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A new pathway for developing *in vitro* nanostructured non-viral gene carriers

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Abstract

Extracellular and intracellular barriers typically prevent the efficient transfection of non-viral gene vectors. The formulation of a gene delivery carrier that can overcome the barriers would be a key for successful gene therapy. We have developed a novel pathway for the preparation of core-shelled DNA nanoparticles by invoking solvent-induced condensation of plasmid DNA (β-galactosidase) in a poor solvent mixture and subsequent encapsulation of the condensed DNA globule in a tri-block copolymer (e.g. polylactide-poly(ethylene glycol)-polylactide, $L_8E_{78}L_8$). The polylactide shell can protect the encapsulated DNA from degradation during electrospinning of a mixture of encapsulated DNA nanoparticles and biodegradable PLGA (a random copolymer of lactide and glycolide) to form a non-woven nanofibrous DNA-containing scaffold. The bioactive plasmid DNA can then be released in an intact form and in sufficient quantity from the scaffold with a controlled release rate and to transfect cells *in vitro*. Further consideration of the stability of the DNA in extracellular and intracellular environments is proposed. In particular, the use of block copolymers with a positively charged block and a hydrophilic block, as well as tri-arm block copolymers with an additional hydrophobic, biodegradable block to stabilize the DNA chain of interest, is discussed.

(Some figures in this article are in colour only in the electronic version)

Dedicated to Sow-Hsin Chen in celebration of his 70th birthday.

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1. Introduction

The basic idea of gene delivery is deceptively simple: integrate a desired gene into cells and then allow those cells to produce their own therapy, mostly in protein production. On the other hand, the actual implementation of this idea is not simple at all. The search for viable alternative approaches to deal with viral DNA delivery (i.e. safety, toxicity, fate of transfected cells, etc) has prompted many researchers to develop non-viral means of delivering genes *in vivo*. These means have included naked DNA, cationic lipids formulated into liposomes and subsequent complex formation with DNA (lipoplexes), complex formation of cationic polymers with DNA (polyplexes), and collagen- or hyaluronan-based DNA gels [1]. In addition, new biomaterials, such as the gene activated matrix (GAM) and biodegradable polymers (e.g., polylactide-co-glycolide, PLGA), were developed that could serve as scaffolds for cell adhesion, proliferation and initiation into new desirable matrices, as well as DNA delivery vehicles [2–4].

Non-viral gene delivery systems, in principle, offer enhanced safety over viral systems. However, they are, at present, plagued with comparatively low gene transfection efficiencies. Furthermore, non-viral gene delivery carriers encounter many practical barriers, both in extracellular and intracellular space, as shown schematically in figure 1. These barriers include the degradation of DNA fragments of interest in plasma, an uptake of DNA by the reticuloendothelial system, lysosomal degradation of the DNA, and a lack of translocation to the nucleus [1, 5, 6]. Thus, for non-viral gene delivery, one must first formulate an effective vehicle that can overcome these physicochemical hurdles. A critical component of the formulation is based on the fundamental understanding of the interactions between DNA and various chemical species in solution. Proper characterization of vectors is an essential step in the development of efficient non-viral gene delivery systems.

It is important to recognize the complexity of a non-viral gene delivery carrier, even though the basic idea of gene delivery is 'simple', because the laws of biology are not fully understood. Few areas of genomic research have produced so many unfulfilled promises as the research on gene delivery. Thus, one should always think carefully before plunging into this type of research. Nevertheless, new hopes for the 'overblown and overhyped' gene delivery research are on the horizon. There are, of course, many different methods being proposed to deliver the gene. For example, if one can physically use a needle to directly deliver the gene to where it is needed, e.g., into tumours or lungs, this could be one aspect of a useful pathway, which, by the way, is currently used in some human gene therapy trials.

The present paper represents our preliminary attempt to develop a unique non-viral gene delivery scheme that has not been demonstrated in the community, and is limited in scope to a specific target. The scheme aims to repair fractured bones that cannot quite heal by either accelerating the repair process or by inducing a union of $\sim$10% of the fractures that are characterized by complications. Genes representing growth (or transcription) factors are used to promote robust osteogenesis and chondrogenesis, resulting in the production of healthy tissue. Again, we let nature produce the needed bone growth. Even in this specific target, we consider only the first few steps of a specific scheme as proof of a concept for non-viral gene delivery. At this level of development, we pay special attention to the toxicity of the vector and its transfection efficiency; but less on cost or its ease of use, although the latter factors should not be forgotten. The presentation can be divided into five sections. Section 2 describes an idealized overall scheme for the ultimate scope and target, and section 3 illustrates the simplified steps that have been implemented and have successfully demonstrated the initial feasibility of the proposed scheme. In section 4, we report how encapsulated DNA nanoparticles with a core-shell structure were formulated by condensing plasmid DNA in a poor solvent mixture, followed by encapsulating the condensed DNA globule in a tri-

