Poly(vinyl alcohol)-Amino Acid Hydrogels Fabricated into Tissue Engineering Scaffolds by Colloidal Gas Aphron Technology

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Summary: A new class of hydrogels made from poly(vinyl alcohol) (PVA) and amino acid was formed into porous tissue engineering scaffolds by the colloidal gas aphron (CGA) method. CGA microfoams are formed using high speed stirring to generate uniform, micrometer scale bubbles. CGAs offer several advantages over conventional scaffold fabrication techniques including room temperature processing, aqueous conditions and utilization of air bubbles to create uniform pores. This technique eliminates the need for toxic solvents and salt templates. In addition, the novel poly(vinyl alcohol) hydrogels are inherently strong, eliminating the need for crosslinkers.

Keywords: aphron; foams; hydrogel; poly(vinyl alcohol); tissue engineered scaffold

Introduction

Tissue engineering seeks to reconstruct or regenerate damaged or missing tissues and organs by the control of living cells and the biological matrices they produce. Tissue engineering uses porous scaffolds to support the growing cells and tissues. There are many review articles and books available on scaffolds for tissue engineering [1-4]. Scaffolds serve numerous functions in tissue engineering: an adhesive substrate onto which cells can attach and proliferate; to shape and structure new tissue; to deliver surface signals for cell processes; to deliver soluble growth factors or other biomolecules.

Many methods can be used to fabricate porous scaffolds. These include salt leachate methods, templating, electrospinning, phase separation (freezing or solvent precipitation) gas foaming and solid free-form fabrication methods.

A new method for porous scaffold preparation is described here, aphron formation[5], that offers many advantages. Air bubbles are the pore formers, obviously non-toxic for

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biomaterials fabrication. The pore size is reasonably uniform and controllable. Finally, this is a low cost processing method, scaleable for production. Aprons are well known in chemical processing but have not been applied for tissue engineering. Aphrons are colloidal foams created with propeller-driven high speed stirring. The propeller rotation produces waves that re-enter the liquid at the baffles creating tiny droplets of gas encapsulated by a liquid shell[5].

Hydrogels made with PVA and amino acids recently discovered in our laboratory were used in this work along with the aphron formation to make scaffolds useful for tissue engineering. We discovered that PVA and any of a number of different amino acids will form hydrogels ranging from soft to hard. The mechanism for the formation of these gels has not yet been elucidated, but some evidence suggests the amino acids may serve as templates to assist non-covalent PVA chain-chain interaction.

Experimental

Materials

Pure glycine, L-proline and L-lysine, pH 7.4 phosphate buffered saline (PBS) tablets, hematoxylin solution and acetone were purchased from Sigma-Aldrich. Poly(vinyl alcohol) (PVA) was obtained as a gift from Kuraray Inc. (Mowiol 28-99, 99% hydrolyzed) and precipitated in acetone. PVA average molecular weight (M_n 17,309 Da, M_w 50,066 Da and PDI 2.89) was determined by gel permeation chromatography [Waters Ultragel 1000 and 250 columns -- OmniSec™ software used to fit results to polystyrene standards to assess PVA molecular weight]. Rat tail collagen type I (3.71 mg/Ml in 0.2 N acetic acid) was purchased from BD Biosciences/Discovery Labware Inc. Ethanol and concentrated hydrochloric acid for preparation of acid alcohol were purchased from AAPER and Fisher Scientific respectively. DMEM high glucose/no L-glutamine growth media, trypsin, Versene, L-glutamine, fetal bovine serum (FBS) and antibiotic/antimycotic solution were purchased from Gibco. C2C12 mouse myoblasts were obtained by gift from Professor Stephen Hauschka's lab at the University of Washington (originally purchased from ATCC). Nanopure water was used for all experiments.

Methods

A spinning disk generator for forming colloidal gas aphrons (CGAs), based upon a published apparatus by Seba, was used to create the CGAs (Figure 1). [5]

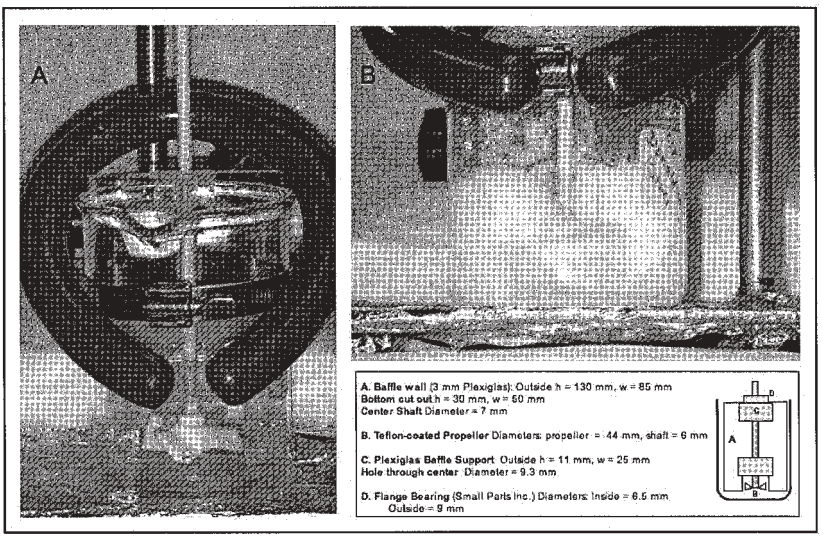

Figure 1. A. Aphron apparatus B. An aphron foam being produced. (Baffle design and dimensions, based on previous work by Felix Seba[5]).

