Published in final edited form as:

Mater Sci Eng C Mater Biol Appl. 2015 March ; 48: 663–672. doi:10.1016/j.msec.2014.12.062.

Dewetting Based Fabrication of Fibrous Micro-Scaffolds as Potential Injectable Cell Carriers

Hokyung Song¹, Liya Yin², William M. Chilian², and Bi-min Zhang Newby^{1,*}

¹Department of Chemical and Biomolecular Engineering, The University of Akron, Akron, OH 44325-3906

²Department of Integrative Medical Sciences, Northeast Ohio Medical University, 4209 SR44, PO Box 95, Rootstown, OH 44272

Abstract

Although regenerative medicine utilizing tissue scaffolds has made enormous strides in recent years, many constraints still hamper their effectiveness. A limitation of many scaffolds is that they form surface patches, which are not particularly effective for some types of “wounds” that are deep within tissues, e.g., stroke, myocardial infarction. In this study, we reported the generation of fibrous micro-scaffolds feasible for delivering cells by injection into the tissue parenchyma. The micro-scaffolds (widths < 100 μ m) were made by dewetting of poly (lactic-coglycolic acid) thin films containing parallel strips, and cells were seeded to form cell/polymer micro-constructs during or post the micro-scaffold fabrication process. Five types of cells including rat induced vascular progenitor cells were assessed for the formation of the micro-constructs. Critical factors in forming fibrous micro-scaffolds via dewetting of polymer thin films were found to be properties of polymers and supporting substrates, temperature, and proteins in the culture medium. Also, the ability of cells to attach to the micro-scaffolds was essential for forming cell/polymer micro-constructs. Both *in vitro* and *in vivo* assessments of injecting these micro-scaffolding constructs showed, as compared to free cells, enhanced cell retention at the injected site, which could lead to improved tissue engineering and regeneration.

Keywords

fibrous micro constructs; injectable constructs; cell retention; dewetting; tissue engineering and regeneration

1. Introduction

Optimal scaffold design/fabrication is an important element in tissue engineering strategies [1], especially to realize the full potential of regenerative medical therapies. Many

© 2014 Elsevier B.V. All rights reserved.

Corresponding author bimin@uakron.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

techniques have been developed for fabricating tissue engineering scaffolds including solution casting [2–4], particulate leaching [2,3,5], thermally induced phase separation (TIPS) [2–5], and electric spinning [2,3,6–9]. They are generally aimed at generating large scaffolds (mm or larger) for applications that would be confined to easily accessible locations, such as the surface of an organ, and they could not be injected or used for a deep wound such as a myocardial infarct or a stroke. For injection based therapy in tissue regeneration, smaller scaffolds are more desirable.

The injection based approach is clinically preferred due to its minimally invasive nature. To this end, development of biomaterial-assisted cell micro-carriers for effective injection is essential. These carriers not only provide supports for cell attachment/proliferation prior to injection, but also, as compared to a biomaterial without cells or cells without a biomaterial, enhance cell retention at the injection sites and have a greater ability to repair damaged tissues [10]. Moreover, it has also been reported that porous injectable cell carriers are superior to the non-porous counterparts in term of cell adhesion rate and tissue regeneration [11,12].

Currently, the most common micro-carriers include: (1) hydrogel based cell encapsulation systems [13–18] – with gelation either prior or post injection; (2) micro particles, micro beads or micro gels, either porous or non-porous, made out of natural and synthetic polymers [5,10–13, 17,19,20] or decellularized matrix [21]; and (3) the combinations of (1) & (2). For hydrogels based micro carriers [i.e. (1) above], the potential lack of sufficient supply of oxygen or nutrient to the entrapped cells that might lead to massive cell death, the non-uniform cell distribution, and the limitation of cell migration and engraftment into tissue are major concerns [10,11,18]. For non-porous spherical carriers – beads or particles, cell infiltration is impossible and modification of these beads/particles is generally needed to enhance cell attachment and matrix-cell interactions [12,22–24]. To enhance cell loading capacity, highly porous microspheres that allowed efficient cell infiltration have been produced. While enhanced cell loading and effective mass transfer of oxygen and nutrients have been achieved using these highly porous microspheres, the process of incorporating cells into the microspheres is tedious [10]. It requires incubation of individual microspheres to avoid aggregation prior to injection. Also, the surface of the scaffold is normally very different from the fibrous structures of native tissue for good cell attachment. To mimic the cellular matrix of native tissue, attempts have been made to generate highly porous hollow microspheres with fibrous structures, but most pores from these attempts are too small for cells to infiltrate. In addition to the above requirements, migration and engraftment of cells from these structures into surrounding tissues *in vivo* are essential. Therefore, suitable techniques for generating fibrous porous carriers that can enhance cell attachment/proliferation prior to injection and serve as effective injection carriers for cell retention/migration/engraftment post injection are still needed.

In this study, we reported a dewetting based approach for generating loosely packed (i.e. highly porous) fibrous scaffolds and subsequently forming micro-constructs as an alternative injectable cell micro carrier (schematic of the process is shown in Fig. 1). A model polymer, biodegradable poly (lactic-co-glycolic acid) (PLGA), and several cell types were used to

illustrate the feasibility of the approach. Also, the fibrous micro-constructs were employed to demonstrate that they could be promising for injection based cell therapy.

