Controllable delivery of non-viral DNA from porous scaffolds

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Abstract

The inductive approach to tissue engineering combines three-dimensional porous scaffolds with drug delivery to direct the action of progenitor cells into a functional tissue. We present an approach to fabricate scaffolds capable of controlled, sustained delivery by the assembly and subsequent fusion of drug-loaded microspheres using a gas foaming/particulate leaching process. DNA-loaded microspheres were fabricated from the copolymers of lactide and glycolide (PLG) using a cryogenic double emulsion process. Microspheres were fabricated in four populations with mean diameters ranging from 12.3\,\mu m to 92.5\,\mu m. Scaffolds fabricated by fusion of these microspheres had an interconnected open pore structure, maintained DNA integrity, and exhibited sustained release for 21 days. Control over the release was obtained through manipulating the properties of the polymer, microspheres, and the foaming process. Decreasing the microsphere diameter or the molecular weight of the polymer used for microsphere fabrication led to increased rates of release from the porous scaffold. Additionally, increasing the pressure of CO\textsubscript{2} increased the DNA release rate. The ability to create porous polymer scaffolds capable of controlled release rates may provide a means to enhance and regulate gene transfer within a developing tissue, which will increase their utility in tissue engineering.

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1. Introduction

The inductive approach to tissue engineering seeks to combine the fabrication of porous polymer scaffolds with drug delivery technology, which has the potential to locally stimulate cellular processes through a controlled release of bioactive factors directly into the cell microenvironment [1]. Scaffolds designed to engineer tissues must have several basic requirements, which include being biodegradable and biocompatible, and having a high surface area/volume ratio with sufficient mechanical integrity. An interconnected open pore structure can provide for cell infiltration from the surrounding tissue, which is important for integration of the engineered tissue with the host. The utility of the scaffold as a drug delivery vehicle can be employed to initiate cellular processes that lead to the creation of a functional tissue that integrates with the body. For example, the release of VEGF in combination with PDGF can induce a vascular network to develop within the polymer scaffold [2]. Alternatively, the delivery of
tissue-specific growth factors can induce differentiation of endogenous or transplanted progenitor cells into the appropriate cell type [3–5].

Polymer scaffolds that release DNA encoding for therapeutic or tissue inductive proteins represent a powerful alternative to the direct delivery of the protein. As cells invade through the pores of the scaffold, they encounter DNA that is either released from or entrapped within the matrix. The transfected cells can subsequently act as bioreactors for the local production of tissue-inductive factors [6]. The polymeric systems developed to deliver biologically active proteins have been adapted to deliver non-viral DNA in order to overcome limitations associated with efficient delivery [7,8]. Encapsulation of DNA within the polymer can protect against its degradation. Collagen-based systems for plasmid delivery have been used for applications in bone regeneration [9,10]. In these applications, collagen functions to entrap the non-viral DNA and also provides a scaffold that supports cell attachment and cell migration. The results suggest that the non-viral DNA is incapable of diffusing through the collagen carrier. Thus, the matrix serves to hold DNA in situ until endogenous fibroblasts infiltrate the scaffold, internalize the DNA, and express the encoded protein. Alternatively, PLG scaffolds with entrapped DNA were fabricated using the gas foaming/particulate leaching process [11,12]. These sustained delivery systems have the potential to overcome difficulties associated with clearance from the tissue and to increase gene transfer. The continued presence of the plasmid during cell division can facilitate entry into the nucleus, during which the nuclear membrane is compromised [13]. These studies illustrate that DNA delivery from polymer scaffolds can enhance tissue formation. However, the ability to fabricate scaffolds capable of controlled, sustained release will provide a means to enhance and regulate gene transfer within a developing tissue, which will enhance their utility toward tissue engineering applications [14].

We previously described the fabrication of polymer scaffolds with an interconnected open pore structure and high porosity by assembling and fusing DNA loaded microspheres [12]. In this report, we describe the procedures to control the release of DNA from these scaffolds by manipulating the processing parameters. DNA-loaded microspheres were fabricated using a cryogenic double emulsion process, which provides high efficiency incorporation and distributes the DNA throughout the polymer. We examined the influence of the polymer properties, the microsphere properties, and the foaming conditions for their effects on the incorporation, release kinetics, and stability of the encapsulated DNA.

