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Incorporation of DNA into Electrospun Nanofibrous Scaffolds: Fundamental Characterization Studies and Gene Delivery

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1. Introduction

The ability to successfully deliver exogenous DNA into cells has provided the scientific community with a plethora of opportunities into the realm of basic, clinical, and translational research. It has afforded us the means to study the structure and function of genes and their products (i.e. proteins), correct genetic or acquired disorders, and generate tremendous amounts of data which researchers around the world continuously work to build upon in order to generate the next "magic bullet". Despite such progress, gene delivery continues to suffer from various limitations, predominantly, low transfection efficiencies when using non-viral approaches (i.e. naked DNA, cationic lipids/liposomes, synthetic and natural polymers, etc.) (Al-Dosari & Gao, 2009) and unacceptable levels of toxicity and immunogenicity when using the more traditional viral vectors. To address these limitations, researchers have focused on a number of innovative alternatives, one of which involves the incorporation of DNA into electrospun nanofibrous, non-woven and biodegradable scaffolds.

Electrospinning is a variation of the electrospray process and enables the experimenter to formulate scaffolds with specified mechanical, biological, chemical and kinetic properties, all readily controlled by alterations of polymer solution composition (i.e. polymer molecular weight and concentration, viscosity, salt concentration, conductivity, surface tension) and processing parameters (i.e. temperature, humidity, electric field strength, distance between spinneret and collector, feed rate and flow rate) (Chiu et al., 2005; Bhardwaj & Kundu, 2010). The resulting nanofibrous scaffolds possess desirable attributes such as high surface area to volume ratio and numerous interconnected pores that mimic the topology of the extracellular matrix (ECM). Obviously these features are a requirement for the transport of oxygen, nutrients, and wastes through the scaffold, as well as supporting robust cell adhesion and viability. Further, the flexible nanofibers and their pliability is beneficial for cell migration throughout the 3D scaffold. As such, electrospinning represents a truly rational and versatile approach for the construction of custom-tailored tissue engineering scaffolds, especially those capable of supporting cell/tissue growth, as well as deliver a range of bioactive molecules, including, DNA, proteins, drugs, etc. These "functionalized" or "biomimetic" scaffolds represent a new paradigm in the design and implementation of tissue engineering strategies.

Since the initial study of Fang & Renneker (1997), demonstrating the possibility of electrospinning pure DNA nanofibers, the use of this technique for a variety of applications has truly exploded. Research has been reported in areas as diverse as scaffolds for tissue regeneration/regenerative medicine (Martins et al., 2007), drug delivery systems (Hadjiargyrou & Chiu, 2008), wound dressing materials (Khil et al., 2003; Kang et al., 2010; Chen & Chian, 2010; Cai et al., 2010), biocatalysis (Nair et al., 2007), cardiovascular grafts (Sell et al., 2009), prevention of post-surgical adhesions (Zong et al., 2004; Bölgen et al., 2007), filtration (Kattamuri et al., 2005), energy and environmental applications (Thavasi et al., 2008), biosensors (Manesh et al., 2007; Kowalczyk et al., 2008; Ding et al., 2010), and protective clothing (Gibson et al., 1998 ; Seungsin & Obendorf, 2006). In fact, there are over 1,650 reports indexed in PubMed for a keyword search of "electrospun or electrospinning". As many of these electrospinning applications are covered in other parts of this book, this chapter will strictly focus on the current literature and latest research on the use of electrospun polymeric scaffolds for fundamental characterization studies on the incorporation and behavior of DNA as well as their application as gene delivery systems.

2. Fundamental studies: Incorporation and characterization of DNA into polymeric nanofibers

The initial and simple study of electrospinning DNA into nanofibers was reported in 1997 by Fang and Reneker, who used commercially available calf thymus genomic DNA that was diluted in a range of 0.3 – 1.5% in a mixture of 70% water and 30% ethanol and electrospun at 12-14 kV onto porous copper grids. The resulting pure DNA fibers had diameters as small as 30 nm that spanned the 50 μm pores of the grids. In addition, the DNA fibers contained beads with diameters from 80 – 300 nm that were a few microns apart on each DNA nanofiber, resulting from DNA retraction into droplets. Lastly, the authors presented a high magnification image of a single DNA fiber that was 62 nm in diameter and speculated that ~600 DNA molecules could pass through each cross section to form the fiber (Fang and Reneker, 1997).

A similar study was conducted using salmon testis DNA with a range of 200-90 kb fragments that was diluted in a range of 0.5 – 2.5 wt% solution in a mixture of 70% water and 30% ethanol and electrospun using a range of voltages (15 – 30 kV) onto mica substrates (Takahashi et al., 2005). The resulting DNA fibers were examined with atomic force microscopy (AFM) and the authors reported the presence of four different types of fibers; twisted shaped, twisted and stretched, curved and straight. Further, the height and length of the fibers ranged from 1.8 nm and 1 μm, respectively, but these slightly varied with the different % DNA solutions and voltages used. The authors concluded that the optimum parameter values for generating ultrathin nanofibers were 1.5 wt% for DNA concentration and 20 kV for the applied voltage.

Aside from electrospinning pure DNA in solution, other investigations were carried out using DNA mixed with various synthetic polymers. For example, one study mixed fluorescently labelled bacteriophage lambda DNA molecules with polyethylene oxide (PEO) and produced polymer nanofibers that contained isolated and stretched DNA molecules (Bellan et al., 2005). These DNA nanofibers had diameters that ranged between 100-350 nm and lengths between 1 and 19 μm, as measured by AFM (Fig. 1). Making use of the fluorescently labelled DNA, this study also examined the relaxation time of the DNA in a viscous solution (bulk PEO) in order to understand the fluid dynamic nature of the DNA.

The authors found that the DNA length vs. time yielded a time constant ranging from 2.1 - 19 seconds, thus adding new knowledge on the dynamic behaviour of the DNA in the PEO solution while in the electrospinning jet.

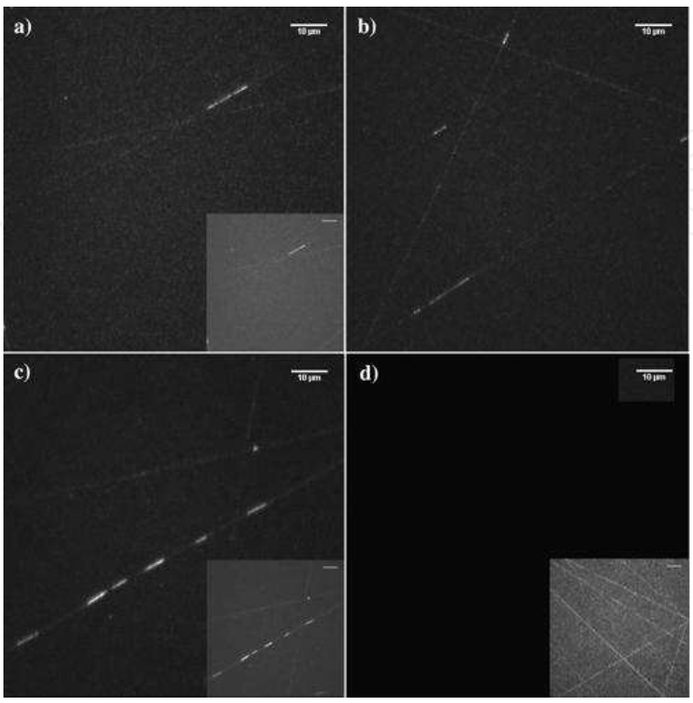

Fig. 1. Fluorescently labelled DNA/PEO nanofibers. Images a-c show the presence of the fluorescently labelled DNA within the PEO nanofibers. Image d shows control nanofibers without any DNA. Reprinted with permission from Bellan et al., 2005, American Chemical Society.