2. Materials and Methods

2.1. Preparation of supporting substrates, PDMS stamps, and imprinted polymer films

Glass slides (cut to 1 cm × 2 cm pieces; Fisher Scientific, Waltham, MA) were cleaned using a freshly prepared piranha solution [i.e. 70/30 (v/v) of 98% H₂SO₄ (Fisher) and 30% H₂O₂ (Fisher)] followed with thorough rinsing using DI water (purified in house with a conductivity value of 0.1 mS or less). Some cleaned slides were modified with 2-[methoxy poly(ethyleneoxy) propyltrimethoxysilane (CH₃O(CH₂CH₂O)₆₋₉ (CH₂)₃Si(OCH₃)₃, PEG-silane; Gelest, Morrisville, PA) according to the previous procedure [25–27], while others were coated with a layer (200–300 μm) of agarose (UltraPure™ agarose, Life Technologies, Carlsbad, CA) gel by spreading a hot (60–80 °C) 1 % (w/w) agarose solution in DI water over the slide and then cooled to room temperature.

Polydimethylsiloxane (PDMS) stamps were fabricated from Sylgard® 184 (Dow Corning, Midland, MI). A piece (12 mm × 10 mm) of silicon wafer (Silicon Quest International, Reno, NV) containing parallel strips fabricated using the fracture-induced structuring method we developed [28] was used as the mold. The mold was first modified with a non-adhesion agent, octadecyltrichlorosilane (Gelest), and the Sylgard® 184 pre-polymer mixture was pour over the mold, de-gassed, cured at ~ 60°C for 4 hours, and then separated from the mold.

For generating an imprinted PLGA (Sigma-Aldrich, Mw = 35–60 kg/mol, St Louis, MO) film, a drop of 2.5 % (w/w) of PLGA solution in acetone (Fisher) was spread on a slide, the PDMS stamp was pressed against the spread drop until the solution dried, and then the stamp was removed (Fig. 2A). Imprinted films of poly (D-lactic acid) (PDLA, Polysciences Inc, Mw = 15 kg/mol, Warrington, PA) and 1 % (w/w) of 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich) in PLGA were also prepared by using 2.5 % (w/w) of the corresponding solution in acetone. The resulting imprinted films were examined using an optical microscope (Olympus IX71, B&B Microscope, Pittsburg, PA) and an atomic force microscope (Multimode NanoScope V, Veeco, Plainview, NY).

2.2. Formation of fibrous micro-scaffolds and cell/polymer micro constructs

In the first approach to form fibrous cell/polymer micro constructs, polymer films imprinted on glass slides and on the PEG-silane modified glass slides were directly seeded with cells and incubated for a period of 1 to 3 days (bottom row of Fig. 1). Five different cell types used in this study were: mouse embryonic fibroblast (MEF, Millipore, Billerica, MA), rat induced vascular progenitor cells (iVPCs, details can be found in an earlier publication, [29]), human embryonic kidney 293A cell line (293A, ATCC, Manassas, VA), human liver cell line (HepG2, ATCC, Manassas, VA), and rat endothelial cells (ECs, Cell Applications, San Diego, CA). Of the five different cell types, MEFs and iVPCs were used for detailed assessments. The morphology of cells on the films was examined using the optical

microscope, and the films, if still remained or formed fibrous constructs, were then separated from glass or the PEG-silane modified glass to be sectioned.

In a second approach, imprinted PLGA films on glass slides were incubated in DI water at ~50 °C for ~2 h to only dewet the thin PLGA strips, and to form fibrous scaffolds (top row of Fig. 1). Then the entire film was floated off and picked up on to a glass slide, a PEG-saline modified glass slide or an agarose coated glass slide. Since the stamp size (12 mm × 10 mm) was slightly larger than the area (~ 10 mm × 8 mm) containing strips; the strips were connected to the rest of the film by their ends. Four edges of the picked up film were then secured down to the slide by using a small amount of 10 % (w/w) PLGA solution as glue. After sterilizing the samples with UV light (Fisher) inside a class II type A2 biological safety cabinet (NuAire, Inc, Plymouth, MN) for 15 min, cells (1×10^5 cells/ml) were seeded on these PLGA fibrous scaffolds in a well of a 24-well plate or a 6-well plate (USA Scientific Inc, Ocala, FL) and incubated inside a 5% CO₂ incubator (NuAire) for 1 to 3 days to form cell/polymer fibrous constructs.

2.3. In vitro injectability evaluation of cell/polymer micro constructs

The cell/polymer fibrous constructs were sectioned into 0.5–1 mm in length by using a pair of surgical scissors (BD, Franklin Lakes, NJ) and dispersed in the culture medium (DMEM + 10 v.% fetal bovine serum (FBS), both from Sigma-Aldrich). The harvested micro constructs (10–20), in ~0.25 ml of culture medium, were injected through a gauge 20 (inside diameter, ID = 584 µm) needle (BD) into, first a 35 mm diameter culture dish (USA Scientific), and then a crude *in vitro* micro-environment (i.e. gel capsule) placed inside a well of a 6-well plate. The micro-environment was created by cross-linking ~ 3 ml of 1 % (w/w) sodium alginate (Sigma-Aldrich) in culture medium with ~ 3 ml of 1 % (w/w) of calcium chloride (Fisher) in DI water for 30 min. All the solutions involved were sterilized prior to making the alginate gel capsule in a well of a 6 well plate, and the gel capsule was thoroughly washed with sterilized DI water prior to injection study. After injecting the constructs into the gel capsule, ~3 ml of culture medium was added into the well and incubated for 7 days.