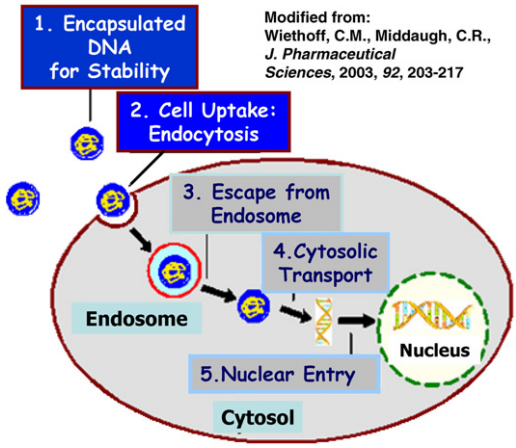

Figure 1. Barriers for non-viral gene delivery.

block copolymer of poly(lactide)-b-poly(ethylene glycol)-b-poly(lactide) (LEL) that could form micelles under the same solvent environment. The encapsulated DNA and PLGA mixed solutions were electrospun together to form a non-woven nanofibrous and nanocomposite scaffold. Preliminary results from the most important performance check are summarized in section 5, and section 6 describes our expectation of this unique pathway for the development of *in vitro* non-viral gene carriers.

2. A new concept to develop nanostructured non-viral gene carriers

It has been noted that, in order to successfully deliver the desired DNA fragments to the nucleus of a cell, the delicate DNA duplex has to be protected over a range of different environments, as shown schematically in figure 1, which represents an idealized overall scheme for DNA delivery. In other words, the DNA chain needs to remain stable, at least in both hydrophilic and hydrophobic solvent conditions, as well as over a range of pH values. In addition, the DNA molecule should be able to penetrate the cell membrane and be protected until it reaches the cell nucleus. Figure 1 shows an arbitrary division of the barriers for a DNA fragment to reach the nucleus. In essence, the DNA fragment should be protected before reaching the nucleus. Thus, several important criteria for successful gene delivery can be summarized here: (1) the DNA is being encapsulated for protection against foreign intrusion; (2) encapsulated DNA has to pass through the cell membrane. However, once inside the cell, the DNA is in a different fluid environment. Although the detailed knowledge for the DNA protection is not yet completely understood in this stage, the remaining criteria could include (3) DNA escape from the endosome, (4) cytosolic transport, and (5) integration of the desired gene in the host cell genome.

The new concept for developing *in vitro* nanostructured non-viral gene carriers is inspired by the pathways of viral gene delivery as illustrated in the idealized scheme (figure 1). Our approach is to simulate some of the essential functions of a virus for a specific and limited target. These functions are as follows: (1) the DNA is compact in a virus shell; (2) the DNA is protected by the virus shell; (3) the size of the virus is fairly small. Our approach in this paper represents a primitive attempt to simulate the three features of a virus, as illustrated in figure 2. It has not yet addressed the steps of (i) how the gene is to enter the cell and (ii) how

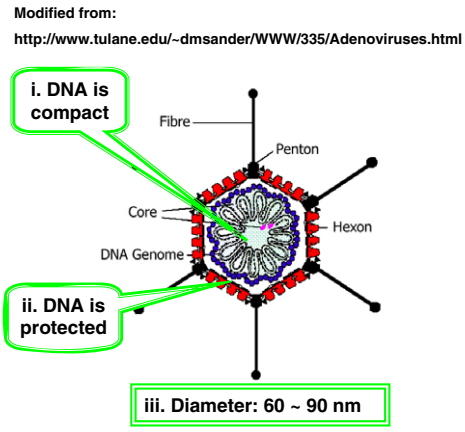

Figure 2. Some essential features of how an adenovirus works.