The same group also electrospun fluorescently labelled DNA and deposited it over photolithographically patterned trenches and gold electrodes and found that the nanofibers displayed diameters of ~27 nm (Bellan et al., 2007). Further, they imaged the nanofibers via a number of imaging techniques, including fluorescence microscopy, scanning electron microscopy (SEM), and AFM. Using AFM, the Young's modulus of a single suspended electrospun DNA fiber was determined by a three point bending test to be 15 ± 2 GPa. In their conclusion, the authors suggest that this approach could conceivably be used to manufacture nanosensors based on such DNA nanofibers, as well as aid in the development of self-assembled DNA devices.

An additional study was reported in the literature that utilized a mixture of DNA/PEO to electrospin nanofibers (Liu et al, 2007). The authors extensively evaluated the basic electrospinning solution properties such as conductivity, surface tension and viscosity (Table 1) and determined that the ionic conductivity of the solution increased significantly with the addition of DNA. In contrast, there was only a slight increase in the ionic conductivity with increasing amounts of PEO, which also decreased the surface tension. On the other hand, the viscosity increased with the addition of either DNA or PEO and as such the authors concluded that solutions containing both DNA and PEO had ideal properties for electrospinning. The electrospun DNA/PEO nanofibers displayed diameters of 50–250 nm

that became thinner with decreased feed rate (tested a range between 50-200 μL/min), increased tip-to-collector distance (10-25 cm range) and thicker with low voltage (5-20 kv range) applied during electrospinning (Liu et al., 2007). As a result of these analyses, it was concluded that these DNA/PEO ultrathin fibers create a 3D porous structure with future potential applications in biosensing as well as cell culturing.

Solution no.	DNA (g)	PEO (g)	Amount of water (g)	Conductivity (S/cm)[a]	Surface tension (mN/m)[b]	Viscosity (mPa s)[c]
1	2	0	98	32.2	64.21	28.2
2	4	0	96	66.7	70.15	49.4
3	6	0	94	74.1	74.51	369.6
4	8	0	92	79.1	77.79	1068.8
5	10	0	90	104.0	74.26	63766.5
6	0	2	98	1.67	66.68	91.82
7	0	4	96	1.75	65.39	233.07
8	0	6	94	1.79	62.12	369.62
9	3	3	94	38.8	64.84	233.07
10	4	4	92	41.4	63.74	1104.14
11	5	5	90	53.6	61.58	2589.68
12	3	7	90	28.8	62.62	2337.77
13	4	6	90	41.9	64.11	2537.88
14	6	4	90	59.2	63.38	1998.76
15	7	3	90	80.4	61.14	8899.07

[a]Determined by a model 20 pH/conductivity meter (Denver Instrument).
[b]Determined by a KSV contact angle analyzer (goniometer, KSV instruments Ltd).
[c]Determined by a DV-II viscometer, shear rate using 20 s^{-1} for viscosity testing.
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Table 1. Properties of aqueous solutions (100 mL) containing DNA/PEO.

Taking further advantage of the unique optical and electronic properties of DNA and thinking along the lines of using DNA as a candidate for device applications (electronics, filtration, sensors), Ner and colleagues (2008), complexed salmon sperm DNA (molecular weight [MW] 500 kDa) with a cationic surfactant (cetyltrimethylammonium chloride, CTMA), as well as a hemicyanine chromophore and electrospun nanofibers. These nanofibers had an average diameter between 250-350 nm and demonstrated amplified emission of the chromophore when compared to thin films of identical composition. When the material properties of these nonwoven DNA-CTMA fiber meshes were tested, it was found that the average initial modulus was 1.44 MPa with a breaking strength of 37.4 kPa, orders of magnitude lower than those of electrospun fluorescently labelled DNA (15 ± 2 GPa), as previously reported (Bellan et al., 2007). The reason for this difference maybe the presence of the CTMA in the DNA fibers, which were capable of only 11% elongation prior to failure, indicating stress-stiffening that may be due to the particular alignment of the DNA strands along the stress axis or the lateral inter-laminar sliding within the fibers. Although the incorporation of dye did not affect the fibers, it did enhance fluorescence that resulted from both the fiber morphology (5–6-fold amplification) and specific interactions (groove-binding) between the chromophore and DNA (18–21-fold amplification). Based on their data the authors suggest that electrospun DNA nanofibers can serve as advanced functional biomaterial for molecular electronics (Ner et al., 2008).

3. Gene delivery: Transfection efficiency

The aforementioned fundamental studies all dealt mostly with processing parameters and characterization of the DNA nanofibers, either electrospun on with pure DNA or in a mixture with various polymers. Moving past the stage of initial characterization and translating into biological feasibility, the issue of transfection efficiency is key for non-viral gene delivery. This was addressed in a number of studies reported in the literature. Thus, I have divided these manuscripts that focus strictly in generating electrospun scaffolds as gene delivery systems into two categories; those that focus on *in vitro* cell based studies and those that report on implanted scaffolds *in vivo* using various animal models.

3.1 *In vitro* studies

Beyond the critical fundamental studies on DNA and polymer interactions, the first demonstration of the ability of electrospun scaffolds to serve as non-viral gene delivery systems using plasmid DNA (pDNA) was conducted in my laboratory in 2003. We prepared polymer solutions of poly-lactide-co-glycolide (PLGA) mixed with either, 10, 12 or 15% of a poly(D,L-lactide)-poly(ethylene glycol) (PLA-PEG-PLA) triblock copolymer and with pDNA, electrospun multiple scaffolds and evaluated the morphology, material properties, pDNA incorporation and release, and transfection efficiency (Luu et al., 2003). These studies showed that increasing the concentration of tri-block copolymer resulted in the formation of larger diameter (~2.5 – 5.0 μm with the 15% triblock) electrospun fibers in comparison to those observed with lower concentrations of block copolymer (250-875 nm and 375-1.1 μm for the 10 and 12% triblock, respectively) (Fig. 2).