Our aphron generator consists of a 3 mm vertical Plexiglas baffle that just fits into a 500 mL beaker. The baffle has a 7 mm center gap, allowing the propeller shaft to pass through (Figure 1). The propeller is positioned just below the solution surface between the two vertical baffles and rotated at 2000 rpm using a variable speed electric stirring motor (Arrow 6000, NJ USA). Propeller and baffle variations affect final PVA scaffold morphology. Experiments detailed here were performed using the set-up shown in Figure 1. Scaffolds were generated using 90mL of PVA solution (8.9 wt% in water) with 10mL of amino acid solution (20 wt% in water) and frothed for 10 minutes. For samples containing collagen I, the protein (1.8 mg collagen I per 1000 mg PVA*) was added to the PVA-AA solution during the last 2 minutes of foaming. Following foam formation, samples were immediately frozen at -80 °C overnight and lyophilized (VirTis, benchtop 6.6) to form a firm, sponge-like material. The final PVA-AA scaffolds contained 80 wt% PVA and 20 wt% AA. All scaffolds were sterilized with ethylene oxide (University of Washington Health Sciences) prior to characterization.

* Minimum level of collagen I found to induce cell adhesion on the PVA-AA materials.

The volumetric swelling ratio (Q) of the polymers was determined by measuring the weight of swollen samples (m_s) after soaking in water for 48 h and the final dry polymer weights (m_{fd}) following lyophilization ($Q = 1 + [(\rho_{polymer} / \rho_{solvent})(m_s / m_{fd})-1)]$)[6]. Where $\rho_{polymer}$ was assumed to equal ρ_{PVA} (1.2619 g/mL) and $\rho_{solvent}$ was ρ_{H2O} (1 g/mL). The tensile modulus and tensile strength of the PBS swollen scaffolds were measured on an Instron™ 5500R using a 2000g load cell. Gauge length 15mm samples (thickness 1.5mm and width 5mm, triplicate samples) were strained at 10mm/min until failure. Merlin™ software was used for Instron™ tests and data analysis.

Preliminary investigations into biocompatibility were performed on PVA-glycine and PVA-glycine-collagen I scaffolds. Data from these experiments will be presented elsewhere, though general conclusions will be summarized here.

Scaffold morphology was investigated using scanning electron microscopy (SEM) (FEI Sirion 30, 3kV beam). In addition, scaffold samples were fixed for 20 h in 3.7% formaldehyde in PBS, rinsed in PBS and 70% ethanol, and paraffin embedded. The embedded samples were sectioned (5 μm), counterstained with hemotoxylin (2 dips in Harris's hematoxylin, 1 dip in acid alcohol, 2 dips in Scott's blue) and imaged using phase contrast microscopy.

Results and Discussion

The colloidal gas aphron methodology can generate solid, microporous PVA-AA materials. SEM analysis of the scaffolds found pores ranging from 50–300 μm (Figure 2). Without amino acid addition, PVA aprons exhibited a poor pore structure. The addition of collagen type I had a modest effect on the pore structure.

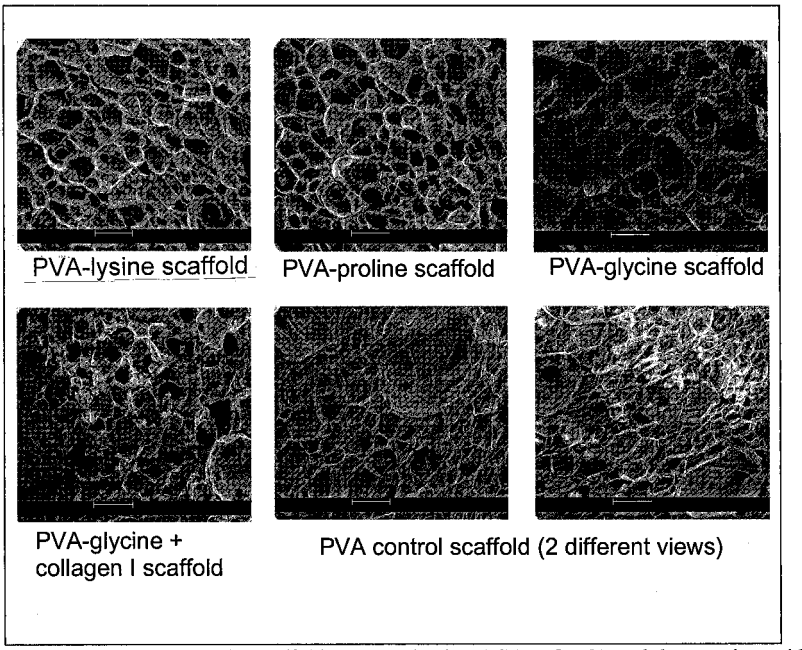

Figure 2. SEM images of a scaffolds prepared using CGAs of PVA and three amino acids. (scale bar = 500 μm)