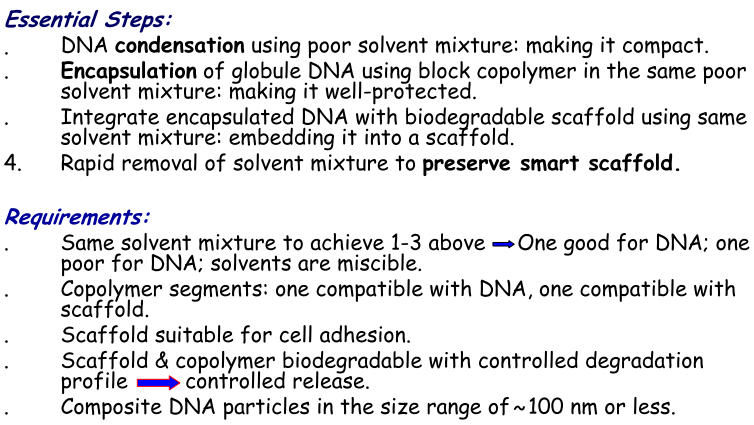

Figure 3. Essential steps and requirements of the simplified scheme.

it can be protected inside the cell before reaching the nucleus. However, such an approach is already quite different from the existing demonstrated pathways for the development of non-viral gene carriers. We shall also briefly discuss our future approach to address the last two steps (i.e. steps 1 and 2 in section 6).

3. Experimental procedures for sample preparation

Within the limited scope, the essential steps and requirements of the simplified scheme to prepare the new nanostructured non-viral gene carriers can be illustrated in figures 3 and 4. Figure 3 outlines the essential steps and the required conditions to preserve a plasmid DNA in a biodegradable scaffold. To achieve this goal, it is essential to consider the means to protect the DNA before it gets incorporated into the scaffold. There are many different ways to protect the DNA. Figure 4 illustrates two simple but effective steps: complex formation (or condensation) and encapsulation of collapsed DNA. They are discussed as follows.

For the condensation step, we can consider the following scenario. The extended double-stranded (ds) DNA helix can form a complex with oppositely charged multi-cations, such as

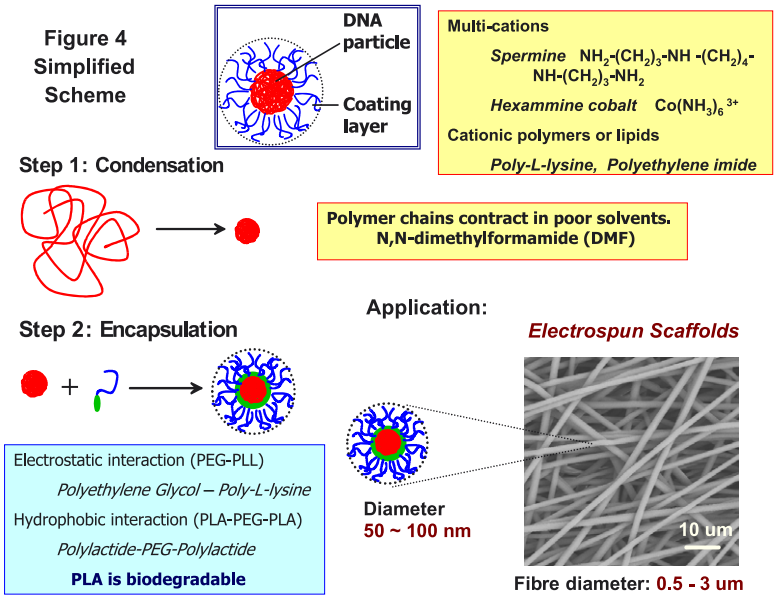

Figure 4. Simplified scheme.

spermine [7, 8] or hexamine cobalt [9, 10], or cationic polymers or lipids, such as poly-L-lysine [11] or polyethylenimine [12], as depicted in the top right panel of figure 4. Alternatively, the DNA fragments can be made more compact by using solvent-induced condensation, as shown in Step 1 of figure 4. Here, we select a solvent mixture that has to satisfy requirement 1 in figure 3. One component of the solvent mixture should be a good solvent for DNA, implying that the solvent component should be aqueous in nature, but not pure water, which is not a truly good solvent for the polyelectrolyte (DNA). Thus, an appropriate buffer solution is used. The second solvent component should be a poor solvent for DNA, but a good solvent for the biodegradable copolymer. These two solvent components should be miscible so that we can vary the solvent compositions to tune the solvent quality, making it sufficiently poor for the extended DNA chain to collapse into a denser chain, such as a globule, and a selective solvent for the block copolymer to form a micelle that can encapsulate the collapsed DNA. Furthermore, the solvent mixture should remain as a good solvent for the biodegradable copolymer, making the polymer solution that contains the encapsulated DNA suitable for the electrospinning process.

For the preliminary experiments, we have chosen N,N-dimethylformamide (DMF) and 1 × TE buffer as the solvent mixture. The reason for using DMF in the solvent mixtures was mainly for convenience, as we had experience in using DMF as a viable solvent for the fabrication of biodegradable nanofibrous PLGA (a random copolymer of lactide and glycolide) membranes by means of the electrospinning technology. It may not be the proper solvent for biomedical applications because of its high boiling point, making complete removal of the solvent from the electrospun membrane after fabrication more difficult. Nevertheless, it should be acceptable for the current experiments on the demonstration of concept.