Despite the variation in fiber diameters, no significant differences in material properties were detected. On average these electrospun scaffolds displayed a tensile modulus of about 35 MPa, 45% tensile strength and ultimate stress of 3.3 MPa, matching those of skin and cartilage. We also showed that the addition of pDNA to the fibers did not change the ultimate strain but did significantly increase both the tensile modulus (by ~10 MPa) and ultimate stress (by ~1 MPa).

Characterizing the incorporation and release of pDNA showed that ~68-80% of the pDNA that was incorporated, was released in two phases over the 20 day study-period. The first phase constituted of an initial burst, where ~18-36% of the pDNA was released within 15 min of immersion of the scaffold into a saline buffer solution and the second phase was a slow and sustained release over the next 20 days. The initial release burst of pDNA indicated that a substantial amount of the electrospun DNA remained on the surface of the nanofibers with the rest embedded within the nanofibers. The integrity of the electrospun pDNA was demonstrated via agarose gel electrophoresis which revealed that the pDNA was indeed structurally intact and more importantly, its bioactivity was demonstrated by directed transfection in cell culture. Sections of the pDNA containing scaffolds placed in culture medium above a monolayer of pre-osteoblastic (MC3T3) cells were able to release active pDNA and transfect cells with the encoded reporter gene (β-galactosidase). The transgene was found to be expressed, albeit, at a very low efficiency in comparison to lipid mediated transfection (Fig. 3). Interestingly, when the released pDNA was complexed with a lipid mediator (Fugene6), the transfection efficiency only reached 20% that of the positive control (Fig. 3), but even 20% is considered a low level of transfection.

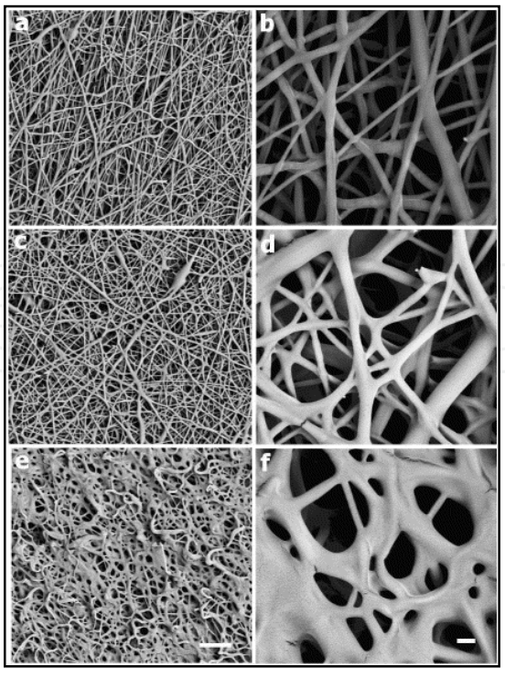


Fig. 2. Morphology of electrospun scaffolds. Highly porous scaffolds shown under low magnification on left, and high magnification on right. 10% block copolymer formulations (a, b) exhibited thin, fairly uniform fibers (250 to 875 nm). 12% block copolymer formulations (c, d) showed larger fibers and a larger distribution (375 nm to 1.1 mm). 15% block copolymer solutions (e, f) displayed thick, plate-like fibers of roughly 2.5 to 5 mm diameter at the junctions. Scale bar for (a), (c), (e) (left) 10 mm and for (b), (d), (f) (right) 1 μm. Reprinted with permission from Luu et al., 2003, Elsevier.

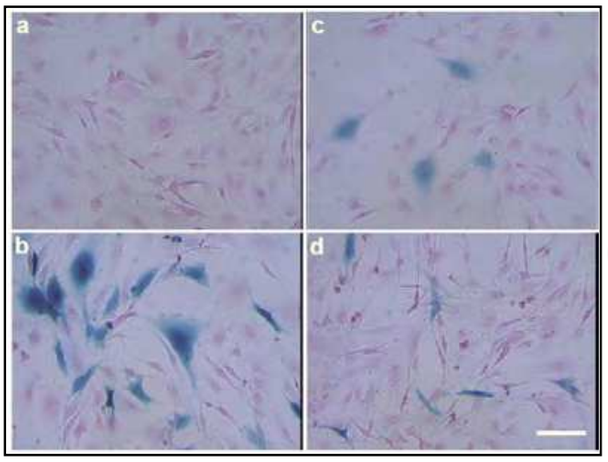


Fig. 3. Bioactivity of released DNA. (a) Naked DNA (2 μg) added directly to cell medium. (b) Cells transfected with complexed control DNA (2 μg) with a lipid-based transfection reagent (Fugene6). (c) Two 1.5 x 1cm sections of scaffold were directly incubated with cells for 4 h, then removed. (d) Released DNA from scaffold (2 μg) complexed with Fugene6. Following transfection (48 hr), cells were fixed and stained for β-galactosidase. Scale bar 100 μm. Reprinted with permission from Luu et al., 2003, Elsevier.

In subsequent experiments we were able to increase the transfection efficiency substantially by plating the cells directly on the electrospun pDNA/PLGA/PLA-PEG-PLA scaffolds (Fig. 4, Liang et al., 2005). This increase in transfection efficiency probably occurred because the cells were physically connected to the surface of the nanofibers where a substantial amount of pDNA was present. As a result, the degradation of the pDNA that probably occurred in our previous study (Luu et al., 2003) where the scaffolds were placed in the culture medium floating above the cells and the pDNA was released into the medium, was eliminated. Unfortunately, we did not conduct a confocal analysis of the scaffolds and thus we were not able to determine the exact transfection efficiency (transfected cells/total cells). Regardless, from the cells on surface shown in these images (Fig. 4), the transfection efficiency appears to be greater than 65%.

