**SHORT COMMUNICATION**

# Initial evaluation of vascular ingrowth into superporous hydrogels

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## Abstract

**There is a need for new materials and architectures for tissue engineering and regenerative medicine. Based upon our recent results developing novel scaffold architecture, we hypothesized that this new architecture would foster vascularization, a particular need for tissue engineering. We report on the potential of superporous hydrogel (SPH) scaffolds for *in vivo* cellular infiltration and vascularization. Poly(ethylene glycol) diacrylate (PEGDA) SPH scaffolds were implanted in the dorsum of severe combined immunodeficient (SCID) mice and harvested after 4 weeks of *in vivo* implantation. The SPHs were visibly red and vascularized, as apparent when compared to the non-porous hydrogel controls, which were macroscopically avascular. Host cell infiltration was observed throughout the SPHs. Blood cells and vascular structures, confirmed through staining for CD34 and smooth muscle α-actin, were observed throughout the scaffolds. This novel soft material may be utilized for cell transplantation, tissue engineering and in combination with cell therapies. The neovascularization and limited fibrotic response suggest that the architecture may be conducive to cell survival and rapid vessel development. Copyright © 2009 John Wiley & Sons, Ltd.**

Tissue engineering, which applies the principles of biology and engineering to the development of functional substitutes for damaged tissues or organs (Langer, 1997), has been increasingly discussed as an option to overcome the limitations of transplanting tissues. While *in vitro* tissue-engineered constructs consist of cells seeded on suitable scaffolds with minimal mortality, *in vivo* cells within these constructs cannot survive by diffusion alone (Folkman, 1995) and constructs must support angiogenesis. Angiogenesis within scaffolds also allows successful engraftment of the construct into the surrounding host tissue (Rucker *et al.*, 2006). To this end, porous hydrogel scaffolds are being investigated (Druecke *et al.*, 2004; Ford *et al.*, 2006; Wake *et al.*, 1994).

The inherent hydrated architecture of synthetic hydrogels imparts mechanical properties similar to soft tissues and extracellular matrix (Peppas *et al.*, 2000), which are natural hydrogels. Natural and synthetic hydrogels have been extensively examined and applied to hard and soft tissue, even when the hydrogel was significantly weaker than the bulk tissue (Lee and Mooney, 2001; Yamamoto *et al.*, 2000). The hydrophilicity, favourable biological recognition properties, and the ability to encapsulate cells within the hydrated network (Burdick and Anseth, 2002; Elisseeff *et al.*, 1999) have suggested the applicability of poly(ethylene glycol) diacrylate (PEGDA) hydrogels for tissue-engineering constructs (Nguyen and West, 2002). Additionally, various strategies have been reported to modulate the degradation of PEGDA scaffolds, which typically aim for degradation of the scaffold in concert with new tissue growth (Hudalla *et al.*, 2008; Kim *et al.*, 2008; Kraehenbuehl *et al.*, 2008). However, the photopolymerization techniques that are commonly used for the preparation of non-porous hydrogels have the disadvantages of relatively large diffusion distances and limited cell–cell interactions within the scaffolds (Nuttelman *et al.*, 2004). While the rate and depth of vascular ingrowth into scaffolds are influenced by the

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presence, size and interconnectivity of pores (Druecke *et al*., 2004; Gerecht *et al*., 2007; Landers *et al*., 2002), engineering interconnected porous architectures within hydrogels is a challenge. Various techniques have been employed for generating a porous architecture (Flynn *et al*., 2003; Ford *et al*., 2006; Landers *et al*., 2002; Liu *et al*., 2000; Weinand *et al*., 2007; Yoon *et al*., 2004) but interconnected pores are not always formed. Without interconnected pores or inherent degradability, cell penetration and proliferation within the scaffolds is inhibited (Chirila *et al*., 1993). In addition to pores being necessary for cell invasion, surface modification, either by conjugating peptides with cell adhesive sequences (Hern and Hubbell, 1998; Hersel *et al*., 2003) or infiltrating the hydrophobic scaffold with a hydrophilic polymer, have been proposed to be required to make hydrophilic scaffolds conducive to cell attachment and migration (Mooney *et al*., 1995). To this end, hydrophilic polyvinyl alcohol (PVA) sponges have been extensively studied as *in vivo* models for wound healing and foreign body reaction (Diegelmann *et al*., 1986; Kyriakides and Bornstein, 2003). While early stages (1–3 weeks) of cell invasion into the PVA sponge are characterized by neovascularization, the later stages (3 weeks) of the response are typical of a foreign body response, leading to a fibrotic encapsulation (Kyriakides and Bornstein, 2003; Nam *et al*., 2004). Formation of this avascular and acellular fibrous capsule can be deleterious to the function of a tissue-engineered construct. The implant cannot further vascularize and the cells within the implant undergo ischaemic death (Anderson, 2006). Additionally, PVA sponges as implant scaffolds require pro-angiogenic factors (Yamamoto *et al*., 2000) or implantation in the highly vascular mesenteric space of a larger animal model, to ensure vascularization (Takeda *et al*., 1995). This limits the use of PVA sponges for long-term implantation and cell encapsulation.