Volumetric swelling ratio (Q) was determined for each scaffold type. The amino acid used and its concentration significantly affects Q. Volumetric swelling decreased upon the addition of amino acids -- the decrease was most pronounced when comparing 100% PVA to scaffolds incorporating L-lysine and L-proline (Q drops over 65%). The decrease in Q from amino acid-free PVA scaffolds to PVA-glycine scaffolds was found to be less pronounced but still significant at over a 20% decrease. Q was not significantly changed by collagen I addition.

Melting temperature (T_m) from differential scanning calorimetry (DSC) was found to decrease upon the addition of an amino acid. Results from these studies will be presented elsewhere.

Tensile modulus for the PVA-AA scaffolds is shown in Figure 3 with a range from 60 to 120 kPa. Tensile modulus changed with different amino acids but was not affected by collagen I incorporation. The 140 ± 40 kPa grey region in Figure 3 shows the expected range for the elastic modulus of human left ventricle (LV) myocardium measured *in*

vitro[7]. The 100% PVA, PVA-lysine and PVA-proline scaffolds all fell within this desired range.

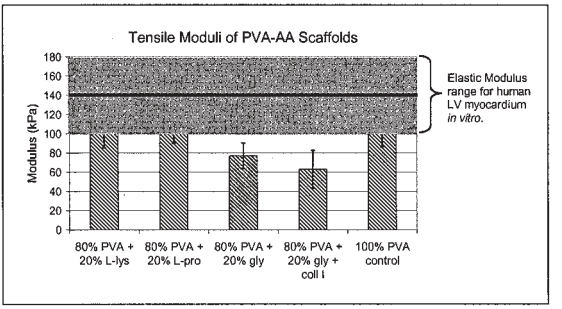

Figure 3. Tensile moduli of PVA-AA scaffolds. Grey region (140 ± 40 kPa) shows the expected range for human LV myocardium measured *in vitro* [7].

The PVA-AA scaffolds were found to have lower Q and T_m values than the 100% PVA scaffold. PVA-lysine and PVA-proline scaffolds were found to have lower Q and T_m values yet higher tensile moduli than their PVA-glycine counterparts. T_m is related to the specific AA used. Scaffold morphology may also drive Q and tensile modulus. Equilibrium swelling ratio measurement for hydrogels can be challenging even on bulk materials. Pores further complicate interpretation. Pores fill with water during swelling. They must be emptied for an accurate measurement of the gel's equilibrium swollen weight. Residual liquid in the pores will artifactually raise the Q value. Assuming this error was present in our experiments, then the Q values may reflect scaffold morphology more than hydrogel swelling. Scaffolds of lower porosity contain less void volume and thus their final swelling ratios would be calculated as lower than those for scaffolds of higher void volume (greater porosity). The lower Q values observed for the PVA-lysine and PVA-proline scaffolds may actually indicate their structure to be less porous than PVA-glycine. Experiments to serial section, image and reconstruct 3D images of the scaffolds are underway and should enable us to accurately compare scaffold morphologies. In addition swelling studies will be conducted on bulk PVA-AA materials to determine more accurate values for Q.

Elastic moduli were found to be comparable for 100% PVA, PVA-Lysine and PVA-Proline scaffolds (around 120 kPa), but decreased for scaffolds made using PVA-glycine (to approximately 70 kPa). This observation is likely attributed to differences in scaffold

morphology more than differences between hydrogels. Scaffolds of lower porosity will contain more hydrogel, translating to thicker pore walls. Tensile testing reports the strength of these pore walls and will be dependent upon wall thickness. This is also true for other calculations (i.e. elastic modulus).

PVA-AA scaffolds were assessed for biocompatibility. Both the PVA-glycine and PVA-glycine-collagen I scaffolds were found to be endotoxin and cytotoxin free. Cell adhesion increased substantially with the addition of collagen I however cell spreading was negligible. We are exploring other extracellular matrix proteins to improve cell interactions and the results of these experiments will be reported elsewhere.

Conclusions

PVA-AA hydrogels foamed by CGA methods offer a new approach to tissue engineering scaffolds. The fabrication method described here avoids many of the limitations of conventional scaffold preparations, including harsh chemicals during pore formation and/or polymer crosslinking and expensive components. CGA scaffolds from PVA-AA contain only PVA, AA and water. The mild conditions of the CGA technique open new possibilities for the incorporation of cells, growth factors and other biological moieties into the scaffolds. Cells will not adhere to the PVA-AA scaffolds as fabricated. The addition of collagen I induces cell adhesion. Through incorporation of the correct combination of biological moieties PVA-AA scaffolds should be tailorable to elicit particular cellular responses. The biodegradability of these hydrogel structures *in vivo* also must be investigated to determine their suitability for specific tissue engineering applications.

Acknowledgments

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