2. Materials and methods

Plasmid DNA was purified from DH5α cultures using Qiagen (Santa Clara, CA) reagents and stored in Tris–EDTA (TE) buffer solution (10 mM of Tris, 1 mM of EDTA buffer, pH 7.5) at −20 °C. The plasmid used in this research was obtained from National Gene Vector Labs with the gene encoding β-galactosidase (pNGVL-1 β-gal). Poly(D,L-lactide-co-glycolide) (PLG, 75: 25 mole ratio of D,L-lactide to glycolide, Resomer 755 and 752) was obtained from Boehringer Ingelheim Chemicals (Petersburg, VA). Resomer 755 has an inherent viscosity in the range of 0.6–0.8 dl/g and will be referred to as the high molecular weight polymer. Resomer 752 has an inherent viscosity in the range of 0.16–0.24 dl/g and will be referred to as the low molecular weight polymer. Poly(vinyl alcohol) (PVA, 88% hydrolyzed, average MW 22,000) was purchased from Acros Organics (Morris Plains, NJ). All other reagents were obtained from FisherBiotech (Fairlawn, NJ), unless otherwise indicated.

2.1. Production of DNA-loaded microspheres

DNA-loaded microspheres were fabricated using a cryogenic double emulsion technique [15]. A DNA solution (100 μl) containing 300 mM of sucrose was added to a 2% solution of PLG in dichloromethane. This mixture was emulsified by sonication, and then selectively frozen by immersion in liquid nitrogen. A PVA solution (50%, 50 ml) containing 300 mM sucrose was subsequently added, and a second emulsion was formed by homogenization at 1000–7000 rpm for 14 s. This solution was diluted in 30 ml of 1% PVA containing 300 mM sucrose and stirred at room temperature for 3 h. Microspheres
were collected by centrifugation, washed three times with deionized water to remove residual PVA, and lyophilized overnight.

2.2. Fabrication of porous scaffolds

Porous PLG scaffolds were fabricated by a gas foaming/particulate leaching process. DNA-loaded microspheres (7 mg) were mixed with 190 mg of sodium chloride crystals (250 μm< diameter <425 μm) and compression molded at 1500 p.s.i. The compressed disk was incubated with 95% humidity at 37 °C for 24 h [16]. After incubation, the mixture was dried under vacuum, and equilibrated with CO2 gas (600 p.s.i., 800 p.s.i.) in a custom-made pressure vessel. After quenching the pressure, the constructs were then immersed in water (1 ml) to leach the sodium chloride. After the leaching procedure, the scaffolds were dried overnight at room temperature.

2.3. Analysis by scanning electron microscopy (SEM)

The pore structure of polymer scaffolds was visualized using scanning electron microscopy (SEM, Hitachi 3500N). The scaffolds were coated with a gold–palladium (60:40 ratio) film (3 nm), and examined using an electron voltage of 20 kV.

2.4. Calculation of DNA encapsulation efficiency

Dried microspheres (10 mg) were dissolved in 600 μl of chloroform and vortexed vigorously. Tris–EDTA buffer (300 μl) was added to the mixture, vortexed, and centrifuged at 3000 rpm for 10 min. The upper aqueous phase was collected, and the procedure was repeated four times. DNA extracted from microspheres was quantified using fluorometer (TD 360, Turner Machines, CA) with the fluorescent dye Hoechst 33258. The encapsulation efficiency of the plasmid in the microspheres was calculated as the mass ratio of plasmid extracted from the microspheres to the plasmid input into the process.

2.5. The integrity of the encapsulated DNA

The integrity of the encapsulated plasmid was analyzed by agarose (0.8%) gel electrophoresis. Ethidium bromide was used to detect DNA. Digital images of the gels were captured with a Kodak gel documentation system. The percentage of DNA in the supercoiled conformation was determined using NIH Image Software.