The morphology of micro-constructs before and after injection was imaged. Free cells were also injected for comparison. Viability of cells before and after injection was assessed by both Trypan blue (Sigma-Aldrich) staining and live/dead – fluorescent staining (fluorescein diacetate/propidium iodide or FDA/PI, Sigma-Aldrich). The fluorescent staining was primarily used for imaging, and the images were obtained using a fluorescent microscope (Olympus IX71). For Trypan blue assays, cells on fibrous scaffolds were trypsinized before staining, and in the case of using alginate gel capsules, the capsules were first dissolved using 10 % (w/w) of sterilized sodium citrate (Sigma-Aldrich) solution in DI water. The percentage of live cells was estimated by counting the stained dead cells.

2.4. In vivo assessment of injected cell/polymer micro constructs

The injectability of fibrous micro-constructs *in vivo* was preliminary demonstrated by injecting the constructs into normal rat hearts and rat hearts with ischemia. In general, ~150 iVPC/polymer micro-constructs consisting of approximately one million cells, or one

million free iVPCs were used for each injection. For the rat myocardial ischemia model, the left anterior descending artery was permanently ligated in an open chest surgery. The constructs or free iVPCs dispersed in 0.25 ml of culture medium were intramuscularly injected into the myocardium of a Sprague Dawley® (SD) rat (Charles River Laboratories International, Inc, Wilmington, MA) within 30 min after ligation by a G20 needle following the animal protocol approved by IACUC from the Northeast Ohio Medical University (NEOMED). To localize the injected cells and micro-constructs, the cells were labeled with Td-tomato fluorescence by lentivirus (Life Technologies). 4 weeks after injection, the rats were sacrificed and heart tissues were harvested, sectioned and imaged using the fluorescent microscope.

3. Results and Discussion

3.1. Dewetting behaviors of imprinted films under incubation conditions

The details of creating the constructs are sketched in Fig. 1. First cells were directly seeded on imprinted polymer thin films and incubated under cell culture conditions (i.e. bottom route in Fig. 1). After incubation for 1 day, different phenomena were observed. Dewetting of imprinted PLGA films on glass slides was observed, but only occurred for the thinner strips of the films (Fig. 3A). Also, dewetting was found to only proceed slightly further at a longer incubation time (> 1 day). Dewetting of PLGA films on PEG-silane modified glass slides (Fig. 3B) and APTES blended PLGA films on glass (Fig. 3C) were hardly noticed, while cell attachment and spreading on these films were observed. On the other hand, poly (D-lactic acid) (PDLA) films (Fig. 3D), both thinner and thicker strips, were completely dewetted into droplets.

In an aqueous environment, many factors could alter the interactions across the polymer film or affect the physical properties of the film, thus influencing dewetting behaviors of polymer thin films [30,31]. For example, adsorption of proteins, from the culture medium, on these film surfaces could lead to surface/interfacial energy and surface potential changes. These changes could greatly affect van der Waals and electrostatic interactions, the two main types of interactions across a polymer film in a liquid medium. We found that proteins adsorbed on PLGA films lead to a decrease of the Hamaker's constant (A), from 6.8×10^{-22} J before protein adsorption to 1.3×10^{-22} J after protein adsorption, across the PLGA film (see details in Supplemental data). A positive value of A , i.e. a van der Waals attraction across the film, would destabilize the film and cause the film to dewet. The smaller A indicated that the destabilization of the PLGA film by van der Waals attractions diminished. Moreover, the surface potential of protein adsorbed PLGA was found to be less negative (e.g. -15 ± 2 mV in PBS) than that of bare PLGA (e.g. -31 ± 2 mV in PBS) (also detailed in the Supplemental data section), indicating the electrostatic interactions across the PLGA film would also become weaker, leading to a less unstable film. In addition to altering interactions across the PLGA film, cells attached and their extracellular matrix proteins deposited on the film would further retard dewetting of the PLGA film. These could be the reasons why the dewetting of PLGA film from glass under culture condition subsided after one day of incubation. For the medium-PLGA film-PEG combination, Hamaker's constants were both negative, i.e. under van der Waals repulsions, before and after protein adsorption

on PLGA, hence no dewetting of PLGA films on the PEG-glass under the culture condition should be resulted from the van der Waals interaction. While the electrostatic interaction might cause dewetting initially, the interplay between the lowered electrostatic interaction by protein adsorption and the enhanced van der Waals repulsion (i.e. a more negative A value after protein adsorption) prevented PLGA films from dewetting from the PEG-glass surface.

