Degradation behaviors of biodegradable macroporous scaffolds prepared by gas foaming of effervescent salts

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Abstract: Biodegradable polymeric scaffolds for tissue engineering were fabricated by a gas-foaming/salt-leaching method using a combination of two effervescent salts, ammonium bicarbonate and citric acid. Poly(D,L-lactic-co-glycolic acid) (PLGA) in a state of gel-like paste was first produced by precipitation of PLGA dissolved in chloroform into ethanol. The polymer slurry was mixed with sieved particles of ammonium bicarbonate, molded, and then immersed in an aqueous solution of citric acid to generate macroporous scaffolds. The scaffolds had relatively homogeneous pore structures throughout the matrix and showed an average pore size of 200 μm and over 90% porosity. By adjusting the concentration of citric acid in the aqueous medium, it was possible to control porosity as well as mechanical strength of the scaffolds. In vitro degradation studies of three different scaffolds having lactic/glycolic acid molar ratios of 75/25, 65/35, and 50/50 exhibited marked swelling behaviors at different critical time points. The swollen matrices had a hydrogel-like internal structure. It was found that massive water uptake into the degrading scaffolds induced matrix swelling, which facilitated the hydrolytic scission of PLGA chains with concomitant disintegration of the matrices. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 55: 401–408, 2001

Key words: biodegradable polymer; PLGA; scaffolds; degradation

INTRODUCTION

Aliphatic biodegradable polymers such as poly(L-lactic acid) and its copolymers with D-lactic acid and glycolic acid have been widely used for temporal scaffolds for tissue engineering. A macroporous structure with uniform pore sizes well over 100 μm is highly desirable for efficient cell seeding and culture. Sufficient supplies of nutrients and oxygen to seeded cells within the porous scaffolds are required for facile tissue formation. There have been a number of fabrication methods for biodegradable scaffolds. They are PGA nonwoven mesh, solvent casting/salt leaching, phase separation, emulsion freeze-drying, gas foaming with pressurized carbon dioxide, 3-D printing, and gas foaming/salt leaching. Very recently we reported that macroporous biodegradable scaffolds successfully could be fabricated using ammonium bicarbonate salt as a gas foaming agent as well as a porogen additive. The gas foaming/salt leaching method is based on the idea that sieved salt particles of ammonium bicarbonate dispersed within a polymer-solvent mixture generate ammonia and carbon dioxide gases within solidifying matrices upon contact with hot water, thereby producing highly porous structures. These scaffolds showed macropore structures of over 200 μm with no visible surface skin layer, which permitted sufficient cell seeding within the scaffolds. Porosities could be controlled by the amount of ammonium bicarbonate incorporated to the polymer. Moreover, it was possible to make various scaffolds with different geometries and sizes by a hand-shaping or molding process because the polymer–salt mixture became a gel paste after a partial evaporation of the solvent.

In this study, the gas foaming/salt leaching method has been improved further by immersing the semi-solidified polymer–salt mixture into an aqueous solution of citric acid for the gas-foaming process at room temperature. Citric acid and ammonium bicarbonate have been known as effervescent salts due to their carbon dioxide evolving property upon contact in aqueous solution. Particularly, various alkalinizing-analgesic oral tablets are commercially available. Different concentrations of citric acid in the aqueous medium were used to control porosities and mechanical strengths of the PLGA scaffolds by modulating the
extent of gas evolution. Furthermore, various PLGA polymers with molar lactic/glycolic ratios of 50/50, 65/35, and 75/25 were used to fabricate scaffolds having different degradation rates. They were incubated in phosphate-buffered saline solution to study their degradation behaviors in vitro. Mass erosion, molecular weight, and water uptake were systematically analyzed as a function of time, with an emphasis of morphologic change in the scaffolds.

MATERIALS AND METHODS

Materials

Poly (DL-lactic-co-glycolic acid) of lactic/glycolic molar ratios of 75/25, 65/35, and 50/50 were purchased from Alkermes (Cincinnati, Ohio). Weight average molecular weights of PLGA 75/35, 65/35, and 50/50 were purchased from Alkermes (Cincinnati, Ohio). Weight average molecular weights of PLGA 75/35, 65/35, and 50/50 were 97,200, 74,300, and 54,200, respectively, as determined by gel permeation chromatography. Ammonium bicarbonate and citric acid were purchased from Sigma (St. Louis, Missouri).

