topography. *Experimental cell research*. 2003; 284(2):274–282. [PubMed: 12651159]
18. Vasita R, Katti DS. Growth factor-delivery systems for tissue engineering: a materials perspective. *Expert review of medical devices*. 2006; 3(1):29–47. [PubMed: 16359251]
19. Curtis AS, Gadegaard N, Dalby MJ, Riehle MO, Wilkinson CD, Aitchison G. Cells react to nanoscale order and symmetry in their surroundings. *IEEE transactions on nanobioscience*. 2004; 3(1):61–65. [PubMed: 15382646]
20. Dalby MJ, Gadegaard N, Riehle MO, Wilkinson CD, Curtis AS. Investigating filopodia sensing using arrays of defined nano-pits down to 35 nm diameter in size. *The international journal of biochemistry & cell biology*. 2004; 36(10):2005–2015.
21. Dalby MJ, Biggs MJ, Gadegaard N, Kalna G, Wilkinson CD, Curtis AS. Nanotopographical stimulation of mechanotransduction and changes in interphase centromere positioning. *Journal of cellular biochemistry*. 2007; 100(2):326–338. [PubMed: 16888806]
22. Dalby MJ, Gadegaard N, Herzyk P, Sutherland D, Agheli H, Wilkinson CD, Curtis AS. Nanomechanotransduction and interphase nuclear organization influence on genomic control. *Journal of cellular biochemistry*. 2007; 102(5):1234–1244. [PubMed: 17427951]
23. Breckenridge LJ, Wilson RJ, Connolly P, Curtis AS, Dow JA, Blackshaw SE, Wilkinson CD. Advantages of using microfabricated extracellular electrodes for in vitro neuronal recording. *Journal of neuroscience research*. 1995; 42(2):266–276. [PubMed: 8568928]
24. Oh S, Brammer KS, Li YS, Teng D, Engler AJ, Chien S, Jin S. Stem cell fate dictated solely by altered nanotube dimension. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106(7):2130–2135. [PubMed: 19179282]
25. Jose MV, Thomas V, Dean DR, Nyairo E. Fabrication and characterization of aligned nanofibrous PLGA/Collagen blends as bone tissue scaffolds. *Polymer*. 2009; 50:3778–3785.
26. Andriano KP, Tabata Y, Ikada Y, Heller J. In vitro and in vivo comparison of bulk and surface hydrolysis in absorbable polymer scaffolds for tissue engineering. *Journal of biomedical materials research*. 1999; 48(5):602–612. [PubMed: 10490673]
27. Ramchandani M, Robinson D. In vitro and in vivo release of ciprofloxacin from PLGA 50:50 implants. *J Control Release*. 1998; 54(2):167–175. [PubMed: 9724903]
28. You Y, Jin Lee S, Min B-M, Park WH. Effect of solution properties on nanofibrous structure of electrospun poly(lactic-co-glycolic acid). *Journal of Applied Polymer Science*. 2006; 99(3):1214–1221.
29. Wang N, Wu XS. Synthesis, characterization, biodegradation, and drug delivery application of biodegradable lactic/glycolic acid oligomers: Part II. Biodegradation and drug delivery application. *Journal of biomaterials science*. 1997; 9(1):75–87.
30. Hohman MM, Shin M, Rutledge G. Electrospinning and Electrically Forced Jets. II Applications. *Physics of Fluids*. 2001; 13:1–16. al., e.
31. Jayaraman K, Kotaki M, Zhang Y. recent Advances in Polymer Nanofibers. *Journal of Nanoscience and Nanotechnology*. 2004; 4:52–65. [PubMed: 15112541]
32. Hohman MM, Shin M, Rutledge G. Electrospinning and Electrically Forced Jets I. Stability Theory. *Physics of Fluids*. 2001; 13:2201–2220. al., e.
33. Yarin AL, Koombhongse S, Reneker DH. Taylor Cone and Jetting from Liquid Droplets in Electrospinning of Nanofibers. *Journal of Applied Physics*. 2001; 91:4836–4846.