The uptake of water into polymer films could decrease their glass transition temperature (T_g) [32,33]. In general, dewetting occurs when the incubation or annealing temperature is greater than the glass transition temperature (T_g) of the polymer films, and a faster dewetting rate occurs at a higher annealing temperature. For PLGA films used in this study, the T_g value was 45–50 °C at its dry state. When the films were submerged in an aqueous medium, water up-take could lead to a 10–15 °C decrease in their T_g [34], meaning the incubation temperature (37 °C) was slightly higher than their T_g to allow dewetting. In the case of PDLA films, the particular PDLA we used had a relatively low molecular weight ($M_w \sim 15$ kg/mol, intrinsic viscosity: 0.2 dl/g), hence a low T_g (~ 24 °C at its dry state, estimated based on a previous study [33]). Upon up-taking of water, a reduction in T_g (e.g. ~ 10 °C from its dry state) would further result [32,33], leading to at least a 20°C difference between the incubation temperature and T_g of immersed PDLA films, hence a much faster dewetting of PDLA films was observed. For APTES blended PLGA, the adhesion promoting properties of APTES and the network APTES molecules created [35,36] would likely anchor the PLGA chains/films on glass to result in less or no dewetting of these films.

3.2. Formation of fibrous micro-scaffolds and fibrous micro-constructs

To minimize complications resulted from proteins presented in culture medium; the possibility of forming fibrous micro-scaffolds by dewetting of imprinted films in deionized (DI) water was then assessed. When imprinted PLGA films were incubated in DI water at 50°C (~ 10 – 20 °C above the T_g of PLGA films in water [34]), thin strips in between thick strips (1–2 μ m thick based on AFM scans, Fig. 2B) started to dewet within minutes. A rough estimation from the dewetted holes of these thin strips showed their thickness was 40–60 nm (Fig. 2C). Dewetting of such thin polymer films (< 100 nm) have been observed extensively in air and under a liquid [30,31,37,38].

The sequence of dewetting of imprinted PLGA films to result in the formation of PLGA fibrous micro-scaffolds is shown in Fig. 4. After 15 min in DI water at 50 °C, some dewetting holes initiated and grew, but most of them had yet to merge with each other (Fig. 4A). After 45 min of incubation, most of the dewetting holes had merged to result in polygonal rims, i.e. the “spider web” features (Fig. 4B). The rims were much smaller than the thick strips that they connected to; as a result, they either quickly broke up due to Rayleigh's instability or retracted into closest thick strips due to the difference in Laplace pressures, leaving behind a relatively clean region in between the thick strips (Fig. 4C). In the meantime, water attached the interface between the thick strips and the underneath glass slide, reducing their interfacial adhesion and causing the strips to delaminate from the slide. As a few adjacent floating strips shifted and bundled together, a fibrous micro-scaffold was formed. In ~ 2 h of incubation, fibrous micro-scaffolds were normally formed throughout the

entire sample (10 mm × 8 mm, Fig. 4D). Then the sample was removed from incubation to ensure no additional breaking down of thick PLGA strips by Rayleigh's instability occurred.

The films containing fibrous micro-scaffolds were floated off and picked up on glass slides or treated glass slides. Cells were then seeded on the films to generate cell/polymer fibrous micro-constructs (Fig. 1, top route). When plain glass slides were used as supports, cells grew nicely on the scaffolds to form cell/polymer fibrous micro-constructs after 1 to 3 days of incubation. However, it was found to be difficult to remove the constructs from the slides, since proteins in the system along with attached/proliferated cells “glued” some portions of the films to the glass slide and some adjacent micro-constructs together. As a result, it was difficult to harvest the fibrous micro-constructs without scrapping them away from the surface or ripping apart the constructs, consequently causing cells detached from the constructs.

To reduce cell attachment and protein deposition to the underneath support, in the first attempt, glass slides modified with a polyethylene glycol (PEG) contained silane [25–27,39,40] were used as supports. Using a non-fouling support was also anticipated to induce preferential cell attachment and growth on the PLGA fibrous scaffolds. While during the initial period (4–6 h) of incubation, cell attachment to the PEG-silane modified glass slides were minimal, as the incubation time increased to 1 day, some cell attachment and proliferation on the PEG-modified glass slides still resulted, which was also confirmed with the MTT assay (Fig. S1A and D). Similar results had been reported previously [40] for surfaces modified with short PEG chains. Therefore, with a long term (e.g. 3 days) incubation time, PEG-modified glass slides, as the supports, still exhibited the same challenges of harvesting the formed fibrous micro-constructs.

Agarose, one of the common non-fouling hydrogels [41,42] was then used. After briefly verifying that the particular agarose we used showed a long term (up to 7 days) inhibition to cell attachment (Fig. S1A and C) and maintained stable under incubation conditions, glass slides were coated with a thin layer (200–300 μm) of agarose. When cells were seeded on PLGA fibrous micro-scaffolds supported on agarose coated glass slides, the cells were randomly and uniformly settled (Fig. 5A). After 4 h of incubation (Fig. 5B), most cells migrated towards the scaffolds and attached to the scaffolds instead of to the agarose coating, and some cell spreading was observed; while those cells settled on the agarose coating showed a more rounded morphology. After 1 day of incubation, cells attached to the scaffolds had appeared to grow into a monolayer (Fig. 5C), with some of them elongated along the direction of the strips. With a higher cell seeding density (e.g. $> 5 \times 10^5$ cells/well, 1 day of incubation) or a longer incubation period (3 days with a cell seeding density of 1×10^5 cells/ml), multilayered cell clusters on the scaffolds also resulted (Fig. 5D). The multilayered cell clustered on the scaffolds was found to be advantageous for shielding cells in the inner layers from shedding away by shear during injection.