2.6. Release kinetics of plasmid DNA

An in vitro release assay was conducted to determine the release kinetics of plasmid DNA from microspheres (7 mg) and foamed scaffolds. The microspheres and scaffolds were immersed in 500 μl of Tris–EDTA buffer (pH 7.5). Solutions were removed with replacement at specified times (4 h, 1, 3, 7, 14, and 21 days). The concentration and integrity of the released DNA were evaluated by fluorometry and agarose (0.8%) gel electrophoresis, respectively. The percentage of the DNA released was calculated as the ratio of the mass of DNA released to the mass of DNA in the polymer. Note that for the release studies from the scaffolds, the quantities released are relative to the amount of DNA remaining in the scaffold after the leaching step.

3. Results

3.1. Microspheres

A cryogenic double emulsion process was employed to fabricate microspheres of controlled sizes and with high efficiency incorporation of plasmid (Fig. 1A). The diameter of the microspheres was dependent on the mixing speed of the homogenizer, which was employed to create microspheres with diameters ranging from 91.5 ± 21.3 μm (1000 rpm) to 12.3 ± 5.0 μm (7000 rpm) (Table 1). The incorporation efficiency of plasmid encapsulated into PLG microspheres (Fig. 1A), which was calculated as the ratio of the quantity of DNA extracted from the microspheres to the initial amount of plasmid input into the process, was also a function of the homogenizer speed. For microspheres fabricated with high molecular weight polymer, the incorporation efficiency ranged from 67.2 ± 6.2% to 83.7 ± 7.4% for the various microsphere diameters. However, for microspheres fabricated with the low molecular weight polymer, the incorporation efficiency ranged...
Fig. 2. Release kinetics of DNA from microspheres fabricated by the double emulsion process. The curves represent various combinations of polymer molecular weight and microsphere diameter: (▲), high MW, 12.3±5.0 μm; (●), low MW, 48.8±17.3 μm; (■): high MW, 91.5±21.3 μm. Cumulative DNA release is defined as the amount of DNA released relative to the amount of incorporated plasmid initially.

The release kinetics of encapsulated DNA from the microspheres were dependent on the microsphere diameter when using the high molecular weight polymer, but not the low molecular weight polymer. Fig. 2 demonstrates the range of release kinetics that were observed from the polymer microspheres. For microspheres fabricated with high molecular weight polymer, the release during the first 4 h ranged from 6.4±2.6% to 23.5±0.3% for the different diameter microspheres, with the largest microspheres providing the smallest initial release (Table 2). The microspheres with the largest mean diameter provided a sustained release for 7 days, and had a cumulative release equal to 27.7±6.5% of the encapsulated plasmid. Conversely, microspheres with mean diameters of 12.3 μm, 22.7 μm or 48.8 μm had sustained release for 21 days and released at least

Table 1
Diameter and incorporation efficiency for microspheres fabricated by the cryogenic double emulsion process

<table>
<thead>
<tr>
<th>Homogenizing speed (rpm)</th>
<th>Mean diameters of microspheres (μm)</th>
<th>Incorporation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High MW</td>
<td>Low MW</td>
</tr>
<tr>
<td>1000</td>
<td>91.5±21.3</td>
<td>83.7±7.4</td>
</tr>
<tr>
<td>3000</td>
<td>48.8±17.6</td>
<td>67.2±6.2</td>
</tr>
<tr>
<td>5000</td>
<td>22.7±11.9</td>
<td>71.8±8.0</td>
</tr>
<tr>
<td>7000</td>
<td>12.3±5.0</td>
<td>71.6±4.6</td>
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</table>
Table 2
Characteristics of DNA release from PLG microspheres fabricated with varying molecular weight and diameter