34. Doshi J, Reneker DH. Electrospinning Process and Application of Electrospun Fibers. *Journal of Electrostatic*. 1995; 35:151–160.
35. Vasita R, Katti DS. Nanofibers and their applications in tissue engineering. *International journal of nanomedicine*. 2006; 1(1):15–30. [PubMed: 17722259]
36. Zong X, Kim K, Fang D. Structure and Process Relationship of Electrospun Biodegradable Nanofiber Membranes. *Polymer*. 2002; 43:4403–4412. al., e.
37. Laoide BM, Courty Y, Gastinne I, Thibaut C, Kellermann O, Rougeon F. Immortalised mouse submandibular epithelial cell lines retain polarised structural and functional properties. *Journal of cell science*. 1996; 109(Pt 12):2789–2800. [PubMed: 9013327]
38. Deitzel JM, Kleinmeyer J, Harris D, Beck Tan NC. The effect of processing variables on the morphology of electrospun nanofibers and textiles. *Polymer*. 2001; 42:261–272.
39. Huang Z-M, Zhang Y-Z, Kotaki M, Ramakrishna S. A review on polymer nanofibers by electrospinning and their applications in nanocomposites. *Composites Science and Technology*. 2003; 63:2223–2253.
40. Zong X, Ran S, Fang D, Hsiao BS, Chu B. Control of structure, morphology and property in electrospun poly(glycolide-co-lactide) non-woven membranes via post-draw treatments. *Polymer*. 2003; 44:4959–4967.
41. Schneider OD, Lohrer S, Brunner TJ, Uebersax L, Simonet M, Grass RN, Merkle HP, Stark WJ. Cotton Wool-Like Nanocomposite Biomaterials Prepared by Electrospinning: *In vitro* Bioactivity and Osteogenic Differentiation of Human Mesenchymal Stem Cells. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. 2007:350–362.
42. Zong X, Ran S, Kim KS, Fang D, Hsiao BS, Chu B. Structure and morphology changes during in vitro degradation of electrospun poly(glycolide-co-lactide) nanofiber membrane. *Biomacromolecules*. 2003; 4(2):416–423. [PubMed: 12625740]

Figure 1. Electrospinning overview. a) Basic apparatus, b) close-up view of polymeric jet formation from a droplet at the end of the needle tip and c) the structure of PLGA, showing lactic acid as the n monomer and glycolic acid as the m monomer of the PLGA polymer.

Figure 2.

SEM image of PLGA fibers electrospun in DMF as the potential is decreased from (a) 20 kV to (b) 19 kV. The decrease in the potential drastically changes the morphology and diameter of the fibers, leading to the formation of beads at the lower potential. When the distance between the needle and collector is reduced, (c) a decrease in the potential has almost no effect on the diameter of the fiber, but fewer beads were formed, while (d) an increase in the feeding rate resulted in thicker fibers that were bead-free.

Figure 3. Surface plots of predicted average diameter of PLGA fibers electrospun in DMF solution. Fiber diameter (Z) is displayed as a function of X and Y: a) % PLGA and Potential, b) % PLGA and Feeding Rate, c) % PLGA and Spinneret to Collector distance and d) Feeding Rate and Potential.

Figure 4.

Effect of HFIP concentration and salt additives on the diameter and morphology of nanofibers. Increasing the PLGA concentration without any salt additives from a) 5wt% to b) 7wt% then c) 9wt%, increases the diameter slightly, however beads are present at all 3 concentrations. d) Addition of 1% MgSO_4 to an 8.5wt% solution did not significantly decrease beading. e) 1% NaCl in an 8.5wt% solution completely eliminated beading and yielded homogenous thin fibers.

Figure 5. 3D Surface plots of predicted average diameter of PLGA fibers electrospun in HFIP solution. Fiber diameter (Z) is displayed as a function of X and Y: a) % PLGA and Potential, b) % PLGA and spinneret-to-collector distance, c) % PLGA and Feeding rate and d) Potential and spinneret-to-collector distance.

Figure 6.

Fibers generated from PLGA-HFIP solutions with 1% NaCl using calculated parameter values from the optimized transfer function. a) Target fiber diameter of 250 nm, actual mean 247.2 nm, parameter settings: 8 wt% PLGA, 10 kV potential, 15 cm spinneret-to-collector distance, 5 μ l/min feeding rate. b) Target fiber diameter of 1200 nm, actual mean 1276.5 nm, parameter settings: 18 wt% PLGA, 12kV potential, 15 cm spinneret-to-collector distance, 16 μ l/min feeding rate.