Scaffold fabrication by gas foaming in acidic aqueous solution

Viscous polymer precipitate in a state of gel paste was prepared first by dissolving 10% (w/v) PLGA in 10 mL of chloroform and then by precipitating it into an excess volume of cold ethanol solution with gentle mixing. Chloroform was a solvent and ethanol was a nonsolvent for PLGA. Sieved ammonium bicarbonate salt particulates (salt particle size was 300–500 μm) were added to the PLGA gel paste and mixed homogeneously by a spatula. The weight ratios of NH₄HCO₃ to PLGA were adjusted at 10:1 and 20:1. Teflon molds having two different dimensions of 10 mm in diameter with 2 mm or 5 mm in thickness were used. A gel paste mixture of polymer/nonsolvent/salt was put into the disk-shaped molds and the nonsolvent (ethanol) was partially evaporated at room temperature to obtain a semi-solidified mass. This was immersed into an aqueous citric acid solution of different concentrations [20, 40, or 60% (w/v), and supersaturated] at room temperature to induce gas foaming as well as salt leaching within the polymer/salt matrices. After the completion of the effervescence, the porous polymeric scaffolds were taken out of the molds, washed with distilled water several times, and then freeze-dried for several days.

Scaffold characterization

Surface and cross-section structures of the scaffolds and the configuration of their inner pores were observed by scanning electron microscopy (SEM; Phillips XL30S, The Netherlands). Before observation, the polymeric scaffolds were coated with gold particles in an argon atmosphere at 5 psia for 5 min under an electric field of 5 mA by using a sputter (HUMMER V, Hummers Techniques, USA). Mercury intrusion porosimetry (PMI 30K-A-1, Porous Materials, Inc., New York) was used to determine total pore volume and porosity of the scaffolds. Duplicate scaffold samples were used for the porosimetry measurement. The mercury filling pressure was applied up to 200 psia, as described previously. The compression modulus of the scaffolds also was measured by Instron 5538 (Instron Corp., USA). A load cell of 10N was hammered vertically at a speed of 2 mm/min on a scaffold specimen, which had a cylindrical shape of 12 mm high and 6 mm in diameter, according to ASTM F451-95.

The amount of residual solvent in polymeric gel paste and dried scaffold was determined by using gas chromatography (Hewlett-Packard 6890). The residual amount of chloroform was quantified with a flame ionization detector (FID) using a HP-1 crosslinked methyl silicone gum capillary column. The obtained data were an average value of at least triplicate samples.

In vitro degradation study

Scaffolds pre-wetted by immersion in ethanol were placed in glass vials containing 20 mL of 33 mM of phosphate-buffered saline (PBS, pH 7.4). The samples were incubated in a 37°C incubator up to 10 weeks under static conditions. The PBS solution was changed daily for the first week, once at day 10 and day 14, and then weekly for the remaining period. At the end of each sampling time point, pH change in the incubation medium was monitored. The retrieved scaffold samples were subjected immediately to measurement of wet weight in a hydrated state, after surface water was removed with a Kimwipes tissue, and then they were freeze-dried. Mass erosion and water uptake of each scaffold sample during the degradation period then were determined by using the wet and dry weights of the sample. Cross-section morphologies of the scaffolds before and after degradation were observed by scanning electron microscopy.

Molecular weight determination

Molecular weight changes of the scaffolds were determined by using gel permeation chromatography. A GPC system composed of a Dynamax Model SD-200 and a refractive index detector (Shodex RI-71) was operated by using two Phenogel 5 columns (pore size 10³ Å and 10⁴ Å, 50 × 7.8 mm; Torrance, California) connected in a series as a gel permeation column. The polymer was dissolved in tetrahydrofuran and eluted through the GPC system at a flow rate 1 mL/min. Polystyrene standards (Polysciences, Warrington, Pennsylvania) were used to obtain a calibration curve.