The model cell types used for this study were mouse embryonic fibroblast (MEF) and rat induced vascular progenitor cells (iVPCs), both had been found to consistently form fibrous micro-constructs (Fig. 6A–C), either with a monolayer of cells (Fig. 6A and B) or with a multilayer of cells (Figs. 5D and 6C). Other cell types, human kidney 293A cell line (Fig.

6D), human liver cell line (HepG2) (Fig. 6E) and rat endothelial cells (ECs) (Fig. 6F), were also tested. Only ECs were found to be unable to form the desired constructs. The exact reasons are unclear, but it could be due to the attachment characteristics of ECs to be different from that of other cell types.

3.3. Injectability of cell/polymer fibrous micro constructs in vitro

A potential application of our cell/polymer fibrous micro-constructs is injection based cell therapy. To assess the feasibility of our constructs for injection, they were sectioned into smaller pieces using a pair of surgical scissors, picked up, along with culture medium, using a blunt tip pipette and placed inside a 1 ml syringe barrel for injection. The micro-constructs dispersed in ~0.25 ml culture medium were injected through a G20 needle into, first, a 35 mm diameter petri-dish. It was noticed that constructs covered with multilayers of cells (e.g. after 3 days of incubation) was easier to be injected. These micro-constructs, after passing through the needle, appeared to be similar to those before injection, only some shape change (e.g. elongated, bended, or cluster more together) was noticed (Fig. 7). As the result, only such micro-constructs, i.e. containing multilayered cells, were used for *in vitro* and *in vivo* studies reported in this manuscript.

The cell viability, before (Fig. 7A and B) and after (Fig. 7C and D) injection through a G20 needle, was assessed using the live/dead fluorescent staining assay and the trypan blue assay. Most cells in the constructs were alive (Fig. 7E). On average, the MEFs/PLGA micro-constructs before injection had an $88.2 \pm 2.8\%$ of live cells, and the dead cells were sparingly distributed on the outer layer of the constructs and at the end regions where the constructs were sectioned by scissors. Viable cells were uniformly distributed throughout the construct, indirectly indicated that the transportation of nutrients and metabolites to and from different locations of the construct was likely not limited to affect cell growth and proliferation. After injection, the cell viability barely decreased to $87.7 \pm 2.2\%$. The few additional dead cells observed at outer regions of the constructs after injection were probably caused by the direct contact of these cells with the needle wall, thus experiencing the maximum shear force during injection to cause damage of these cells.

The viability of free cells by direct injection was also evaluated for comparison. The viability of injected free MEF cells reduced by ~5% from that before injection (~98%). Similar viability results were obtained for free iVPCs and iVPCs/PLGA constructs (Fig. 7E). The relatively high cell viability after injection could be due to the larger needle (G20, ID = 0.584 mm) we used, which could reduce the extensional flow the cells experienced as compared to smaller needles (G25 to G27) used by others [43].

When the fibrous micro-constructs were injected into the alginate gel capsule and incubated for 7 days, it was found that relative positions of the constructs remained unchanged and proliferation of cells on the scaffolds was observed (Fig. 8A and B). With a majority (> 85%) of cells were viable after injection, the injected micro-constructs were able to retain at the injected site, and cells in the constructs continued to proliferate, the potential of an *in vivo* evaluation was warranted.

3.4. Injectability of cell/polymer fibrous micro constructs in vivo

To demonstrate the potential of the injectable cell/polymer fibrous micro constructs in cell based therapy by injection, the micro constructs containing iVPCs or free iVPCs were injected into myocardium of SD rats. First, we injected free iVPCs (stained by Td-red) or iVPCs /PLGA micro constructs mixed with microspheres (labeled with FITC) into normal rat heart to check for cell survival and cell retention. Fig. 8C and 8D shows fluorescent images of sections of left ventricle stained with DAPI (blue) after injection of free iVPCs (Fig. 8C) or iVPC/PLGA micro-constructs (Fig. 8D). More iVPCs aligned with the micro-constructs, suggesting iVPC/PLGA micro-constructs resulted in a better cell retention and/or cell survival than free iVPCs. When the cells or micro constructs were injected in a myocardial infarction model, similar observations were made (Fig. 8E and 8F). Specifically, for free iVPCs injected, only sparsely dispersed individual cells were observed (Fig. 8G, after enhancing the image to show the red cells). By comparison, many more cells were noticed when they were delivered with constructs (Fig. 8F and H), and most of them were easily visualized as a band along the direction of injection, where the micro construct would have been situated. A rough estimate by counting cells using ~10 images showed that at least 10× cells, as compared to injected free cells, retained at the injected site when injected with constructs. The better survival and retention of these cells suggested that the fibrous micro constructs improved cell survival/retention after injection. Also, the loose scaffolds and cells located on these loose scaffolds would more likely allow the cells to interact better with the microenvironment than cells being encapsulated, leading to increased survival. More importantly, as the polymer scaffolds degrade at a later time point, the cells could migrate to where they needed to be with the cue of tissue repairing.