Figure 7.

PLGA nanofibers can support the survival, growth and 3D-like epithelial morphology of salivary gland cells. a) Proliferation assay for SIMS submandibular cells cultured on flat glass, PLGA flat films, or PLGA nanofibers for the indicated time points, detached and counted using a hemocytometer. b) Cell viability on different surfaces was assayed using trypan blue staining and hemocytometer counting at the indicated time points. c) SIMS cells were seeded on flat glass, PLGA flat films, or PLGA nanofiber for 48 hrs. were stained with calcein to detect live cells (1 μ M, 488nm excitation) and ethidium homodimer-1 to stain dead cells (2 μ M, 543 nm excitation). Images were obtained using a Zeiss CellObserver microscope. Scale, 20 μ m. d) SIMS salivary gland cell morphology on multiple surfaces. SIMS cells were cultured overnight on flat glass, flat PLGA, and PLGA nanofibers (average diameter ~250 nm), respectively, and images of fixed cells were captured by SEM. Scale, 10 μ m.

Table 1

The effect of DMF and HFIP on the morphology of the electrospun fibers: solution concentration, potential, feeding rate were modulated.

Solvent	PLGA (wt%)	Potential (Kv)	Feeding rate ($\mu\text{l}/\text{min}$) ¹	Spinneret-to-collector distance (cm)	b.p. (°C)	v.p. ² mm Hg	Fiber diameter, average (nm)	Fiber diameter, range (nm)	Beads (+/-)
DMF	30	20	7	18	153	2.7	967	763-1165	-
	30	19	7	18			176	94-332	+
	35	15	6	15			954	661-1283	-
	30	18	7	15			230	131-328	+
HFIP	30	19	8.5	20			709	440-943	+
	5	12	16	10	58	120	262	107-347	+
	7	12	16	10			1159	470-1487	+
	9	12	16	10			1821	1699-2037	-

¹ All of the runs were performed using a needle tip of 0.41 mm inner diameter.

² v.p.=vapor pressure at 20°C.

Table 2

The effect of NaCl and MgSO₄ on the morphology of the electrospun fibers. The potential and the spinneret-to-collector distance were also modulated, while other parameters such as the solvent, PLGA concentration, and feeding rate were kept constant.

Salt	PLGA (wt%)	Potential (kV)	Feeding rate (μl/min) ¹	Spinneret-to-collector distance (cm)	Fiber diameter, average (nm)	Fiber diameter, range (nm)	Beads (-, ++, +++)
MgSO ₄	8.5	12	16	10	367	60-653	++
	8.5	15	16	10	379	68-883	++
	8.5	20	16	10	329	38-737	++
	8.5	15	16	15	315	68-786	++
	8.5	20	16	15	337	122-547	++
NaCl	8.5	12	16	15	278	136-410	-
	8.5	15	16	15	183	42-452	+
	8.5	20	16	15	167	24-553	++
	8.5	12	16	10	137	36-286	+
	8.5	15	16	10	135	36-321	+
	8.5	20	16	10	110	19-185	+

The “-” indicates that no beads were observed, “+” indicates that a very small number of beads were observed, and “++” indicates that beads were present throughout the sample.

Table 3

DOE regression coefficients for the average diameter of PLGA nanofibers electrospun in HFIP.

Term	Coefficient	Std. Dev. Coefficient	P
PLGA (%)	160.56	38.75	0.000
Potential (kV)	-307.94	320.32	0.345
Feeding rate (μl/min)	0.05	43.21	0.999
Spinneret-to-Collector (cm)	-71.16	72.35	0.336

Table 4

System and process parameters computed by Minitab's optimizer for target average diameters of 250 nm and 1200 nm, respectively.

Predicted Average Diameter (nm)	PLGA (%)	Potential (kV)	Feeding rate (μl/min)	Spinneret-to-Collector (cm)	Experimental Average Diameter (nm)	Experimental Range of Diameter (nm)
250 ±50	8	10	5	15	247.2	210-283
1200 ±200	18	12	16	15	1276.5	1168-1401