The second step deals with the encapsulation of collapsed DNA by using a block copolymer, as depicted in figures 3 and 4. One can use the complex formation by means of electrostatic interaction or take advantage of hydrophobic interaction to stabilize the DNA, or both, as demonstrated by the bottom left panel in figure 4. In addition, the hydrophobic block

(PLA) is biodegradable, so the resultant protective 'shell' can be degraded by hydrolysis in order to expose the DNA for transfection. The example here is PLA–PEG–PLA. Both PLA and PEG are soluble in DMF. However, in the presence of a small amount of buffer solution, PEG prefers to be miscible with water while PLA is hydrophobic, resulting in water-induced micelle formation that has a buffer-containing PEG core, useful for the encapsulation of the collapsed DNA.

It is certainly not advisable to have DNA in contact with DMF over long periods of time. Next, we want to remove the DMF very quickly but to preserve the encapsulated DNA for storage. This step can be accomplished by using the electrospinning process [13], where the solvent in the solution jet stream is being evaporated over a very short time period along a flight path of the order of about 20 cm between the spinneret and the collector, as illustrated in figure 4, in the application for an electrospun scaffold with the encapsulated DNA being embedded. The scaffold can be designed to be suitable for a specific type of cell adhesion. Thus, the approach relies on cells adhering to the scaffold and there is no need for a homing device for the DNA to go to the cells. In other words, the DNA-containing scaffold becomes the new temporary extracellular matrix, capable of bringing the cells in close proximity to the released DNA. Further, we can change the chemical composition and membrane morphology to control the scaffold degradation rate which can be correlated with the imbedded DNA release rate.

4. Results and discussion

4.1. Experimental findings on DNA condensation and encapsulation

Experiments on the quality of solvent-induced DNA coil-to-globule transition and subsequent encapsulation in a tri-block copolymer have been carried out. The results are very encouraging for the specific application and have been recently published [14]. The published experimental data will not be reproduced. Only the key findings are summarized in what follows.

4.1.1. DNA condensation. DNA condensation has attracted a great deal of attention in recent years due to its biological importance in DNA packing in the virus head as well as in the development of gene delivery vehicles [15, 16]. The extended DNA chains could be effectively collapsed into compact particles through charge neutralization by cationic polymers [17] or polyamines [8]. DNA condensation has been reported in the literature, showing that double-stranded DNA in the extended coil conformation could be compacted by complex formation, based on strong electrostatic interactions, upon mixing with spermine [18, 19], Ca^{2+} [20], propanol [21] or other cationic reagents [22]. Upon condensation, the DNA molecules could also undergo a conformational transition. Zhang *et al* [23] reported that calf thymus DNA transformed from B-form to C-form when mixed with didodecyldimethylammonium bromide (DDAB) at a high DDAB to DNA ratio. Quaternary diammonium dications, $R(CH_3)_2N^+(CH)_nN^+(CH_3)_2R$, depending on their intercharge distance, were able to transform giant T4 DNA from B-form to A-form [24]. However, DNA molecules would remain in B-form when mixed with Ca^{2+} [25] or chitosan [26]. We chose an alternative route of using a *poor* solvent mixture to condense DNA. There are two advantages of using a poor (or non-) solvent for DNA condensation: no contamination and easy solvent removal through evaporation. The chosen poor solvent was 94% (in volume) N,N-dimethylformamide (DMF) + 6% 1× TE buffer (to be denoted as 'water' hereafter). Experiments (not shown) prove that the plasmid DNA could remain intact in DMF over short time periods and its bioactivity could be maintained.

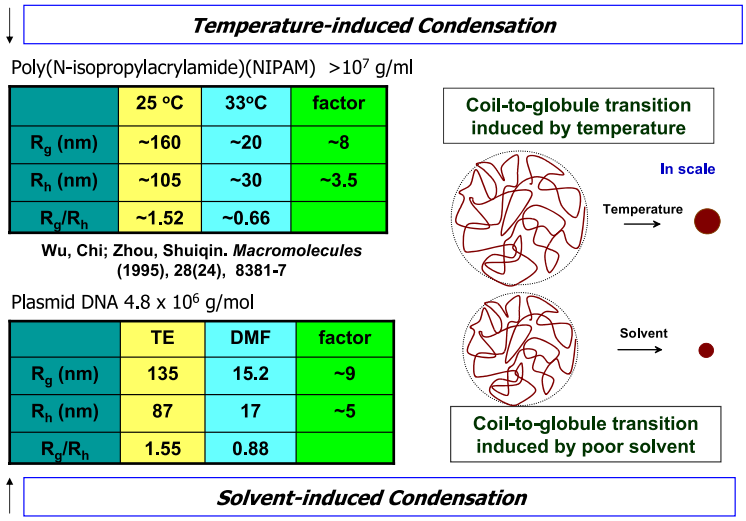

Figure 5. Temperature-induced versus solvent-induced condensation.