RESULTS AND DISCUSSION

Figure 1 shows a schematic representation of polymeric scaffolds prepared by a gas foaming/salt leach-
ing method. Previously, poly(l-lactic acid) (PLLA) was dissolved in chloroform to make a viscous gel phase into which sieved ammonium bicarbonate particles were mixed to produce polymer/salt gel paste for a gas foaming process in hot water. The gel formation of PLLA in chloroform was likely to be caused by the physical crosslinking of crystalline domains between PLLA chains under the condition of highly concentrated PLLA polymer solution. While this method was good enough for fabrication of semicrystalline PLLA scaffolds, it was difficult to fabricate PLGA scaffolds because amorphous PLGA dissolved in chloroform did not readily become a gel phase even at high concentrations. Thus as an alternative method PLGA first was dissolved in chloroform and then precipitated in nonsolvent ethanol. The recovered PLGA precipitates had a resilient, gel-like property, and when mixed with ammonium bicarbonate particles, a gel paste state could be obtained. This mixture of precipitated PLGA and salt particles was hand-shapeable and moldable. The gel paste that had been filled into the Teflon mold, after a partial evaporation of ethanol under ambient temperature became semi-solidified, sufficiently enough to handle. Then it was immersed into aqueous solution containing different citric acid concentrations for a simultaneous process of gas foaming and salt leaching, after which it was freeze-dried.

Surface and cross-sectional morphologies of the resultant scaffolds are shown in Figure 2. It can be seen that the surface has a highly porous structure and does not have a dense skin layer, which often was observed in the scaffolds prepared by a salt leaching method. Pores on the surface and in the internal region of the scaffolds are uniformly macroporous, with sizes ranging from 200 to 500 μm. The pores also are well interconnected throughout the scaffold matrix.

Pore sizes and morphologic shapes in the scaffold were determined primarily by the amount of ammonium bicarbonate particles and by the extent of gas foaming upon contact with aqueous citric acid solution. In a previous report, it was shown that porosity of the scaffolds can be controlled by varying the fractional amount of ammonium bicarbonate particles incorporated into the gel paste. Since aqueous citric acid solution was used in this study instead of hot water for the gas foaming process, it was of interest to see the effect of citric acid concentration in the aqueous medium on the porosity of scaffolds. It was expected that controlling the extent of carbon dioxide evolution within solidifying gel matrices would affect the porosity of the scaffolds. Figure 3 shows the porosity change of PLGA 65/35 scaffolds as a function of citric acid concentration. The porosity was determined by a mercury intrusion porosimetry. Increasing the citric acid concentration from 20% (w/v) to a supersaturated condition, while the weight ratio of ammonium bicarbonate to PLGA was set at 10/1, caused the porosity gradually to increase from 87.0 to 94.1%. This suggests that the porous volume of scaffolds can indeed be modulated by controlling the extent of acid-base reaction between citric acid and ammonium bicarbonate. More vigorous gas evolution during the

Figure 1. Schematic representation of polymeric scaffolds prepared by gas-foaming/salt-leaching method.
gas foaming process visually could be observed with increasing citric acid concentration. When the weight ratio of ammonium bicarbonate to PLGA was adjusted at 20/1 and supersaturated citric acid was used in the aqueous medium, as high as 96.3% porosity could be achieved. This result is consistent with the previous finding that scaffold porosity can be controlled by the amount of incorporated salt particles.

Compression modulus values of these scaffolds prepared by using different citric acid concentrations were analyzed (Fig. 4). The compression modulus value decreases as the citric acid concentration increases, indicating that more porous scaffolds have weaker mechanical strength, as expected. Controlled pore sizes in the scaffolds were also confirmed by SEM images (Fig. 2).

Figure 2. SEM images of PLGA scaffolds (thickness 3 mm): (A) surface; (B) magnified view of surface; (C) magnified view of cross-section.

Figure 3. Porosity of PLGA scaffolds prepared in aqueous media with different citric acid concentrations.