<table>
<thead>
<tr>
<th>PLG Microsphere sizes (µm)</th>
<th>Initial burst&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Initial release rates&lt;sup&gt;b&lt;/sup&gt; (%/h)</th>
<th>Total cumulative release&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.16–0.24</td>
<td>91.5±21.3</td>
<td>14.3±1.2</td>
<td>41.0±4.0</td>
</tr>
<tr>
<td></td>
<td>48.8±17.3</td>
<td>18.0±2.0</td>
<td>47.3±4.3</td>
</tr>
<tr>
<td></td>
<td>22.7±11.9</td>
<td>13.0±2.0</td>
<td>44.3±1.3</td>
</tr>
<tr>
<td></td>
<td>12.3±5.0</td>
<td>12.0±2.0</td>
<td>43.4±2.9</td>
</tr>
<tr>
<td>0.6–0.8</td>
<td>91.5±21.3</td>
<td>6.4±2.6</td>
<td>27.7±6.5</td>
</tr>
<tr>
<td></td>
<td>48.8±17.3</td>
<td>10.0±0.0</td>
<td>66.0±4.0</td>
</tr>
<tr>
<td></td>
<td>22.7±11.9</td>
<td>20.0±1.0</td>
<td>69.0±7.0</td>
</tr>
<tr>
<td></td>
<td>12.3±5.0</td>
<td>23.5±0.3</td>
<td>76.3±0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total DNA released through 4 h (burst).
<sup>b</sup> The average release rate through 1 day.
<sup>c</sup> Total cumulative release after 21 days.

66% of the encapsulated DNA. For microspheres fabricated with the low molecular weight polymer, the release kinetics were similar for microspheres of all diameters (Table 2). An initial burst between 12.0±2.0% and 18.0±2.0% of the incorporated DNA was observed and the total mass of DNA released from these microspheres ranged from 41.0±4.0% to 47.3±4.3% (Table 2).

The DNA released from the microspheres during the 21 day release study retained its integrity; however, there was a reduction in the fraction of supercoiled DNA that was more pronounced for the low molecular weight polymer. For microspheres fabricated with the high molecular weight polymer, DNA released from the microspheres at the earliest time point had the largest percentage in the supercoiled conformation (79.9%), which decreased during the release study to a final amount equal to 27.9% (Fig. 3A). A similar trend was observed for microspheres fabricated from the low molecular weight polymer. However, the percentage of DNA in the supercoiled conformation at all time points was lower than that observed for the microspheres with the high molecular weight polymer (Fig. 3B).

3.2. Porous scaffolds

Scaffolds with an interconnected, open-pore structure were fabricated from the DNA-loaded microspheres using a gas-foaming/particulate leach process (Fig. 4A). These scaffolds are more than 94% porous and the pore size is regulated by the size of the salt crystals, which was determined based on the volume and mass of the scaffold and the density of the polymer [12,17]. Scaffolds were fabricated from polymers at two molecular weights, microspheres with a range of mean diameters (12.3, 22.7, 48.8, 72.7, 119, 123μm) were fabricated from the DNA-loaded microspheres. The other lanes are representative for plasmid released at: lane 2, 1 day; lane 3, 3 days; lane 4, 7 days; lane 5, 14 days; lane 6, 21 days; lane 7, unincorporated plasmid DNA.
polymer had an increased quantity of DNA lost during the leaching step, which was not statistically different for the various microsphere sizes ($P > 0.29$, mean for all diameters equals $23.5 \pm 2.1\%$) (Fig. 4B).

Microsphere diameter was a determinant of the quantity of DNA released from the porous scaffold when using the high molecular weight polymer at a high pressure (800 p.s.i.). Fig. 5 demonstrates the range of release kinetics that were observed from the polymer scaffolds. The amount of DNA released ranged from 20% to 90% and was dependent upon the polymer molecular weight, the microsphere diameter, and pressure CO$_2$ used for foaming. The characteristics of the release profile from scaffolds are summarized in Table 3. At a pressure of 600 p.s.i., no significant differences were observed in the release profile for scaffolds fabricated from microspheres of varying diameters. A mean initial burst during the first 4 h of $5.6 \pm 1.3\%$ was followed by a sustained release of plasmid, with a total amount of DNA released that ranged from $28.1 \pm 3.8\%$ to $31.4 \pm 3.7\%$. For the high molecular weight polymer processed at 800 p.s.i., the release profile of DNA from the scaffold was dependent upon the diameter