Our group has recently reported that unmodified PEGDA superporous hydrogels (SPH) allow human mesenchymal stem cell loading, survival for up to a month and stimuli-induced differentiation (Keskar *et al*.). PEGDA SPHs are highly permeable due to their high water content, which may hold advantages over hydrophobic polymer-based scaffolds. With these observations, we hypothesized that the highly interconnected porous architecture would be favourable for cellular infiltration and angiogenesis *in vivo*. We further hypothesized that the limited fibrotic response to non-porous PEGDA-based hydrogels would be similar when the porous network was implanted.

PEGDA (MW 3400 g/M) SPH scaffolds were prepared and characterized as previously reported (Keskar *et al*.). Briefly, PEGDA solution, foam stabilizer (Pluronic® F127, double-distilled water, the initiator pair, N,N,N′,N′-tetramethylethylene-diamine (TEMED) and ammonium persulphate, were added sequentially to a glass vial. Saturated citric acid solution was used for pH adjustment. The precursor solution was mixed and heated gently to 37 °C for approximately 2 min. Sodium bicarbonate (200 mg) was added with constant stirring to evenly distribute the salt and evolving gas. The SPHs were then removed from the vial and allowed to swell in double-distilled water to remove traces of unpolymerized monomers and salt before dehydrating in 80% ethanol, followed by overnight dehydration in absolute ethanol. The hydrogels were then dried in a food dessicator and stored in an airtight container for further use. To make the non-porous PEGDA hydrogels (NPH), 200 mg sodium bicarbonate was replaced with sodium hydroxide solution. The precursor solution was pipetted into 96-well plates, with each well containing an equivalent volume to the size of the total volume of the porous hydrogel. Polymerization was allowed to proceed for 30 min. The NPHs were then rinsed with double-distilled water to remove traces of unpolymerized monomers, dried and stored in an airtight container for further use. Scanning electron microscopy of the interior surface of the dehydrated SPHs revealed interconnected pores in the range 100–600 μm, with an average pore size of $250 \pm 94$ μm (Figure 1a). The hydrated SPH had a larger pore diameter and broader distribution in pore diameter ($395 \pm 107$ μm), as estimated from brightfield images (Figure 1b).

To investigate the potential of the SPHs for *in vivo* angiogenesis, they were implanted in the dorsal skin fold of SCID mice (Fox Chase SCID, Charles River
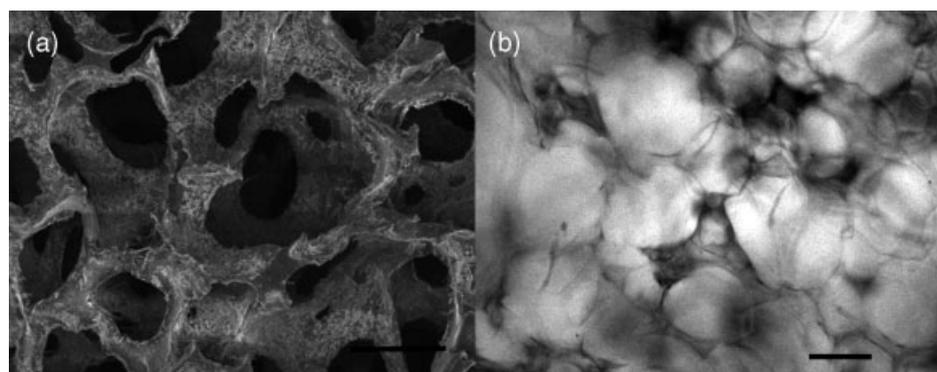