Laser light scattering, including both static laser light scattering (SLS) and dynamic laser light scattering (DLS), was employed to monitor the changes in DNA size and conformation. Zimm plots of pCMVβ plasmid DNA (7164 bp) encoding β-galactosidase in 1× TE buffer (10 mM Trisbase and 1 mM EDTA in pure water, pH = 8.1) and in 94% DMF + 6% 'water' (denoted as DMF) are shown in figure 5, respectively. From a Zimm plot, the weight average molecular weight (M_w), the radius of gyration (R_g), and the second virial coefficient (A_2) can be obtained. The value of M_w of plasmid DNA in the solvent mixture was only slightly larger than that in 1× TE buffer, suggesting a very small amount of aggregation. However, the R_g value was significantly reduced from 135 nm (in 1× TE buffer) to about 15 nm, indicating the occurrence of *chain condensation*. The hydrodynamic radius, R_h, obtained from the CONTIN [27] analysis of dynamic light scattering data, was also dramatically decreased from 87 nm in 1× TE buffer to about 17 nm in 94% DMF + 6% water. The relationship between the conformation of polymer chains and the R_g/R_h ratio has been well established. Typically, a random coil has an R_g/R_h value of 1.5, and a solid sphere has a value of 0.775 [28]. As shown in figure 5, plasmid DNA had a R_g/R_h ratio of 1.55 in 1× TE buffer, suggesting essentially a random-coil conformation, while in 94% DMF + 6% water, the value was about 0.88, close to 0.775, indicating a relatively compact solid-sphere conformation. During the solvent-induced coil-to-globule transition process, the conformation of DNA molecules may be changed from B-form. However, after the removal of solvent by electrospinning and subsequent release of encapsulated DNA in an aqueous medium, the DNA conformation was able to recover, at least substantially, as evidenced by gel electrophoresis. Polymer chain collapse could also be induced by temperature changes as for poly(*N*-isopropyl acrylamide) in water [29]. The collapse induced by changing the solvent quality, however, is more pronounced, as illustrated in figure 5. It is noted that the PNIPAM sizes were larger because of its higher initial molar mass.

4.1.2. Aggregation of $L_8E_{78}L_8$ and interaction of plasmid DNA with LEL in poor solvent. The tri-block copolymer, $L_8E_{78}L_8$ (abbreviated as LEL), was synthesized according to a known scheme [30]. In brief, PEG and D,L-lactide with known ratio were mixed together and the

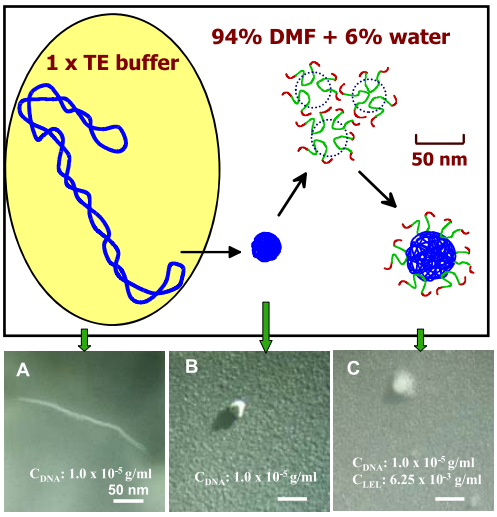

Figure 6. Plasmid DNA condensation and encapsulation.

polymerization process was accomplished at 130 °C for 15 h using stannous octoate as the catalyst. Before mixing plasmid DNA and LEL together, the solution behaviour of LEL in 94% DMF + 6% water was investigated. With the known molecular weight of PEG block (3.4 k), the average length of PLA block could be estimated from proton NMR (data not shown), which was about 0.6 k. Therefore, the average repeating unit of each block was estimated to be $L_8E_{78}L_8$ (abbreviated as LEL). Gel permeation chromatography (GPC) showed that the weight average molecular weight of LEL was about 5.9 k.

The molecular scale interaction of DNA and LEL is depicted schematically in figure 6. The figure also shows (TEM) experimental evidence for the condensation of plasmid DNA (TEM panels (A) and (B)), the aggregation of LEL, and the encapsulation of DNA by LEL in 94% DMF + 6% water, panel C). In 94% DMF + 6% water, the DNA chain was condensed to form a much smaller globule with a diameter of ∼30 nm, also shown in figure 5.