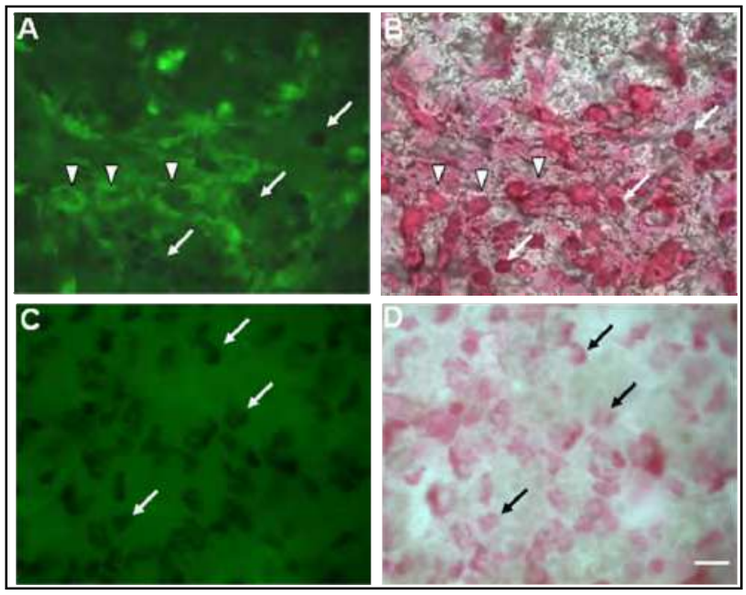


Fig. 4. Transfection of MC3T3 cells by nanofibrous electrospun scaffolds. Panels A-D show the increased transfection by plating MC3T3 cells directly on GFP pDNA/PLGA/10% block copolymer scaffolds. (A) Fluorescent image of cells 24 hr post-plating. Arrowheads indicate representative transfected GFP-expressing cells and arrows indicate non-transfected cells. (B) Light micrograph of cells (shown in A) stained with nuclear fast red. Arrowheads and arrows indicate identical cells in A. (C) Fluorescent image of cells on a control scaffold containing no DNA and indicating no fluorescent (green) cells (arrow). (D) Light micrograph of cells (shown in C) stained with nuclear fast red. Arrow indicates identical cells in C. This transfection efficiency reflected that of cells present only on the exterior surface of the scaffold and not for all cells within the scaffold. Scale bar: 10 μm. Reprinted with permission from Liang et al., 2005, Oxford University Press.

We also investigated the interactions between the polymers and pDNA using laser light scattering (LLS). Specifically, we determined that in a solution of N,N-dimethylformamide (DMF), the organic solvent used to dissolve the PLGA polymer, pDNA condenses into sphere-like structures and in the presence of the triblock copolymer, the formation of a micellar structure occurs with DNA being encapsulated by a shell made from the PLA-PEG-PLA triblock copolymer. When electrospun, these micelles are deposited randomly on and

into the PLGA fibers, with a significant amount located on the surface (Fig. 5), which accounts for the observed rapid burst release of pDNA (Luu et al., 2003; Liang et al., 2005). Finally, it was also established that the presence of the polylactide shell encapsulating the pDNA, protects it during electrospinning, as in its absence, the pDNA was completely degraded.

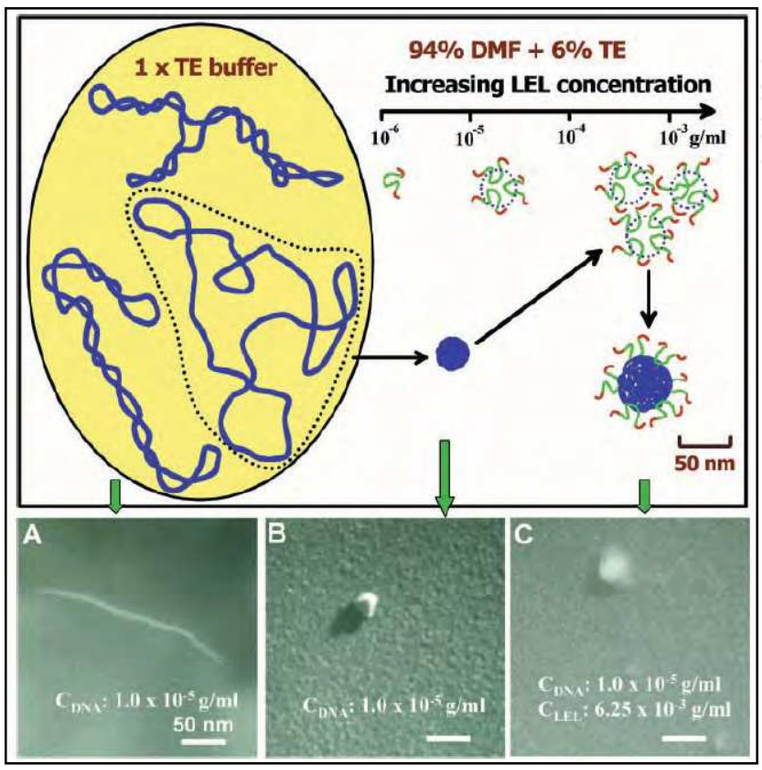


Fig. 5. Schematic presenting the condensed pDNA, the aggregation of LEL and the encapsulation of DNA by LEL. (A–C) Shows transmission electron microscopy (TEM) images of pDNA in 1x Tris-EDTA (TE) buffer, DNA in 94% DMF + 6% TE and encapsulated DNA in 94% DMF + 6% TE, respectively. Reprinted with permission from Liang et al., 2005, Oxford University Press.

Following our initial reports on the fabrication and use of electrospun scaffolds for gene delivery, a number of other studies were published that further expanded on the field, some with impressive increases in pDNA transfection efficiency. For example, Nie and Wang (2007) fabricated PLGA/hydroxyapatite (HAp) composite electrospun scaffolds using different HAp contents (0, 5, and 10%) in three compositions: 1) using only naked pDNA; 2) encapsulation of pDNA/chitosan nanoparticles; and 3) encapsulation of pDNA/chitosan nanoparticles mixed with a PLGA/HAp solution. The scaffolds generated were non-woven, with nano to micron fiber structures composed predominantly of PLGA with the HAp dispersed within the fibers (Fig. 6, white arrows). Scaffolds with the encapsulated pDNA/chitosan nanoparticles mixed with a PLGA/10%HAp showed the largest fiber diameters (100 nm). In addition, these scaffolds exhibited a much higher tensile strength (>4 times) than those with either naked pDNA or encapsulated with pDNA/chitosan nanoparticles (Nie and Wang, 2007).

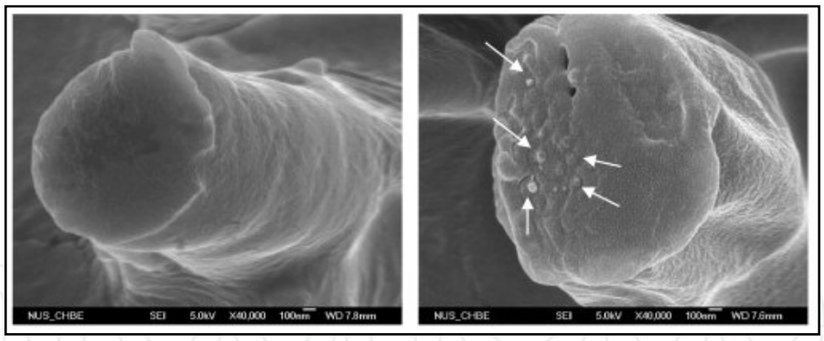


Fig. 6. The morphology observed in fiber cross sections containing pDNA/chitosan nanoparticles (left panel) and pDNA/chitosan nanoparticles mixed with a PLGA/HAp solution (right panel). This image illustrates clearly the encapsulated pDNA/chitosan nanoparticles (arrows) at the cut section of the fiber. Reprinted with permission from Nie and Wang, 2007, Elsevier.