4. Conclusion

In this study, an approach based on polymer thin film dewetting was developed to create loosely packed fibrous scaffolds for tissue engineering and regeneration. We optimized conditions: dewetting temperature and time, non-fouling properties of the underneath support, and cell incubation time, to form most constructs having a size of < 100 μm and loosely packed. Utilizing such scaffolds to create injectable micro constructs was demonstrated, and both *in vitro* and *in vivo* injection feasibility evaluations of the constructs showed that cells survival and retention at the injected site was greatly enhanced as compared to injected free cells. Our micro fibrous constructs would also allow direct and better cell/microenvironment interaction *in vivo* under both physiological and pathological (ischemia) conditions. Therefore, they could be an attractive cell carrier alternative (as oppose to hydrogel and microsphere based cell carriers) for direct injection based tissue engineering and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Ms. Molly Enrick and Ms. Kelly Stevanov at the Department of Integrative Medical Sciences, NEOMED, for assistance with animal studies. Financial supports from Austen BioInnovation Institute in Akron, Ohio (award: ORSSP #R8505 or Project: P002988) and from the National Institutes of Health, the National Institute of General Medical Sciences under award number 1R15GM097626-01A1 (to BMZN) and the National Heart, Lung and Blood Institute under award number 1R15HL115540-01 (to LY) are acknowledged.

References

- Hollister SJ, Maddox RD, Taboas JM. Optimal design and fabrication of scaffolds to mimic tissue properties and satisfy biological constraints. *Biomaterials*. 2002; 23:4095–4103. [PubMed: 12182311]
- Liao S, Chan CK, Ramakrishna S. Stem cells and biomimetic materials strategies for tissue engineering. *Mater. Sci. Eng. C*. 2008; 28:1189–1202.
- Subramanian A, Krishnan U, Sethuraman S. Development of biomaterial scaffold for nerve tissue engineering: biomaterial mediated neural regeneration. *J. Biomed. Sci*. 2009; 16:108. [PubMed: 19939265]
- Hutmacher DW. Scaffold design and fabrication technologies for engineering tissues –state of the art and future perspectives. *J. Biomater. Sci. Polym. Ed*. 2001; 12:107–124. [PubMed: 11334185]
- Salerno A, Domingo C. A novel bio-safe phase separation process for preparing open-pore biodegradable polycaprolactone microparticles. *Mater. Sci. Eng. C*. 2014; 42:102–110.
- Holzwarth JM, Ma PX. Biomimetic nanofibrous scaffolds for bone tissue engineering. *Biomaterials*. 2011; 32:9622–9624. [PubMed: 21944829]
- Beachley V, Wen X.j. Fabrication of nanofiber reinforced protein structures for tissue engineering. *Mater. Sci. Eng. C*. 2009; 29:2448–2453.
- Jin, G.; Li, K. The electrically conductive scaffold as the skeleton of stem cell niche in regenerative medicine. *Mater. Sci. Eng., C*. 2014. <http://dx.doi.org/10.1016/j.msec.2014.06.004>
- Kai, D.; Liow, SS.; Loh, XJ. Biodegradable polymers for electrospinning towards biomedical applications. *Mater. Sci. Eng., C*. 2014. <http://dx.doi.org/10.1016/j.msec.2014.04.051>
- Huang CC, Wei HJ, Yeh YC, Wang JJ, Lin WW, Lee TY, et al. Injectable PLGA porous beads cellularized by hAFSCs for cellular cardiomyoplasty. *Biomaterials*. 2012; 33:4069–4077. [PubMed: 22386922]
- Liu, X.h.; Jin, X.b.; Ma, PX. Nanofibrous hollow microspheres self-assembled from star-shaped polymers as injectable cell carriers for knee repair. *Nat. Mater*. 2011; 10:398–406. [PubMed: 21499313]
- Zhang, Q.c.; Tan, K.; Ye, Z.y.; Zhang, Y.; Tan, W.s.; Lang, M.d. Preparation of open porous polycaprolactone microspheres and their applications as effective cell carriers in hydrogel system. *Mater. Sci. Eng. C*. 2012; 32:2589–2595.
- Hou, Q.p.; De Bank, PA.; Shakesheff, KM. Injectable scaffolds for tissue regeneration. *J. Mater. Chem*. 2004; 14:1915–1923.
- Mooney RJ, Vandenburgh H. Cell delivery mechanisms for tissue repair. *Cell Stem Cell*. 2008; 2:205–213. [PubMed: 18371446]
- Radhakrishnan J, Krishnan UM, Sethuraman S. Hydrogel based injectable scaffolds for cardiac tissue regeneration. *Biotechnol. Adv*. 2014; 32:449–461. [PubMed: 24406815]
- Prabhakaran MP, Venugopal J, Kai D, Ramakrishna S. Biomimetic material strategies for cardiac tissue engineering. *Mater. Sci. Eng. C*. 2011; 31:503–513.
- Song, K.d.; Yang, Y.f.; Li, S.x.; Wu, M.l.; Wu, Y.x.; L, M.; Liu, T.q. In vitro culture and oxygen consumption of NSCs in size-controlled neurospheres of Ca-alginate/gelatin microbead. *Mater. Sci. Eng. C*. 2014; 40:197–03.
- Toh, WS.; Loh, XJ. Advances in hydrogel delivery systems for tissue regeneration. *Mater. Sci. Eng., C*. 2014. <http://dx.doi.org/10.1016/j.msec.2014.04.026>
- Hernandez RM, Orive G, Murua A, Pedraz JL. Microcapsules and microcarriers for in situ cell delivery. *Adv. Drug Del. Rev*. 2010; 62:711–39.