and 91.5 $\mu$m), and two pressures of CO$_2$ (600 p.s.i., 800 p.s.i.). During the leaching step, scaffolds fabricated with the high molecular weight PLG had decreasing losses of DNA as the microsphere diameter increased. The mean percentage of DNA lost during leaching decreased from 15.7% to 1.7% as the mean microsphere diameter increased from 12.3 to 91.5 $\mu$m (Fig. 4B). A similar trend was observed at lower pressures, but with lower amounts of DNA lost to the leaching step. The lower molecular weight polymer had an increased quantity of DNA lost during the leaching step, which was not statistically different for the various microsphere sizes ($P > 0.29$, mean for all diameters equals $23.5 \pm 2.1\%$) (Fig. 4B).

Fig. 4. (A) Scanning electron photomicrograph of the surface of a polymer scaffold that was fabricated from 12.3$\pm$5.0 $\mu$m microspheres at 800 p.s.i. (Magnification, $\times60$; electron voltage, 20 kV). (B) Percentage of DNA lost from the scaffold during leaching process. The percentages of DNA lost are relative to the amount of DNA within the polymer scaffold prior to the leaching process, which is equal to the amount of DNA within 7 mg of microspheres.

Fig. 5. DNA release from polymer scaffolds fabricated from microspheres using the gas foaming process. The curves represent various combinations of polymer molecular weight, microsphere diameter and CO$_2$ pressure: (▲): low MW, 12.3$\pm$5.0 $\mu$m, 800 psi, (●): low MW, 91.5$\pm$21.3 $\mu$m, 800 psi, (■): high MW, 12.3$\pm$5.0 $\mu$m, 800 psi, (♦): high MW, 12.3$\pm$5.0 $\mu$m, 600 psi, (♦): high MW PLG, 91.5$\pm$21.3 $\mu$m, 800 psi. The cumulative DNA release is relative to the amount of DNA within the scaffold after the leaching process.
Table 3 Characteristics of DNA release from PLG scaffolds fabricated for varying molecular weight, microsphere diameter, and CO₂ pressure

<table>
<thead>
<tr>
<th>PLG i.v. (dl/g)</th>
<th>CO₂ pressure</th>
<th>Microsphere sizes (µm)</th>
<th>Initial burst (a) (%)</th>
<th>Initial release rates (b) (%/h)</th>
<th>Total cumulative release (c) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.16–0.24</td>
<td>800 p.s.i.</td>
<td>91.5±21.3</td>
<td>28.9±4.3</td>
<td>1.9</td>
<td>76.3±4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.8±17.3</td>
<td>26.7±4.1</td>
<td>1.8</td>
<td>76.3±10.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.7±11.9</td>
<td>30.7±3.4</td>
<td>2.0</td>
<td>81.7±8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3±5.0</td>
<td>29.1±1.9</td>
<td>2.0</td>
<td>90.0±3.9</td>
</tr>
<tr>
<td>0.6–0.8</td>
<td>800 p.s.i.</td>
<td>91.5±21.3</td>
<td>5.3±0.1</td>
<td>0.4</td>
<td>23.4±1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.8±17.3</td>
<td>4.9±0.2</td>
<td>0.4</td>
<td>28.0±1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.7±11.9</td>
<td>9.6±0.9</td>
<td>0.6</td>
<td>42.8±4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3±5.0</td>
<td>10.9±0.7</td>
<td>0.7</td>
<td>47.4±2.2</td>
</tr>
<tr>
<td>0.6–0.8</td>
<td>600 p.s.i.</td>
<td>91.5±21.3</td>
<td>6.6±0.9</td>
<td>0.7</td>
<td>30.2±0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.8±17.3</td>
<td>3.7±1.0</td>
<td>0.4</td>
<td>28.1±3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.7±11.9</td>
<td>5.7±0.6</td>
<td>0.4</td>
<td>29.1±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.3±5.0</td>
<td>6.5±0.5</td>
<td>0.5</td>
<td>31.4±3.7</td>
</tr>
</tbody>
</table>