These structural differences also translated into differences in pDNA release kinetics and transfection efficiencies. For the composite scaffold with just naked pDNA, an initial burst release of ~80-85% pDNA was observed for the first 7-9 days with the remaining pDNA released in the following 5 days. The scaffold with the encapsulated pDNA/chitosan nanoparticles did not show a burst release, but rather a slight delayed release with an overall cumulative release of 100% after ~24-30 days. In contrast to both of these scaffold types (just naked pDNA; pDNA/chitosan nanoparticles), those with encapsulated pDNA/chitosan nanoparticles mixed with a PLGA/HAp solution displayed a more linear and longer release curve with a 95% cumulative DNA release in 45-55 days. Further, for all three different scaffold types, the released DNA appeared to be structurally intact, as monitored by gel electrophoresis. When the authors measured the transfection efficiency of their scaffolds using human mesenchymal stem cells (plated directly on the scaffolds), they show that the second type of scaffold (encapsulated with DNA/chitosan nanoparticles) increased the *in vitro* expression of the delivered transgene (BMP-2), but unfortunately, the exact % transfection efficiency was not quantified. In addition, it was reported that these scaffolds resulted in reduced cell viability over time, which was attributed to the invasive transfection of the pDNA/chitosan nanoparticles as a result of the initial burst release (Nie and Wang, 2007). These results highlight the problem of balancing the need for high transfection efficiency with maintenance of cell viability. Thus, a scaffold that can do both, namely increase transfection efficiency while maintaining robust cell viability, would serve as an ideal gene delivery system.

A different method was utilized to generate electrospun pDNA containing scaffolds; surface immobilization of PEI/pDNA on electrospun PLA scaffolds using a layer by layer approach as a means of effectively controlling the functionality of the scaffold (Sakai et al., 2008). In this particular study, pDNA (pGL3 encoding luciferase) was incorporated on individual nanofibers via successive immersion into a solution of PEI and increasing concentration of pDNA. Results showed that the concentration of pDNA deposited on the electrospun nanofibers can be increased with increasing layers. Surprisingly, when tested with COS cells plated directly onto scaffolds, the scaffold with the highest plasmid concentration displayed decreased transfection efficiency as compared to one with a lower concentration (Fig. 7). Unfortunately, this study did not report transfection efficiency as a total percentage of cells.

Rather, they reported data normalized to the quantity of cellular protein which only approximated the number of transfected cells. Lastly, the authors also show that with longer incubations, the overall transfection efficiency did increase. Taken together, this study clearly showed that transgene expression can be controlled by changing both the concentration of DNA (via the number of immersions), as well as the incubation time with the cells (Sakai et al., 2008).

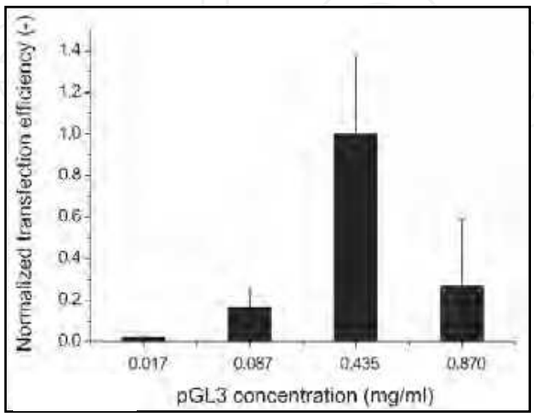


Fig. 7. Effect of pGL3 concentration in the immersion solution on transfection efficiency of PLA-(PEI/pGL3)$_2$PEI fibrous mat after 30 h of cultivation. Bars represent mean +/- SD (n= 4 – 10). Reprinted with permission from Sakai et al., 2008, John Wiley and Sons.

Gene delivery was also investigated using nanofibrous scaffolds that were generated using coaxial electrospinning. These scaffolds have a sheath/core fiber morphology whereby two immiscible polymer solutions are used to fabricate the individual fibers. Coupled to coaxial electrospinning, this study investigated the effect of four parameters: a) Poly(εcaprolactone) (PCL) concentration; b) PEG MW; c) PEG concentration; and d) DNA concentration (Saraf et al., 2010). By varying the amounts of these four components, the authors fabricated eight groups of scaffolds and then directly compared them by examining scaffold morphology, DNA incorporation and release and transfection efficiency. The various scaffolds exhibited fiber diameter distribution of ~200 nm to 4 μm, with higher fiber diameters obtained with increasing PCL concentration, followed by increased PEG MW, PEG concentration and higher pDNA concentration. More importantly, the investigators evaluated pDNA release over 60 days and observed a pattern that could be divided into four groups: a) burst release (0 – 24 hrs); b) Phase 1 (2 - 10 days); c) Phase 2 (11 - 28 days); and d) Phase 3 (35 - 60 days). Even though there were large differences between the groups for the burst release of pDNA, none of the fabricated scaffolds achieved release profiles with Phases 1-3 (Fig. 8).

The average transfection efficiency (in rat fibroblasts) over 60 days for the eight groups of coaxial electrospun scaffolds, was ~15%. Despite the low level of transfection, in comparison to scaffolds carrying pDNA alone without rhodamine-tagged PEI-HA, all experimental groups showed significance increases in transfection efficiency (~15% vs. ~5%, respectively). It is important to note that despite the rapid release kinetics, with some coaxial electrospun scaffolds, transfection efficiencies approached ~40%, a substantial level for a non-viral approach. Lastly, the cells remained viable for the duration of the 60 day transfection experiment (Saraf et al., 2010) which represents a significant improvement given the lower viability results reported in the Nie and Wang study (Nie and Wang, 2007).

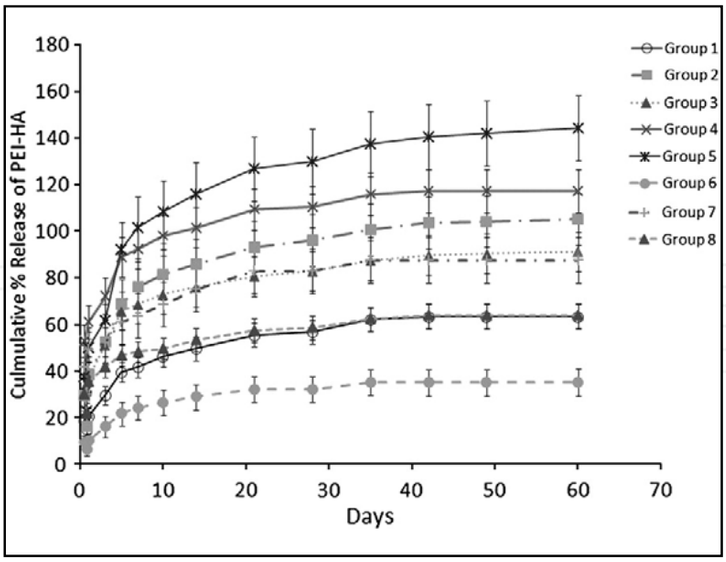


Fig. 8. Cumulative release of rhodamine-tagged PEI-HA (r-PEI-HA) from electrospun coaxial fiber meshes at 37 °C in PBS with agitation at 115 rpm. Error bars represent standard deviation for n=4. Reprinted with permission from Saraf et al., 2010, Elsevier.