20. Liu W, Li Y.q. Zeng Y, Zhang X.y. Wang J.y. Xie L.p. et al. Microcryogels as injectable 3-D cellular microniches for site-directed and augmented cell delivery. *Acta. Biomater.* 2014; 10:1864–1875. [PubMed: 24342043]
21. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, Schup-Mogoffin PJ, Christman KL. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials.* 2009; 30:5409–5416. [PubMed: 19608268]
22. Chun KW, Yoo HS, Yoon JJ, Park TG. Biodegradable PLGA microcarriers for injectable delivery of chondrocytes: effect of surface modification on cell attachment and function. *Biotechnol. Prog.* 2004; 20:1797–1801. [PubMed: 15575714]
23. Tan, H.p.; Wu, J.d.; Huang, D.j.; Gao, C.y. The design of biodegradable microcarriers for induced cell aggregation. *Macromol. Biosci.* 2010; 10:156–163. [PubMed: 19714563]
24. Verma S, Kumar N. Effect of biomimetic 3D environment of an injectable polymeric scaffolds on MG-63 osteoblastic-cell response. *Mater. Sci. Eng. C.* 2010; 30:1118–1128.
25. Papra A, Gadegaard N, Larsen NB. Characterization of ultrathin poly(ethylene glycol) monolayers on silicon substrates. *Langmuir.* 2001; 17:1457–1460.
26. Choi, I.h.; Kang, S.K.; Lee, J.j.; Kim, Y.h.; Yi, J.h. In situ observation of biomolecules patterned on a PEG-modified Si surface by scanning probe lithography. *Biomaterials.* 2006; 27:4655–4660. [PubMed: 16701869]
27. Cai, Y.j.; Zhang Newby, B.-m. Dewetting of polystyrene thin films on poly(ethylene glycol) modified surfaces as a simple approach for patterning proteins. *Langmuir.* 2008; 24:5202–5208. [PubMed: 18407678]
28. Cai, Y.j.; Zhang Newby, B.-m. Fracture-induced formation of parallel silicone strips. *J. Mater. Res.* 2010; 25:803–809.
29. Yin, L.y.; Ohanyan, V.; Pung, Y.F.; DeLucia, A.; Bailey, E.; Enrick, M., et al. Induction of vascular progenitor cells from endothelial cells stimulates coronary collateral growth. *Circ. Res.* 2012; 110:241–252. [PubMed: 22095729]
30. Verma A, Sharma A. Enhanced self-organized dewetting of ultra thin films under water-organic solutions: fabrication of sub-micrometer spherical lens arrays. *Adv. Mater.* 2010; 22:5306–5309. [PubMed: 20872412]
31. Verma A, Sharma A. Submicrometer pattern fabrication by intensification of ultrathin polymer films under a water-solvent mix. *Macromolecules.* 2011; 44:4928–4935.
32. Siemann U. The influence of water on the glass transition of poly(DL-lactic acid). *Thermochim. Acta.* 1985; 85:513–516.
33. Steendam R, van Steenberg MJ, Hennink WE, Frijlink HW, Lerk CF. Effect of molecular weight and glass transition on relaxation and release behavior of poly(DL-lactic acid) tablets. *J. Controlled Release.* 2001; 70:71–82.
34. Shah SS, Cha Y, Pitt CG. Poly(glycolic acid-co-DL-lactic acid): diffusion or degradation controlled drug delivery? *J. Controlled Release.* 1992; 18:261–270.
35. Choi S-H, Zhang Newby B.-m. Suppress polystyrene thin film dewetting by modifying substrate surface with aminopropyltriethoxysilane. *Surf. Sci.* 2006; 600:1391–1404.
36. Patel NG, Cavicchia JP, Zhang G, Zhang Newby B.-m. Rapid cell sheet detachment using spin-coated pNIPAAm films retained on surfaces by an aminopropyltriethoxysilane network. *Acta Biomater.* 2012; 8:2559–2567. [PubMed: 22475785]
37. Reiter G. Dewetting of thin polymer films. *Phy. Rev. Lett.* 1992; 68:75–78.
38. Sharma A, Reiter G. Instability of thin polymer films on coated substrates: Rupture, Dewetting, and Drop formation. *J. Colloid. Interface Sci.* 1996; 178:383–399.
39. Faucheux N, Schweiss R, Lutzow K, Werner C, Groth T. Self-assembled monolayers with different terminating groups as model substrates for cell adhesion studies. *Biomaterials.* 2004; 25:2721–2730. [PubMed: 14962551]
40. Zhang M, Desai T, Ferrari M. Proteins and cells on PEG immobilized silicon surfaces. *Biomaterials.* 1998; 19:953–960. [PubMed: 9690837]
41. Amedee J, Bareille R, Jeandot R, Bordenave L, Remy M, et al. Evaluation of cell colonization on biomaterials: preventing cell attachment to plastic containers. *Biomaterials.* 1994; 15:1029–1031. [PubMed: 7841292]

42. Su G, Zhao Y, Wei J, Han J, Chen L, et al. The effect of forced growth of cells into 3D spheres using low attachment surfaces on the acquisition of stemness properties. *Biomaterials*. 2013; 34:3215–222. [PubMed: 23439133]
43. Anuado BA, Mulyasmita W, Su J, Lampe KJ, Heilshorn SC. Improving viability of stem cells during syringe needle flow through the design of hydrogel cell carriers. *Tissue Eng. A*. 2012; 18:806–15.