(a) Total DNA released through a 4 h (burst).
(b) The average release rate through 1 day.
(c) Total cumulative release after 21 days.

des of the microsphere used in fabrication. For scaffolds fabricated from microspheres with the largest mean diameter (48.8, 91.5 µm), the release profile at 800 p.s.i. was similar to that observed at 600 p.s.i. However, scaffolds fabricated from the smaller microspheres (mean diameters equal to 12.3, 22.3 µm) released more than 40% of the incorporated DNA. Consistent with the results for microsphere release (Table 2), scaffolds fabricated from the smallest microspheres released the largest total amount of plasmid. Note that the total amount of plasmid released from the scaffold, however, was less than that of plasmid released from the microspheres alone. Studies also examined the pressure release rate (average of 20 p.s.i./min, 40 p.s.i./min) for changes in the release kinetics of DNA; however, no significant effects were observed (data not shown).

Decreases in the polymer molecular weight resulted in increases in the quantity of DNA released from the scaffold and were independent of the mean diameter of the microsphere (Table 3). For the low molecular weight polymer, an initial burst between 26.7±4.1% and 30.7±3.4% of encapsulated plasmid was released after 4 h. Following this initial release, a sustained release of plasmid was observed, with the quantity of encapsulated DNA released at 21 days ranging from 76.3±4.9% to 90.0±3.9%. For scaffolds fabricated with the low molecular weight PLG, the quantity of DNA released from the scaffold was greater than the quantity released from the microspheres.

The gas foaming process allowed for the release of intact DNA from the scaffold at all time points examined; however, the percentage of DNA in the supercoiled conformation was reduced relative to that observed for the release from microspheres (Fig. 6). Similar to the microsphere release, the DNA released at the earliest time points had the largest percentage in the supercoiled conformation, with decreases in this percentage observed during the 21 days of the study. Scaffolds fabricated from microspheres with the smallest diameter (12.3 µm) and the highest molecular weight retained the greatest fraction of supercoiled DNA (Fig. 6A, B). Increasing the diameter of the microsphere served to decrease the percentage in the supercoiled conformation (Fig. 6C). Scaffolds fabricated using the lowest molecular weight polymer had the lowest percentage of DNA in the supercoiled conformation (Fig. 6D, E).
4. Discussion

Previous reports demonstrated the potential for assembling and fusing drug-loaded microspheres into a drug-releasing scaffold using a gas foaming/particulate leaching process [2,12]. This report demonstrates the means to control release rates from the scaffold through regulation of the polymer properties, the microsphere properties, and the processing conditions. DNA-loaded microspheres were fabricated in diameters ranging from 12.3±5.0 μm to 91.5±21.3 μm. Incorporation efficiencies ranging from 37.4±7.6% to 83.7±7.4% and a maximal loading of 8.5 μg DNA per mg of polymer were obtained using a cryogenic double emulsion process. Interconnected open pore scaffolds could be fabricated from the DNA-loaded microspheres. DNA was released for times ranging from 7 days to 21 days, and the release rate can be controlled through the properties of the polymer, microspheres, and foaming process. Increasing the pressure of CO₂, decreasing the molecular weight of the polymer, and decreasing the size of the microsphere were found to increase the release of DNA from the porous scaffolds. Analysis of the released DNA throughout the 21 day study by gel electrophoresis demonstrated that the DNA retained its integrity with conversion to the open conformation at later times of release.

Scaffolds capable of sustained release of growth factors [2,5,18–21] and DNA [10,11,22] have shown potential in stimulating and directing the processes within tissue formation and regeneration. The release of transfection-competent DNA within the scaffold has been employed to stimulate tissue formation through the local and transient production of growth factors [1,11]. Transfected cells are thought to function as bioreactors for the localized production of tissue inductive factors. Extending the utility of DNA-releasing scaffolds for the engineering of tissues in vivo and in vitro will require the development of systems that efficiently transfect cells on the scaffold. Naked DNA released from polymer scaffolds has been shown to transfect large numbers of cells in vivo when sufficient quantities of plasmid are delivered (more than 100 μg) [10,11,22]. Polymeric release is hypothesized to mediate the bioavailability by maintaining elevated levels of DNA for extended times. However, the dose and release rate required