**Figure 1. Interconnected porous architecture can be seen where each pore was internally connected to the adjacent pores. (a) Representative scanning electron micrograph of the interior of dried PEGDA SPHs. (b) Representative light micrograph of the interior of a hydrated PEGDA SPH. Scale bars = 200 μm**
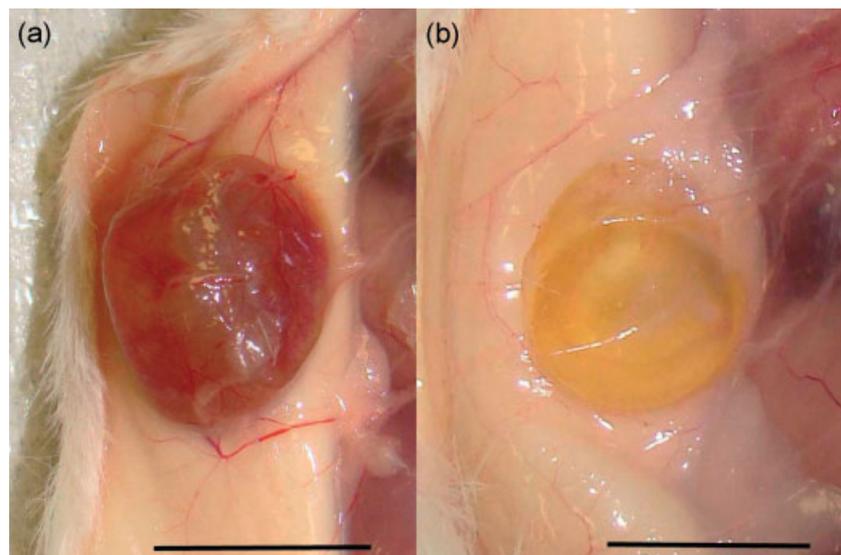
**Figure 2. Gross images of the hydrogels extracted after 4 weeks of subcutaneous implantation in the back of SCID mice. (a) Superporous hydrogel attached to the skin, red in appearance, indicating possible vascularization. A very thin fibrous layer can be seen enclosing the SPH. (b) In comparison, the non-porous hydrogel appears avascular. In both images, the pale skin (at the centre) and muscles (at the edge) are apparent. Scale bar = 1 cm**

Laboratories). SCID mice were chosen as the model for vascularization as part of a larger experimental design involving human cells incorporated within the SPH where the tissue growth and vascularization within the SPH will be evaluated. All animal experiments were approved by the institutional animal care committee at the University of Illinois at Chicago. Experimental design for the study was developed using power analysis of published data of porous polymer implants (Arinzeh *et al*., 2005; Hidetsugu *et al*., 2007). Briefly, the mice were divided into two groups of seven mice each. The mice were anaesthetized with intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine. Subcutaneous pockets were opened in the back of the mice, using a blunt probe. Two hydrogels, either SPH or NPH, were inserted within either side of the pocket. The incision was closed and the animals were monitored for 4 weeks. At the end of four weeks, the mice were sacrificed by an overdose of carbon dioxide followed by cervical dislocation. The hydrogels were removed *en bloc*. Fixed samples were embedded in paraffin, sectioned and processed further for histological evaluation of cellular infiltration and vascular ingrowths.