Figure 4. Compress moduli of PLGA scaffolds prepared with different citric acid concentrations in aqueous media.
folds are highly desirable for inducing selective cell migration into the scaffolds since various types of cells used for \textit{in vivo} tissue regeneration migrate and infiltrate porous materials in a pore-size-dependent manner.\textsuperscript{2,18} There are insignificant variations in morphologic characteristics with changing the lactic/glycolic composition of PLGA (50/50, 65/35, and 75/25) and with varying scaffold dimensions (3 and 5 mm in thickness). For all the scaffolds, pores sizes were around 200 \textmu m and porosities were about 90\%. The SEM observation of the scaffolds also confirmed no detectable difference in the morphology. These results reveal that the gas foaming/salt leaching method is very effective in producing a consistent quality in various PLGA scaffolds regardless of polymer composition and dimension. It should be noted that the above data of pore size and porosity might be underestimated because they were determined by a mercury intrusion porosimetry method. The mercury intrusion method is based on the assumptions that pores are a cylindrical shape and that they are well interconnected.\textsuperscript{14} Some dead-end pores and micropores within the scaffolds could not be taken into account. The SEM pictures in Figure 2 show much larger pores—well over 200 \textmu m—in contrast to those determined by the mercury-intrusion method. Residual solvent in the scaffolds raises major safety concerns when the scaffolds are used for cell culture and are implanted into the body. The amount of residual chloroform, as quantified by gas chromatography, shows a rapid elimination of the solvent with the progression of a series of fabrication steps. These data are listed in Table I. After the gas foaming process, the residual amounts of chloroform in the scaffolds are well below 60 ppm, an upper limit value of chloroform amount in FDA-approved medical devices.\textsuperscript{16} In particular, there was no residual chloroform detected in completely dried scaffolds after 7 days of freeze-drying.

Three PLGA scaffolds having different lactic/glycolic acid compositions were prepared and incubated in phosphate-buffered saline solution (pH 7.4) at 37°C under static condition in order to investigate their degradation behaviors. Although there are many reports about \textit{in vitro} and \textit{in vivo} degradation studies of nonporous biodegradable PLLA and PLGA matrices in various geometries,\textsuperscript{18–28} there are few studies about degradation behaviors of porous PLGA scaffolds fabricated for tissue engineering.\textsuperscript{28,29} It has been reported that a heterogeneous bulk degradation for nonporous PLLA and PLGA matrices occurred due to the accumulation of hydrolyzed PLGA chains within the interior region. The enriched PLGA oligomers with a terminal carboxylic acid autocatalyze the faster degradation inside rather than outside of the matri-

<table>
<thead>
<tr>
<th>Preparation Step</th>
<th>Residual Chloroform (ppm)</th>
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<tr>
<td>Polymeric gel</td>
<td>263.13 ± 25.16</td>
</tr>
<tr>
<td>Molding and before gas foaming</td>
<td>113.37 ± 11.25</td>
</tr>
<tr>
<td>After gas foaming</td>
<td>24.87 ± 9.45</td>
</tr>
<tr>
<td>Rinse with water</td>
<td>8.81 ± 6.63</td>
</tr>
<tr>
<td>Complete drying</td>
<td>0.00 ± 4.91</td>
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Table I. Residual Chloroform in the Polymeric Gels and Scaffolds Prepared by a Gas-Foaming/Salt-Leaching Method During a Series of Fabrication Steps

Figure 5. Photographs of different PLGA scaffolds after hydrolytic degradation in PBS at 37°C.
It was postulated that in contrast to nonporous matrices, highly porous PLGA scaffolds might have different degradation behaviors because the exchange of aqueous fluid between within and outside of the scaffolds can take place in a more facilitated manner.

Figure 5 shows photographic pictures of various scaffolds during the degradation. It can be seen that while the slowest degrading PLGA 75/25 scaffolds did not show any evidence of physical disintegration, the fastest degrading PLGA 50/50 scaffolds started to disintegrate physically at day 21. The PLGA 65/35 scaffolds, which were expected to degrade with a rate between the above two scaffolds, tended to disintegrate at day 63. It is of particular interest to note that the PLGA 75/25 scaffolds gradually collapsed from day 21 to day 49, and then suddenly started to swell at day 63. The PLGA 65/35 scaffolds exhibited the swelling behavior at day 21 without showing the collapse of the scaffolds. The PLGA 50/50 scaffolds did not show any detectable swelling behaviors. From the results of physical appearance for the three scaffolds, it is obvious that porous PLGA scaffolds undergo significant changes in dimension during the degradation depending on the molar ratio of lactic/glycolic acid.