Water is a good solvent for PEG but not for PLA. In the solution state, water molecules prefer to stay together with PEG blocks instead of PLA blocks. Even though DMF is a good solvent for *both* PEG and PLA, their solubility in the solvent mixture could be different, causing the tri-block copolymer molecules to aggregate. The average diameter of the aggregates was around 80 nm at concentrations above 1×10^{-3} g ml^{-1} (data not shown). Given that the bond lengths of C–C and C–O are 0.154 and 0.143 nm, respectively, the maximum backbone length of $L_8E_{78}L_8$ should be less than 30 nm even if the polymer chain were fully stretched. Therefore, each LEL aggregate could contain more than one PEG domain (figure 6), while the PEG domains should contain most of the water molecules. Upon mixing with condensed DNA, which is hydrophilic, such PEG/water domains could serve as a 'shelter' for the plasmid DNA. Since DNA dislikes PLA, one could then expect the LEL chains to accommodate the condensed DNA particles to form a core–shell structure. In other words, the condensed DNA particles were encapsulated by LEL, which was verified by laser light scattering results [14] as well as TEM imaging at various stages (super-coiled plasmid, condensed plasmid, and encapsulated DNA nanocomposite particle), as shown schematically and experimentally in figure 6. In the mixtures of DNA with different amounts of $L_8E_{78}L_8$, only one component was observed by dynamic laser light scattering, indicating the formation of DNA complexes. The apparent

hydrodynamic radius of those DNA complexes, depending on the polymer-to-DNA ratio, was larger than that of the globule DNA itself at the same concentration [14]. After being air dried from 1× TE buffer, plasmid DNA was rod-like with a length of more than 200 nm (figure 6, panel (A)), very close to the shape of a compact random coil with high super-helical content; while in 94% DMF + 6% water, each plasmid DNA formed essentially a solid sphere with an average diameter of ∼30 nm. There could be a few multimers, i.e., more than one single DNA molecule in the collapsed globule. However, the number should be relatively low, as depicted by the average DNA molecular weight based on static light scattering measurements. The core–shell structure, as shown in figure 6, was quite different from condensed DNA structures, typically toroids [31, 32] and rods [33], formed in aqueous solutions upon mixing with multivalent cations, including cationic polymers and polyamines [16]. Further studies on this finding are in progress.

4.2. Experimental findings on scaffold fabrication

As mentioned briefly before, the encapsulated DNA fragments are in a fluid medium containing mostly DMF. As a gene carrier, it is essential to remove all the DMF and yet to preserve the structure of the supra-molecular complex that we just created. To achieve this goal, we took advantage of the electrospinning process, which can provide two unique features.

- The solvent in the jet stream of a polymer solution is removed very rapidly by evaporation along the flight path in the presence of an applied electric field. The DNA-containing fibrous material is essentially in a dry state, as shown schematically as well as by scanning electron microscopy in figure 4, by the time it reaches the ground collector in an electrospinning process. The residual solvent, especially DMF, can be removed by evaporation under mild vacuum at relatively low temperatures. It is interesting to note that the jet stream velocity can be accelerated to approach the speed of sound under an applied electric field. Thus, an estimated evaporation time could be in the millisecond range while the effective spin draw ratio could reach a value of the order of 10^6.
- The resultant DNA-containing non-woven nanofibrous scaffold in the dry state can be stored for reasonable periods of time without fear of degradation.

DNA-containing nanoparticles have been incorporated successfully into a non-woven nanofibrous PLGA scaffold for controlled release. The PLGA scaffold acts as a matrix for cells because it is designed for cells to adhere to the scaffold, to penetrate into it, and to proliferate (data not shown). The LEL tri-block copolymer was used to encapsulate the DNA molecules and to protect them from degradation during the electrospinning process. However, with LEL tri-block copolymers, the encapsulated DNA should burst out in an aqueous environment after it is released from the fibres in the scaffold.

The electrospun nanofibrous biodegradable scaffolds possess many controllable properties, such as high surface-to-volume ratio, appropriate porosity, and malleability to conform to a wide variety of sizes and shapes, which make them superior scaffolds for tissue engineering [34], as well as for the delivery of cells and bioactive agents, including drugs [35, 36], protein [37] and DNA [38, 39].