Upon implantation, the hydrogels could be palpated easily. Daily monitoring did not reveal any weight loss or any apparent signs of toxicity, such as inflammation or reddening of the skin, in the test animals. At the end of 4 weeks, the hydrogels could be seen attached to the inside of the dorsal skin. Gross appearance revealed the red, vascularized superporous hydrogels (Figure 2a) and the pale yellow avascular non-porous hydrogels (Figure 2b). Haematoxylin and eosin (H&E) staining of the SPH sections revealed host cell infiltration throughout the scaffold (Figure 3a), which was absent in the non-porous hydrogels. Thus, the porous architecture of the SPH seemed to provide a favourable environment for
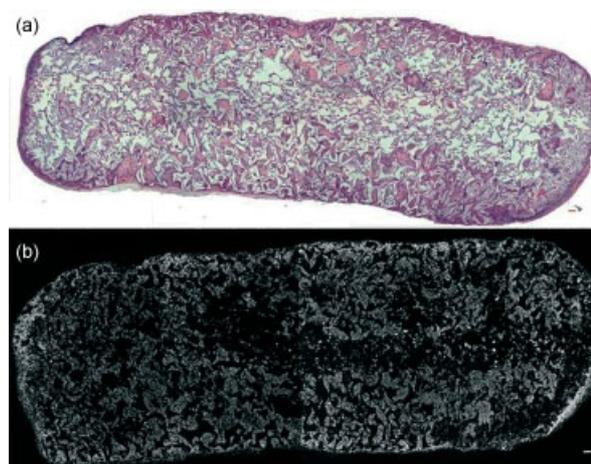


**Figure 3. (a) H&E-stained sections of the hydrogel taken from the centre of the scaffold. Cellular infiltration (purple nuclei and pink cytoplasm) can be seen throughout the SPH, indicating that the host cells were able to penetrate right up to the core. (b) Combined epifluorescent images of H33258 staining within the SPH. Scale bars = 100 μm**

micro-vessel formation. While it has previously been demonstrated that the rate and the depth of vascular ingrowths within scaffolds are influenced by the presence of pores (Gerecht *et al*., 2007), the number of vascular ingrowths are limited by the interconnectivity of the pores within the scaffolds (Druecke *et al*., 2004; Landers *et al*., 2002).

To further elucidate the presence of host cells, paraffin-embedded sections were deparaffinized in xylene, hydrated with serial concentrations of ethanol and stained with Hoechst 33 258 nuclear stain (Latt and Stetten, 1976) (Figure 3b). A fibrotic, vascularized capsular layer surrounding the SPH was formed (Figure 4). Extracted SPH implants showed the presence of blood cells and
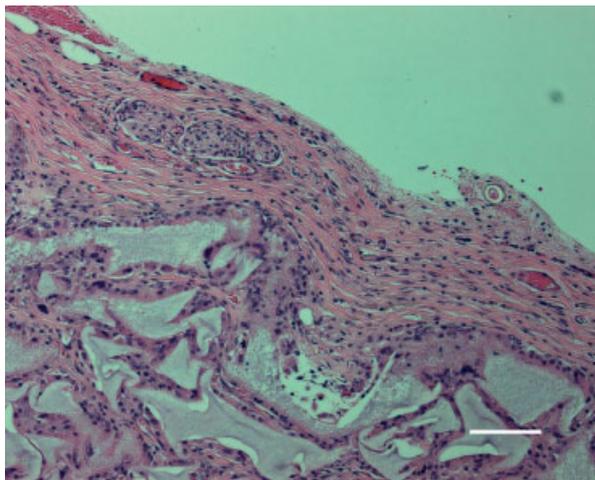
**Figure 4. H&E-stained section of the thin vascularized fibrotic tissue seen encapsulating the SPH. Scale bar = 100 μm**

vascular structures. A representative image taken from the centre of the SPH section revealed the presence of these ingrowths to the core of the SPH (Figure 5a). Lumen-like structures filled with blood cells (Figure 5b) confirmed the presence of a functional microvasculature.

To further confirm the presence of microvasculature, CD34, an early stage endothelial cell marker (Civin *et al*., 1990), and $\alpha$-smooth muscle actin ($\alpha$-SMA), a vascular smooth muscle cell marker (Van Gieson *et al*., 2003), were examined. Briefly, the deparaffinized and hydrated sections were blocked with 1% bovine serum albumin in phosphate-buffered saline, pH 7.4, for 30 min. The sections were then incubated with primary rat polyclonal antibodies against CD34 and mouse polyclonal antibodies against smooth muscle $\alpha$-actin (Santa Cruz Biotechnology) for 2 h, followed by incubation with FITC-conjugated goat anti-rat and goat anti-mouse (Molecular Probes), respectively, for 30 mins. Hoechst 33 258 was used as the nuclear stain. CD34-positive endothelial cells were localized throughout the SPHs (Figure 6a). In addition, vessel-lining musculature (Owens, 1995) was

observed in the form of $\alpha$-SMA positive cells associated with the vascular growths (Figure 6b). These results suggest that the ingrowths observed within the implanted acellular SPHs are neovasculature.