Fig. 6. Integrity of plasmid following release from PLG scaffolds. The mean microsphere diameter, polymer molecular weight, and pressure used in fabrication are (A) 12.3±5.0 μm, high MW, 800 p.s.i.; (B) 12.3±5.0 μm, high MW, 600 p.s.i.; (C) 91.5±21.3 μm, high MW, 800 p.s.i.; (D) 91.5±21.3 μm, low MW, 800 p.s.i.; (E) 12.3±5.0 μm, low MW, 800 p.s.i.; Lane 1, molecular weight marker. Lanes 2–6, plasmid released at 1, 3, 7, 14, and 21 days, respectively. Lane 7, unincorporated plasmid.
for in vivo transfection are not well understood, which provided the motivation for the fabrication of scaffolds capable of controlled DNA release. Low levels of transfection are observed following release in vitro and may be limited to those cells in close proximity or direct contact to the polymer, possibly as a result of micro-emulsions formed during the fabrication process [22]. The incorporation and polymeric release of DNA complexes with transfection reagents (e.g., cationic lipids, cationic polymers) may extend the application of these scaffolds to in vitro studies and potentially enhance transfection in vivo.

The cryogenic double emulsion process was used to achieve high incorporation efficiencies and loadings of intact DNA. Previous approaches to fabricate DNA-releasing scaffolds from DNA-loaded microspheres employed a standard double emulsion process for microsphere development. The standard double emulsion process typically provides incorporation efficiencies ranging from 20% to 30% [12]. Additionally, these microspheres had a complete release of DNA within 24 h, likely due to surface-association of the DNA. For the cryogenic double emulsion process, increased incorporation efficiencies were obtained; however, the incorporation efficiency varied depending upon microsphere diameter and the polymer molecular weight. A sustained release of DNA was observed from microspheres fabricated by the cryogenic process, suggesting that the DNA was incorporated throughout the polymer microsphere. The increased incorporation efficiency translates into increased quantities of DNA in the scaffold. Importantly, the integrity of the DNA was retained during fabrication and subsequent release, which is consistent with the initial report for this process [15].

Several processes exist to fabricate scaffolds with interconnected open pore structures, such as phase separation/sublimation [23], sintering [24], and solvent casting [25]. The gas foaming/particulate leaching process avoids the use of organic solvents and high temperatures in creating the three dimensional structure, which may be advantageous for the fabrication of drug-releasing scaffolds [11,26]. Although CO₂ has a long history in polymer processing, it has only recently been applied to the fabrication of scaffolds [17,27–29]. After equilibration in a high pressure CO₂ environment, quenching of the pressure causes the individual microspheres to fuse into an interconnected matrix. Foaming via pressure quenching [30–34] mimics the effect of raising the temperature [35]. By lowering the pressure at a fixed temperature, the Tₚ begins to rise and eventually reaches the point where the polymer Tₚ is higher than the foaming temperature: at this point the cellular structure can grow no further and is locked in [30,35]. CO₂ is a desirable plasticizer because it can be used at low temperatures, which maintains the activity of the incorporated drug [11,21,26,35–38]. Polymer composition and molecular weight are established determinants of the mechanical and structural properties of a polymer; however, these properties also affect CO₂ dissolution [35]. The solubility of CO₂ is greater in amorphous polymers than crystalline polymers and plasticization is greater for low molecular weight polymers. Previous studies with poly(methyl methacrylate) have demonstrated that changing the exposure time and pressure of CO₂ can be used to tailor the properties of the foam [32].