The random copolymer PLGA (M_w = 75 k, LA/GA = 75/25) and plasmid DNA, with or without LEL, could be mixed to form a viscous solution. Then, the scaffold could be fabricated using the mixed DNA-containing polymer solution by using the electrospinning process. Figure 7 shows an example of PLGA scaffolds having ∼10% w/w LEL and DNA. The scaffolds contain a distribution of submicron-sized diameter fibres that were annealed together because the presence of block copolymer lowered the glass transition temperature

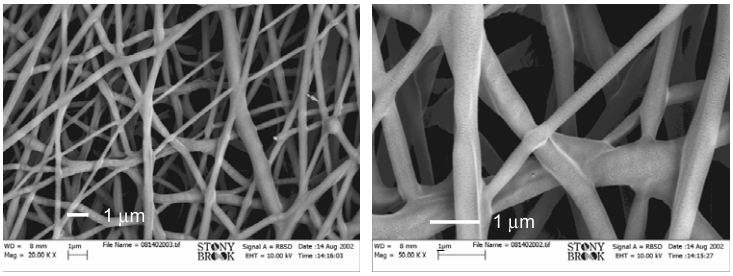

Figure 7. Biodegradable electrospun scaffolds containing PLGA (90% PLGA, 10% BC)/β-gal plasmid DNA.

of the blend. The SEM image of the PLGA scaffold without LEL (data not shown) showed no visual difference in the overall morphology, porosity and fibre diameter. Thus, the presence or absence of block copolymers has revealed no obvious physical difference on the overall morphology of these scaffolds.

After removing the solvent (94% DMF + 6% water) under vacuum, the scaffold allowed cells to adhere and proliferate. DNA released from the scaffold was analysed by using agarose gel electrophoresis. Prior to electrophoresis, sections of scaffold were incubated in $1\times$ TE buffer solution, and the concentration of the released DNA from solution was analysed via the use of a Pico Green stain assay. Thus, we were able to quantitatively determine the amount of released DNA time point. Our results showed that DNA-containing scaffold samples without the block copolymer had degraded the DNA during the electrospinning process. In contrast, in the presence of block copolymer, the DNA released from the scaffold was essentially intact structurally, with no apparent degradation. Further experiments should be carried out for a better comparison under the same conditions of DNA concentration, pH, and ionic strength for the released DNA.

To clarify how the DNA/LEL nanoparticles were incorporated in the PLGA fibres, we measured the sustained release of intact DNA from the PLGA scaffold over a seven-day time period. During the gene release process, where a dry scaffold was immersed into an aqueous solution, LEL aggregates were disassembled and the LEL chains would likely form flower-like micelles with PLA being the core and PEG being the corona (data not shown). This inside-out transformation would instantly cause the release of the encapsulated DNA. Therefore, the DNA release rate could also be influenced by the diffusion of aqueous solution into the PLGA scaffold. The biodegradation of the scaffold is also partially affected by the presence of encapsulated DNA.

5. DNA transfection

There was a *difference* in the structural quality of the released DNA from the two types of scaffold (with and without LEL block copolymer). Results from gene transfection experiments using pre-osteoblastic MC3T3 cells are presented. Previously, we showed that when compared with naked DNA itself in solution, where no gene transfection was observed, the PLGA scaffold

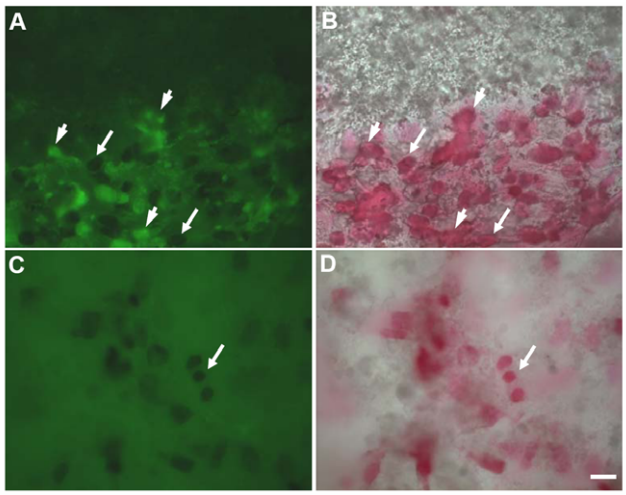

Figure 8. Increased transfection of MC3T3 cells plated directly on a GFP plasmid DNA-containing scaffold.