# 1. Conclusion

Tissues generated *in vitro* or *in vivo* must survive and function *in vivo*. However, *in vivo* tissue viability and function is limited by the ability of the scaffold to support functional microvasculature and overcome subsequent transport limitations. The unmodified PEGDA SPHs utilized in this study were able to support initial *in vivo* vascularization upon implantation in SCID mice. The presence of a highly interconnected porous architecture within the PEGDA SPH provided an open network, which facilitated cellular infiltration from the host and vascular ingrowth throughout the scaffold; PEGDA scaffolds with discontinuous pores should, however, be employed in future studies to elucidate the importance of pore interconnectivity and the rate of *in vivo* vascularization. In summary, we present a platform scaffold technology that should be further examined for tissue-engineering applications. The approach of *in vivo* implantation of the acellular SPH, or seeding cells within the SPH scaffolds before *in vivo* implantation, could both benefit from rapid thorough vascularization. Future studies will be directed at further understanding the dynamics of vascular infiltration and the stability of the newly formed vasculature.
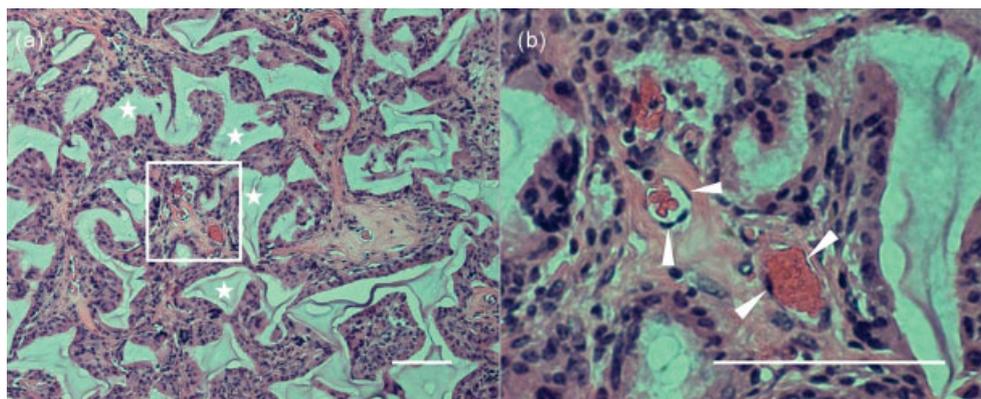
## Acknowledgements

**Figure 5. H&E staining within the SPH sections, showing presence of vascular ingrowths. (a) Representative image showing extensive host cell infiltration and areas of vascularization within the SPH (\*). The hydrogel sections have a faint staining and have dehydrated and thus pulled away from the cells. (b) A higher magnification image of the area highlighted in (a) clearly shows the endothelial lining of the microvessels stained blue (marked by white arrows) and the lumen filled with bright red, anucleated blood cells. Scale bars = 100 μm**
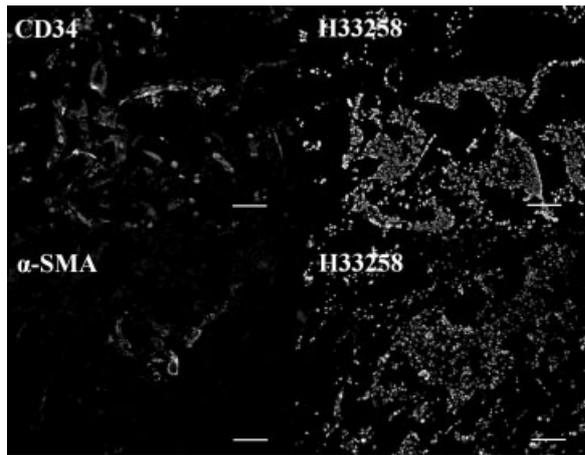
**Figure 6. Immunohistochemical analysis of the SPH sections. (top) CD34$^+$ endothelial cells can be seen throughout the sections with a lumen visible. (bottom) The microvessels stain positive for smooth muscle $\alpha$-actin ($\alpha$-SMA), indicating mature blood vessels. In both sections, H33258 was used as the nuclear stain. Scale bars = 100 $\mu$m**

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