A very recent study further investigated the relationship between DNA transfection via electrospun scaffolds and cell viability. Yang et al. (2011) utilized emulsion electrospinning to fabricate core-sheath structured fibers with a core loading of pDNA or pDNA polyplexes inside the fibers with either PEI or PEG. Four specific formulations of electrospun scaffolds were prepared with fibers consisting of: 1) pDNA/poly(DL-lactide)-PEG (PELA); 2) pDNA/PELA-PEI; 3) pDNA-PEI/PELA; and 4) pDNA-PEI/PELA-PEG. Morphological studies revealed that in comparison to the electrospun pDNA-loaded fibers (~500 - 600 nm diameter), the emulsion electrospun PELA fibers displayed a much wider size distribution, ranging between 700 and 1000 nm. In addition, the release rate also varied, with the pDNA/PELA fibers showing a two-phase profile, with an initial burst (~22% of pDNA) followed by a sustained one (~67%) over 3 weeks. On the other hand, adding PEI led to a higher burst release (~35%) of pDNA but lower cumulative release over the 3 weeks. Even lower burst and cumulative pDNA release resulted from the pDNA-PEI/PELA scaffold. Interestingly, pDNA-PEI/PELA scaffolds containing different MW PEG led to faster and more sustained release as a result of the molecular chains of high MW PEG forming larger channels thereby accelerating dissolution (Yang et al., 2011).

Similar to our studies (Luu et al., 2003; Liang et al., 2005), Yang et al (2011) also examined the integrity and bioactivity of the released pDNA and found it to be conformationally intact (attributed to the protective effect of core-sheath fibers on the encapsulated pDNA), as well as bioactive since it retained its activity for transfection and expression of the transgene (GFP) in NIH3T3 fibroblasts. But again the exact transfection efficiency was not reported. Instead, data were presented as relative light units, normalized to total cellular protein. Lastly, despite good cell attachment on some of their scaffolds, the authors did report cytotoxic effects on cells and poor adhesion on scaffolds that contained higher MW PEG, as well as with variable concentration of the pDNA polyplexes. As such, the study concludes that pDNA-polyplex loaded scaffolds containing 10% PEG show the best performance in balancing transfection efficiency and cell viability (Yang et al., 2011).

3.2 *In vivo* studies

Despite the presence of numerous manuscripts reporting on the transfection efficiency of DNA-loaded electrospun scaffolds using *in vitro* studies, only two very recent studies have published results dealing with *in vivo* applications of the electrospun scaffolds to deliver DNA. In addition, my laboratory has also conducted a study (Zhao et al., 2008) on bone regeneration (using a critical size drill defect) with electrospun scaffolds (PLGA mixed with a PLA-PEG diblock copolymer and GFP pDNA) and some of our results are also presented here.

Prior to the two recent studies, my laboratory performed experiments to test the efficacy of electrospun scaffolds to serve as gene delivery biomaterials (Zhao et al., 2008). Composite scaffolds of PLGA mixed with a 10% PLA-PEG diblock copolymer and GFP pDNA were prepared, cut in 5 x 1 cm strips and sterilized by brief ethanol immersion. Once in ethanol, the scaffolds shrank uniformly and considerably (~40 - 50%) and were then rolled into a 4 mm plugs (Fig. 9A). Once dried, we observed that these scaffold plugs were hard, similar in consistency to plastic. Using the unilateral 4 mm tibial plateau critical size defect rat model, the scaffold plugs were embedded in the defect and held in place by a pair of sutures. After 1, 2 and 3 weeks, the tibias were harvested and we analyzed the integration of the scaffold into the tissue as well as the GFP expression by the surrounding cells. Gross examination of a 3 week implanted electrospun scaffold indicates that it did not integrate well within the tissue (Fig. 9B). This was verified histologically, with a clear demarcation between the scaffold and surrounding tissue (Fig. 9C). In addition, we did not detect any GFP expression in the surrounding cells (data not shown). Based on these results we conclude that the scaffold sterilization employed in this study (100% ethanol) causes the release of a substantial amount of DNA that is present on the surface of the nanofibers, as observed in our previous studies (Luu et al., 2003; Liang et al., 2005) and the scaffolds to substantially shrink and thus alter their structural morphology, with porosity decreasing substantially and reducing the scaffolds advantageous high surface to volume area. The ethanol also likely increased the hydrophobicity of the scaffolds, which may have prevented the cells from attaching and thus decreased the overall scaffold biocompatibility. Thus, these results, are inconclusive in answering the question of whether these electrospun scaffolds can successfully be used as tissue engineering scaffolds (as prepared in this study) to deliver DNA and the experiments need to be repeated following development of a more suitable means of scaffold sterilization.

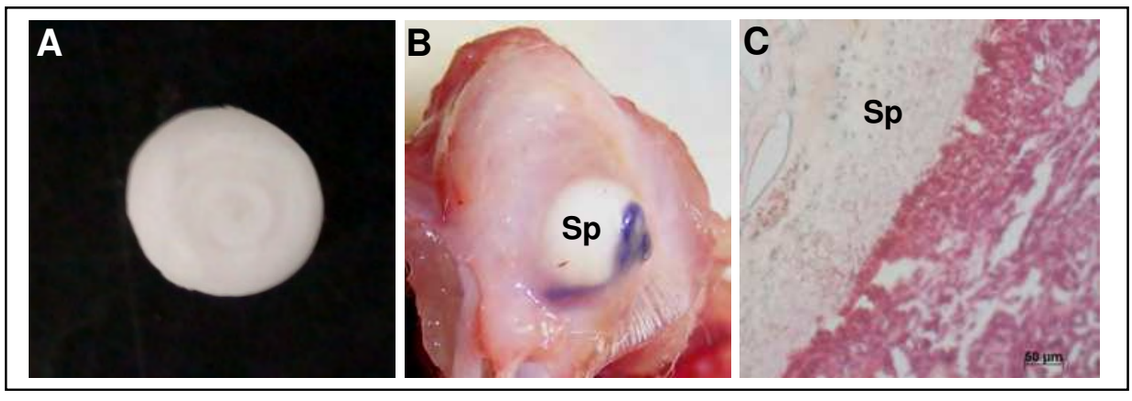
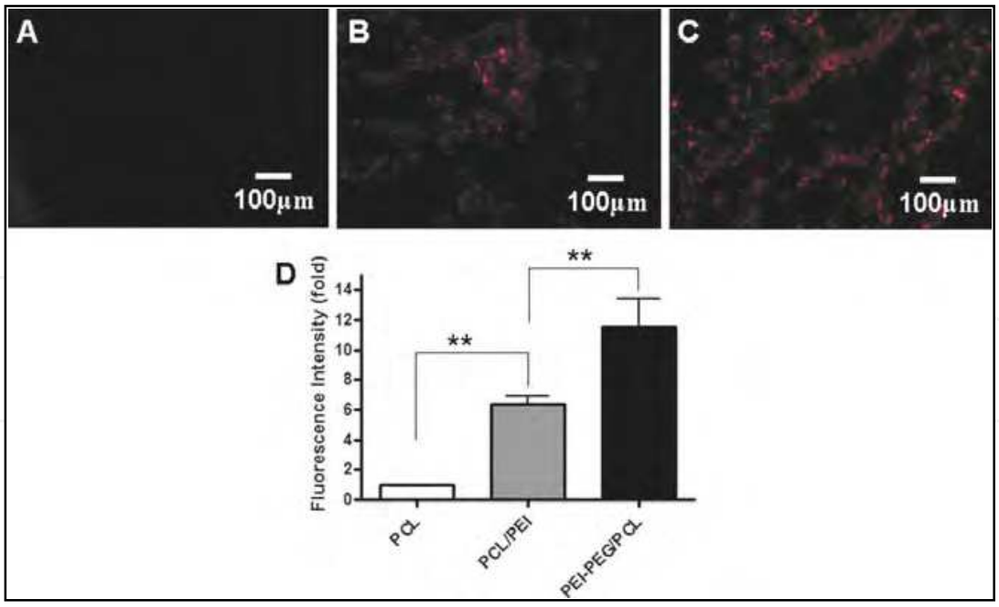