Highlights

1. Fibrous micro-scaffolds fabricated by dewetting of polymer thin films
2. Micro-scaffolds by dewetting formed with proper conditions/material properties
3. Cells attached/grew on micro-scaffolds yielded cell/polymer micro-constructs
4. Micro-constructs injected through a G20 needle with minimal cell loss/damage
5. Enhanced cell retention achieved in both *in vitro* and *in vivo* injection studies

Fig. 1.

The schematic illustrates the formation of cell/polymer fibrous micro-constructs from the imprinted films contain strips and utilizing a dewetting process. The polymer fibrous micro-scaffolds might be first formed by dewetting, and then cells are seeded on these micro-scaffolds to form the micro-constructs (top row). Alternatively, the cells might be seeded directly to the imprinted polymer films, which might dewet during cell incubation, leading to the formation of cell/polymer micro-constructs.

Fig. 2.

The process of imprinting polymer thin films (side views) using a PDMS mold containing parallel strips is shown in (A). (B) is an AFM topographic scan of the imprinted film and a cross-sectional view showing the thick strips have roughly a height of $\sim 1 \mu\text{m}$ and a spacing of $13 \mu\text{m}$. (C) is an AFM topographic scan of an imprinted PLGA film incubated in DI water at 50°C for 15 min to allow partial dewetting and a cross-sectional along a thin strip to show the thickness of the thin strip was 40–60 nm. The scan rate used for both scans was 5 Hz.

Fig. 3.

Different stabilities of imprinted films under MEF cell culture condition were observed after the films were incubated for 1 day: (A) PLGA film on glass; (B) PLGA film on a PEG-modified glass slide, (C) PLGA with 1% (w/w) of APTES film on glass, and (D) PDLA film on glass. The scale bars denote 100 μm .

Fig. 4.

The representing optical microscope images of imprinted PLGA films at different stages of dewetting at 50 °C in DI water are shown: (A) hole formation on the thin strips (15 min), (B) rim formation (45 min), (C) shifting thick strips due to their weakened adhesion with substrates (90 min), and (D) fibrous micro-scaffolds formed over the entire sample at the end of the dewetting process (~2 h). The scale bars for (A), (B), and (C) are 50 μm and for (D) the entire region consisting of fibrous micro-scaffolds is $\sim 10\text{ mm} \times 8\text{ mm}$, and the scale bar is 2 mm.

Fig. 5.

The formation of cell/polymer fibrous micro-constructs with MEF cells at different incubation durations is illustrated: (A) Right after seeding with 100k cells/ml, (B) 4 h after seeding cells – cell attachment occurred, (C) after incubation for 1 day – a single layer of cells grown on the scaffolds, and (D) after incubation for 3 days – scaffolds with multilayer cells “glued” to the scaffolds with their ECM. The scale bar in each image is 100 µm.

Fig. 6.

The formation of cell/PLGA fibrous micro-constructs with different cell types incubated on the PLGA micro-scaffolds. (A) MEFs, 1 day of incubation, (B) iVPCs, 1 day of incubation, (C) iVPCs, 3 days of incubation, (D) human kidney 293A cells, 3 days of incubation, (E) human liver HepG2 cells, 3 days of incubation, and (F) mouse endothelial cells, 3 days of incubation. The seeding cell density used was $\sim 1 \times 10^5$ cells/ml. The scale bar in each image is 100 μm .

Fig. 7.

Optical images of cell/polymer fibrous micro-constructs (left: bright field, right: florescent) before, (A) and (C), and after, (B) and (D), they were injected through a pipette connected to a G20 hypodermic needle into a 35 mm dish containing culture medium. The florescence images show that the majority of the cells are alive (green) and retained on the scaffolds after injection. Cell viability before (light gray bars) and after (darker gray bars) injection, of both cell/PLGA micro-constructs and free cells, only differed slightly (E). The scale bar in each image is 100 μ m.

Fig. 8.

The representative images of injection results. (A) immediately after and (B) 7 days after injecting the MEF/PLGA micro constructs into an alginate gel capsule; and injection of (C) & (E) free iVPCs and (D) & (F) iVPCs/PLGA micro constructs in normal rat hearts (C & D) and ischemic myocardia (E & F) four weeks after LAD ligation followed by the injection therapy. (G) and (H) are the enlarged sections boxed in (E) and (F), respectively, with images further enhanced to show the distribution of the iVPCs. iVPCs were labeled with tdTomato (Td-red) while the nuclei of other cells were stained by DAPI (blue) to help with visualization. For injection into normal rat hearts, green fluorescent microspheres (see C & D) were blended in the cell or micro construct suspension to help locating the injected cells after four weeks. The scale bar in (A) and (B) is 500 μm , in (C) and (D) is 200 μm , and in (E) and (F) is 400 μm .