The release rate of DNA from the polymer scaffold was observed to increase with a decrease in the polymer molecular weight, a decrease in the microsphere diameter, and an increase in the pressure of CO₂ used for gas foaming. These observations are consistent with a link between the release rate of the DNA and the extent of polymer plasticization by CO₂, which is required for microsphere fusion. At a pressure of 600 p.s.i., the release rate of DNA was independent of microsphere size and the mean amount of DNA released at 21 days ranged from 28.1% to 31.4% (Table 3). Increasing the pressure to 800 p.s.i. increased the total release from the scaffolds fabricated from the smallest microspheres (12.3 μm, 22.7 μm) to more than 40% (Table 3). A similar effect of the microsphere size was observed with respect to the amount of DNA lost in the leaching step (Fig. 4B). The effect of microsphere size on release may result from the increased surface area for small microspheres relative to large microspheres given the same quantity of polymer. The increase in CO₂ pressure likely increases the amount of solubilized CO₂ within the polymer, which would increase the plasticization. Subsequent experiments with the lower molecular weight polymer demonstrated an increased release rate, which is consistent with plasticization having a
greater influence on polymers with low molecular weight than high molecular weight. Interestingly, the scaffolds fabricated from the low molecular weight polymer released 81.1±6.5% of incorporated DNA after 21 days, while the microspheres released approximately 44.0±2.6% of incorporated DNA. Note, however, that the dependence of release rate on polymer molecular weight is also consistent with polymer degradation contributing to the release. Polymers with a molecular weight between 80 and 97 kDa have demonstrated a 3–15% mass loss after 12 weeks in vitro [39,40]. Polymers with lower molecular weight (8 kDa) exhibited a 78.5% decrease in molecular weight after 14 days in vitro [41].

Total DNA release from the polymer scaffolds results from a combination of erosion (subsequent to hydrolysis) and diffusion [42]. The total release of DNA from the scaffold increased as the microsphere diameter decreased (for high pressure CO₂ processing) and as the molecular weight decreased. These properties are consistent with the effects of plasticization and polymer degradation on the release rates. The dependence of total release on microsphere diameter differs for release from the scaffold and from the individual microspheres (Table 2 versus Table 3). For release from microspheres, the high molecular weight polymer had a larger cumulative release relative to the low molecular weight polymer. The cumulative release of DNA from these polymeric systems ranged from 23% to 90% of the incorporated DNA, which is consistent with several published reports [43,44]. Complete release of the incorporated DNA may be possible under the appropriate conditions; however, polymeric systems must be developed to stabilize the DNA integrity for long-term release.

The integrity of the incorporated DNA is retained after incorporation and throughout the 21 day release; however, the percentage of DNA in the open conformation increases after scaffold fabrication and during the subsequent release. For DNA released from the scaffold during the first day, the percentage of DNA in the supercoiled conformation (25.0% to 47.2%) is less than that observed in the microspheres prior to foaming (50.3% to 79.9%). The DNA released subsequent to this first day showed a gradual increase in the percentage of DNA in the open conformation with increased time of release from the polymer. Hydrolysis of the PLG can decrease the pH within the polymer, which is known to affect DNA stability [45]. The initial decrease in the percentage of supercoiled DNA immediately after foaming may not result entirely from polymer hydrolysis, as the extent of polymer degradation may be limited within the initial 24 h. Degradation of the high molecular weight polymer, for example, occurs on a timescale of weeks [39,40]. Sucrose is used as a cryoprotectant during the microsphere processing. Sucrose could be employed as the porogen in place of sodium chloride, which may serve to enhance the stability of DNA within the polymer. Alternative approaches that are being developed to stabilize DNA are the incorporation of an antacid, such as Mg(OH)₂, or the complexation with cationic polymers prior to encapsulation [46–48].

5. Conclusion

We have fabricated three-dimensional porous polymer scaffolds capable of controlled sustained delivery of DNA, by the assembly and fusion of DNA-loaded microspheres using a gas foaming/particulate leaching process. This process provides the versatility to employ multiple microsphere populations to provide a controlled release of one or more factors from the matrix. The release of DNA from the scaffold can be controlled through the microsphere properties, the polymer properties, and the processing conditions. The combination of synthetic polymer scaffolds with controlled drug (e.g., protein, DNA) delivery may be used to enhance both the inductive and cell transplantation approaches to tissue engineering, which are based on the ability to direct cellular processes within a developing or regenerating tissue [14].

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References