Figure 6 shows the mass erosion, molecular weight, and water uptake profiles of the three scaffolds during the degradation period. It can be observed that the mass loss patterns of PLGA scaffolds demonstrate different lag periods, depending on the molar ratio of lactic/glycolic acid, before starting physically to erode. This observation is consistent with the previous finding that during the lag period, hydrolyzed PLGA polymer chains are retained within the matrices until they reach critical molecular weights of water-soluble PLGA oligomers. Molecular weight values decrease monotonically for PLGA 50/50 and in a multiphasic mode for PLGA 65/35 and 75/25. Water uptake profiles, which measure the swelling extent of scaffolds, show a latent period followed by a marked water uptake (swelling) period. After extensive swelling, the scaffolds lose their water uptake capacity due to physical disintegration and fragmentation of the scaffolds. The latent periods of the scaffolds for water uptake depend on the molar composition of lactic/glycolic acid, but they are not exactly matched with the lag periods observed in the mass erosion profiles. During the swelling period, it seems that the molecular weight of PLGA decreased in an accelerated fashion with physical disintegration occurring simultaneously, resulting in the multiphasic profiles for PLGA 65/35 and 75/25. From the above degradation profiles, it is conceivable that hydrolytic scission of PLGA skeletal backbone in the scaffolds gradually produces short-chain PLGA species that progressively can absorb more water molecules from the aqueous medium in the pores. Since the volume fraction of PLGA skeleton relative to the total volume of scaffolds was only around 10%, even a small extent of water uptake in the polymer skeleton would lead to significant overall swelling of the scaffolds. Thus three dif-
different consecutive events—hydrolytic chemical scission of PLGA, water uptake and swelling of PLGA skeleton, and physical disintegration of the scaffolds—are combined to produce the dimension change of the scaffolds as shown in Figure 5. The water uptake profiles in Figure 6 agree well with the results of the visual examination shown in Figure 5.

For the fastest degrading PLGA 50/50 scaffolds, the three events are likely to occur simultaneously as soon as they are incubated in the medium. This might be the reason there is no visible swelling effect in the scaffolds in Figure 5, that is because the swelling and disintegration processes were synchronized. For the slowest degrading PLGA 75/25 scaffolds, hydrolytic chain scission of PLGA should take place before swelling, and they show the longest lag period. The apparent shrinking behaviors of PLGA 75/25 scaffolds before the swelling process might be due to the partial collapse of the macropores within the scaffolds. Figure 7 shows cross-sectional observations of the scaffolds during the degradation. It can be seen that significant swelling of the scaffolds produced a hydrogel-like microporous structure with the disappearance of macropores.

The above in vitro degradation studies were carried out without seeding cells within the scaffolds. Although it is clear that PLGA scaffolds, depending on their polymer compositions, undergo significant morphologic and dimensional changes during degradation in vitro, it is still questionable whether they will exhibit similar changes when cells are seeded. Excretion of extracellular materials by proliferative cells, such as proteoglycan and collagensous proteins, into the interior region of the degrading scaffolds might affect the dimension of regenerated tissues. In order to maintain the same dimension of regenerated tissue as that of the initial scaffold, it is very important to judiciously select biodegradable PLGA polymers having appropriate degradation rates. For this purpose, it is highly desirable to synchronize the swelling process with the physical disintegration process. For instance, PLGA 50/50 scaffolds in the present study show minimum change of the dimension in the scaffolds because the swelling and disintegration processes occurred at the same time.

CONCLUSIONS

Macroporous PLGA scaffolds successfully were prepared by a modified gas foaming/salt leaching
method. A combination of effervescent salts, citric acid, and ammonium bicarbonate was used to prepare the scaffolds at room temperature. The porosity and mechanical strength could be controlled by the adjustment of an acid-base gas evolving reaction between the two salts. The degradation behaviors of three PLGA scaffolds with different compositions were characterized, revealing that PLGA scaffolds exhibit significant dimensional changes during degradation depending on their degradation rates. For tissue engineering applications, PLGA scaffolds should be selected carefully to minimize unwanted morphologic and dimensional variations when implanted in vivo, or used as temporal templates for tissue regeneration in vitro.

References