Fig. 9. (A) 4 x 4 mm electrospun Scaffold "Plug"; (B) 3 week post-implantation scaffold plug within rat tibial critical size defect (purple material is the suture used to hold scaffold within defect); (C) Tissue section showing the clear boundary between the implanted scaffold and surrounding tissue. Sp, scaffold plug.

The study by Zhang et al. (2010) generated three different kinds of electrospun scaffolds; one containing pure PCL, another with PCL/PEI and the third with PCL/PEI-PEG. The pDNA was added following fabrication of the electrospun scaffolds by immobilization (absorption) onto the nanofibers. Prior to the *in vivo* experiments, the authors tested the incorporation and release of DNA, as well as cell viability and transfection efficiency. Results indicated that approximately 15% and 50% of DNA was absorbed by the pure PCL and PCL/PEI scaffolds, respectively (the PCL/PEI-PEG also showed 50% maximum absorption). Despite the differences in DNA absorption both types of scaffold showed ~60% DNA release over 72 hrs. The transfection efficiency of these electropsun scaffolds was determined using two different cell types, a human embryonic kidney cell line (HEK293) and rat primary mesenchymal stem cells (MSCs). In HEK293 cells, the transfection efficiencies using PCL/PEI-PEG and PCL/PEI were ~70% and 38%, for each scaffold, respectively. Not surprisingly, with MSCs, the transfection efficiencies were lower at ~44.5% and 19%, for the PCL/PEI-PEG and PCL/PEI electrospun scaffolds, respectively. Further, the viability of these cells were also determined on both scaffolds and it was found that the PEG modification of PEI improved the biocompatibility, but still cell viability only reached ~50-80% of cells plated on PCL scaffolds. Following the *in vitro* characterization, the authors implanted the three different types of pDNA loaded scaffolds subcutaneously in mice and results showed that both pDNA containing PCL/PEI and PCL/PEI-PEG scaffolds successfully transfected surrounding tissue cells, with the highest measured fluorescent intensity obtained with PCL/PEI-PEG (Fig. 10, Zhang et al., 2010). As such, the absolute number of transfected cells and thus the exact effectiveness of these scaffolds to transfect with high efficiency *in vivo* remains to be determined.



**Statistical significance at p <0.01. Reprinted with permission from Zhang et al., 2010, John Wiley and Sons.

Fig. 10. Representative fluorescence images of *in vivo* transfection 14 days after subcutaneous implantation. (A) PCL/pDNA. (B) PCL/PEI/pDNA. (C) PCL/PEI–PEG/pDNA. (D) Averaged fluorescence intensity from three independent experiments.

In the second study, Kim and Yoo (2010) devised a clever approach to deliver DNA for the treatment of diabetic ulcers. Specifically, they reasoned that because diabetic ulcers contain high concentrations of matrix metalloproteinases (MMPs), they functionalized their PEI based electrospun scaffolds to contain an MMP-cleavable linkage. The idea was very simple; in the presence of MMPs, the linkerPEI (LPEI)/pDNA complex would be cleaved from the nanofibrous scaffolds, thereby transfecting the surrounding cells. This synthesis scheme is outlined in Fig. 11. In addition, the authors tested the incorporation of various amounts of DNA, its release and transfection efficiency *in vitro* prior to the *in vivo* experiments.
Various amounts of pDNA were loaded in the LPEI nanofibers and demonstrated that the DNA incorporation efficiency significantly increased with additional amounts of pDNA (from 17.4% to 79.5%) and this was attributed to the high cationic charge densities present on the nanofiber surface with respect to the anionic charge densities of the DNA. When the authors investigated pDNA release in the presence or absence of MMP-2, they found that depending on the amount of pDNA, MMP-2 treatment resulted in ~40 - 80% being released from the scaffolds (in the absence of MMP-2, the amount of released pDNA was below 30%). Further, the transfection efficiency of the released pDNA was found to be dependent on the charge ratio of the incorporated pDNA into the scaffolds rather than the actual amount of pDNA, but the data do not reveal what percent of the cells were transfected. Instead, it represents a comparison between the released pDNA with that of LPEI/DNA scaffolds.