with encapsulated DNA (also in solution) enhanced the DNA transfection [39]. When we plated cells directly onto a scaffold containing a green fluorescence protein (GFP) DNA plasmid, there was a dramatic increase in DNA transfection, as exhibited by the presence of green cells (figure 8, panel (A) and panel (B)). In contrast, no green cells were apparent in a scaffold without any GFP DNA (figure 8, panel (C) and panel (D)). The increase in DNA transfection observed in this experiment probably occurred in two ways: the scaffold brought cells closer to the released DNA by allowing cell adhesion directly onto the nanofibres, and the released DNA maintained a relatively higher concentration around the scaffold surface due to substrate immobilization [40]. In figure 8, panel (A) shows the fluorescent image of cells on GFP plasmid DNA containing a PLGA/10% block copolymer scaffold at 48 h post-plating. Arrowheads indicate representative transfected GFP-expressing cells and arrows indicate non-transfected cells. Panel (B) is the light micrograph of cells (shown in (A)) stained with nuclear fast red. Arrowheads and arrows indicate identical cells in (A). Panel (C) shows the fluorescent image of cells on a control scaffold containing no DNA and indicating no fluorescent (green) cells (arrow). Panel (D) is the light micrograph of cells (shown in (C)) stained with nuclear fast red. The arrow indicates identical cells in (C). In figure 8, the scale bar $= 10\ \mu$m.

6. Conclusions

Together with our previous studies [39], five concluding remarks can be listed from our experimental results.

(1) By understanding and capitalizing upon the molecular interactions of block copolymers and plasmid DNA in solution, novel structures and additional functionality can emerge.
(2) By considering the molecular self-assemblies and the electrospinning process, it is anticipated that one can develop an effective gene delivery vehicle.
(3) In the present case, *in vitro* gene delivery to cells was accomplished by attracting the cells to a scaffold that was capable of supporting new cell adhesion and growth. Thus, the incorporation of a homing device could be of secondary importance.

(4) The volume contraction of a condensed DNA molecule was of the order of a *thousand-fold*. Although the volume contraction was less than what a virus could accomplish, it permitted us to demonstrate, at least in principle, how the *condensed* plasmid DNA could be encapsulated and protected by its incorporation into an electrospun scaffold.
(5) Finally, the scaffold with its network structures reduced the mobilization of released DNA before transfection. Thus, judicious control on the structure and morphology of the fabricated gene delivery scaffold could be developed in terms of the scaffold material properties and processing procedures, ultimately leading towards improved transfection efficiency.

There are several thoughts on how to proceed that should also be included here. Although we have demonstrated a unique pathway with simplified steps to develop a non-viral gene delivery vehicle, the work is only just getting started because after the encapsulated DNA has been released from the scaffold, it is not well protected. There is also no mechanism to promote the DNA duplex to pass through the cell membrane. Furthermore, once it is inside, a different mechanism for its protection and release is needed. Our proposed next step is to use complex formation via the route of electrostatic interactions. By using such a combination, we hope to stabilize the collapsed DNA chain by complex formation using a block copolymer having one hydrophilic block and one positively charged block, such as PEG-PLL. The degree of neutralization could be used to stabilize the DNA before the complex is 'encapsulated'.

Block copolymers of EB could form the starting point of future research, with E and B being, respectively, a hydrophilic E block (e.g., polyethylene oxide (PEO)) and a charged B block (e.g., poly-L-lysine (PLL)). The purpose of incorporating PEO, a hydrophilic block, is to improve the solubility of the PLL/DNA complex. In fact, we can combine the electrostatic and hydrophobic interactions into a more versatile variation by using a tri-arm, tri-functional block copolymer that could have the following advantages.

- The presence of a positively charged B block (e.g., poly(L-lysine), neither a single cationic charge nor a long chain of cations, but only a relatively short chain of cations, and as denoted by PLL or B) is intended for the complex formation of B with the negatively charged DNA.
- The presence of a hydrophilic block (e.g., polyethylene glycol and as denoted by PEG or E) is to increase the solubility of the charged complex in the aqueous environment. We have chosen a solvent mixture of DMF and water that has a unique property for PEG because PEG is also soluble in DMF, but its interaction with water via hydrogen bonding is stronger.
- After the tri-arm block copolymer has interacted with DNA by means of strong electrostatic interactions, the two remaining blocks (E and L) are similar to the LEL (or EL) block copolymers. The use of blends of EBL and LEL tri-blocks is to sustain what has already been accomplished, but in addition, to protect the DNA nanoparticles after their release from the scaffold in an aqueous environment. The PLL/DNA complex with attached soluble blocks is intended as a partial and temporary protective coating.
- The DNA/PLL complex should provide a stronger stabilization advantage for the DNA nanoparticles during the electrospinning process.
- When the nanoparticles are released from the scaffold, the DNA/PLL complex will prevent the DNA from bursting out into the aqueous environment for a desirable pre-designed period of time.
- It is noted that the use of blends (EBL and LEL) permits us to control the degree of stability for the complex in both the hydrophobic and aqueous environments.

It should be interesting to find out if our ultimate goal of delivering the DNA duplex of interest to the cell nucleus can be achieved by implementing the above more complex pathways.

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