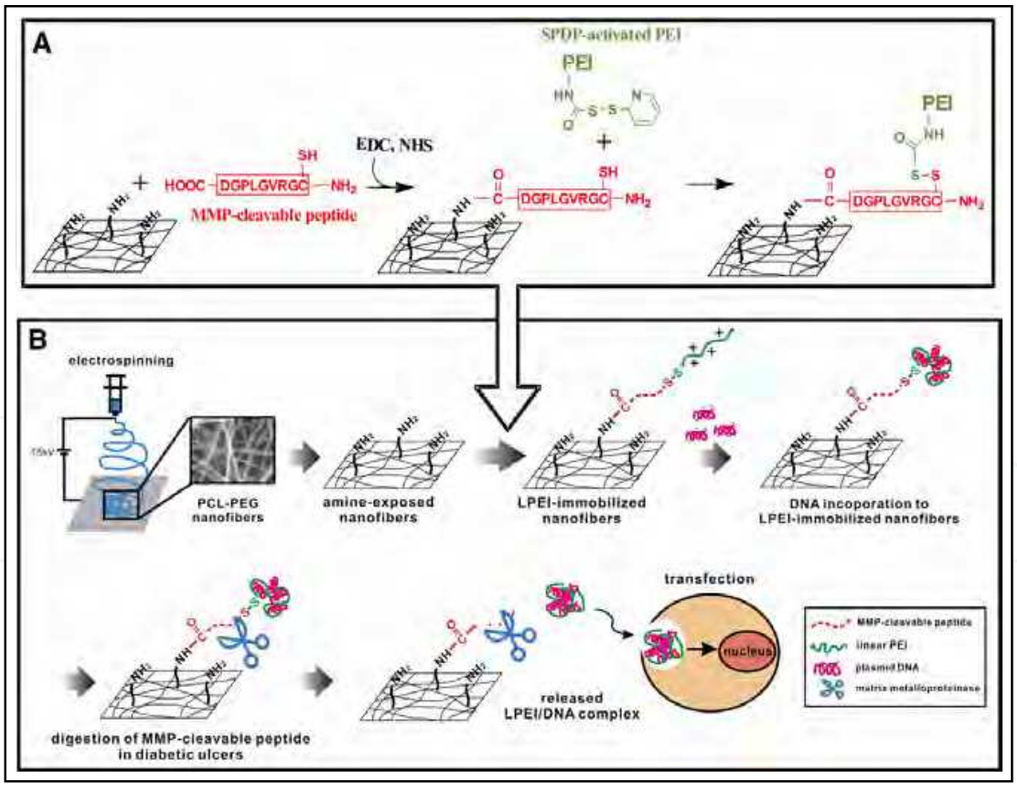


Fig. 11. (A) Synthetic scheme of LPEI immobilization on the surface of nanofibrous matrix via an MMP-cleavable linkage. (B) Schematic diagram of MMP-responsive electrospun nanofibrous matrix for gene delivery. Reprinted with permission from Kim and Yoo, 2010, Elsevier.

To investigate the performance of these MMP-responsive scaffolds (PCL-PEG and LPEI) in their ability to deliver GFP pDNA *in vivo*, a diabetic dorsal dermal ulcer mouse model was utilized. Results from these experiments showed that the highest level of GFP expression was detected in the dermal wounds treated with LPEI 3 days post-treatment. In contrast, very little GFP expression was detected with either naked DNA or with the PCL-PEG (no LPEI) scaffolds (Fig. 12). Similar results were also obtained with application of the scaffolds to normal, non-diabetic dermal wounds, although GFP expression with the LPEI containing scaffolds was increased in the diabetic wounds, as compared to the normal wounds (Fig. 12). Based on these results, the authors conclude that these MMP responsive DNA based electrospun scaffolds can be potentially applied for local gene delivery for the treatment of diabetic ulcers.

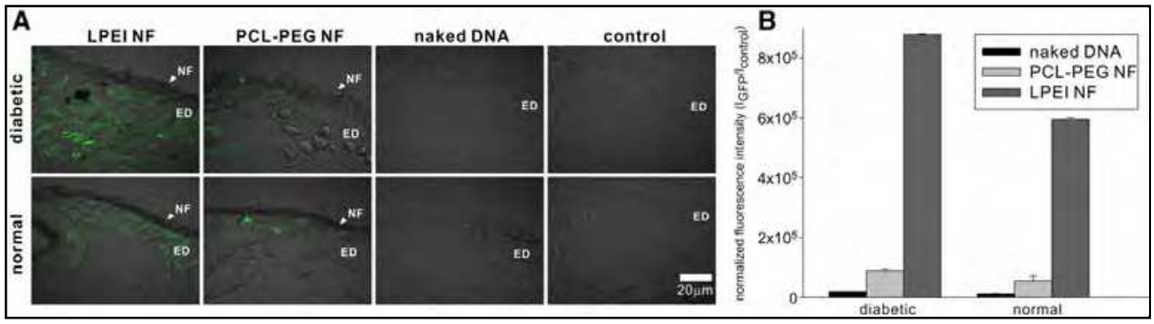


Fig. 12. Cross-sectional images of diabetic and normal wound tissue administered with DNA-incorporated nanofibrous matrix (NFs) at day 3. DNA/LPEI-immobilized nanofibrous matrix [LPEI NF] (NF 16) via a MMP-cleavable linker; DNA/PCL-PEG nanofibrous matrix without LPEI [PCL-PEG NF]. NF and ED indicate nanofibrous matrix and epidermis, respectively. GFP was visualized by confocal microscopy. (B) Image-analysis results of quantifying fluorescence intensity. Image-analysis methods based on an intensity based accumulation of the computer software (Image-Pro 6.0) was employed to quantify each image and average values and standard deviation were obtained (n=3). Reprinted with permission from Kim and Yoo, 2010, Elsevier.

4. Conclusion

The application of electrospinning in the fundamental studies of DNA and polymer behavior and gene delivery remains an active area of research. Although successful studies have been reported on the interactions between DNA and various polymers, the incorporation of DNA within nanofibers and its release, the structural characterization of the nanofibrous scaffolds and more importantly, demonstrated high transfection efficiency in both *in vivo* and *in vitro* settings, to date no definitive data supports the notion that electrospun scaffolds can serve as effective non-viral gene delivery systems that rival the efficiency of viral vectors. As such, there is a continual need to explore the nature of these electrospun scaffolds in order to be able to fine tune: a) the type(s) and combination(s) of polymers; b) the amounts of incorporated pDNA; c) the electrospinning parameters; d) the release rate of pDNA; e) the structural integrity of the released pDNA; and most of all, f) quantifiable and high transfection efficiency both *in vitro* and *in vivo*. Greater insight on the interactions between DNA and various polymers, understanding of the formation of

complex structures and their properties, the process of electrospinning, as well as DNA transfer into cells, can all play a significant role in enabling us to generate effective gene delivery systems with higher transfection efficiencies than currently achievable. If successful, the field of gene therapy will be enhanced and more widely embraced than it has been so far as a result of the shortcomings of viral gene delivery systems that are widely used to deliver the therapeutic genes. This is even more important now since regulatory molecules such as microRNAs and RNA interference (RNAi) are beginning to find their way to the clinic. Thus, electrospun scaffolds with all of the functional versatility could prove to be the next "magic bullet" in the realm of molecular medicine.

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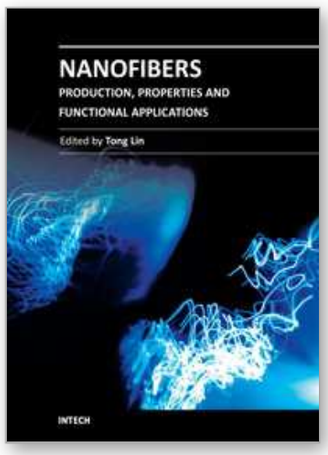

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As an important one-dimensional nanomaterial, nanofibers have extremely high specific surface area because of their small diameters, and nanofiber membranes are highly porous with excellent pore interconnectivity. These unique characteristics plus the functionalities from the materials themselves impart nanofibers with a number of novel properties for advanced applications. This book is a compilation of contributions made by experts who specialize in nanofibers. It provides an up-to-date coverage of in nanofiber preparation, properties and functional applications. I am deeply appreciative of all the authors and have no doubt that their contribution will be a useful resource for anyone associated with the discipline of nanofibers.

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