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Characterization of Poly(vinyl alcohol)/Chitosan Hydrogels as Vascular Tissue Engineering Scaffolds

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Summary: In this study, PVA/Chitosan hydrogels were fabricated by freeze/thaw cycles and further crosslinking with a KOH/Na_2SO_4 coagulation bath and the effect of freeze/thaw cycle number on cell behaviour was evaluated. The surface of the hydrogels were further modified with Collagen type I adsorption and seeded with bovine aortic vascular smooth muscle and endothelial cells. Increasing the number of freeze/thaw cycles resulted in a marked change in surface morphology, hydrophilicity and protein adsorption. Collagen coating caused an increase in initial attachment and proliferation. We concluded that hydrogels that have undergone 3 freeze/thaw cycles were optimum for cell attachment both in the presence and the absence of coating.

Keywords: chitosan; freeze/thaw; hydrogel; PVA; tissue engineering

Introduction

PVA, a hydrophilic, biodegradable and biocompatible synthetic polymer, has been widely used in different areas of the biomedical field.[1] In tissue engineering, several PVA based scaffold forms, such as thin films, foams, electrospun fibers and hydrogels have been produced for different target tissues.[2–4] For vascular tissue engineering, the main aim is to develop substitutes to currently available artificial grafts, which are not reliable for small diameter vessel replacements. One relevant scaffold form is the PVA hydrogel; due to mechanical properties comparable to that of vasculature, which would decrease compliancy related problems.[5] PVA based hydrogels can be produced via chemical crosslinking, photopolymerization, physical crosslinking or irradiation.[6] Freeze/thawing is one of the physical hydrogel production methods, in which repeated freezing and thawing cycles result in higher degree of crystallization of PVA chains and produce a highly elastic hydrogel. As the number of cycles increases, the percentage of crystalline regions within the hydrogel increases too; resulting in a stiffer structure.[5] Hydrogels produced by this method have been shown to have mechanical properties similar to that of aortic root.[7]

Even though PVA hydrogels have remarkable mechanical characteristics, the highly hydrophilic nature of PVA makes it a subpar surface for cell attachment and spreading. Previously, this problem has been tackled by either covalent immobilization of attachment promoting molecules[8] or preparation of composite hydrogels that contain additives and are much more conducive to cell attachment.[9] Previously, in our group, it has been shown that vascular smooth muscle cell and vascular endothelial cell attachment and proliferation can be achieved by adding chitosan into the hydrogel structure.[10] Our current focus is the evaluation of the response of the vascular cells to the changes in hydrogel structure by increasing freeze/thaw cycle number.

Materials and Methods

PVA (10%, Molecular Weight (MW) 78400, Vassar Brothers, USA) and water soluble chitosan (85% deacetylation, Jinan

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Haidebi Ltd, China) solutions were prepared in distilled water and mixed at a ratio of 9:1 w/w. For even distribution of chitosan within PVA solution, mixture was stirred at 60 °C for 2 h. The mixture was then poured into custom-made persplex templates with a thickness of 500 μm and frozen at −20 °C overnight. Hydrogels were obtained by thawing the frozen solutions at room temperature for additional 12 hours (1st cycle). Same procedure was repeated for another set of hydrogels 3 times (3rd cycle). Hydrogels were further crosslinked by immersion into a coagulation bath composed of 7.5% KOH and 1 M Na_2SO_4 for 1 hour. Membranes were finally washed with dH_2O several times and stored at 4 °C up until use.

The effect of coagulation and additional cycles on hydrogel physical characteristics were evaluated by determination of the water content of hydrogels, measurement of water contact angle on hydrogels, SEM imaging and uniaxial tensile strength tests. The water content was measured by determination of the weight loss after the hydrogels were completely dried. Water contact angles were determined by sessile drop method on hydrogels blotted dry. Tensile strength tests were conducted by a Universal tester (Zwick-Roell, Germany) at a strain rate of 60%/min following an initial preconditioning cycle at the same loading rate up to 60% strain ($n \geq 6$). Samples were tested up to failure. Protein adsorption onto the hydrogels were quantified by Bicinchoninic acid assay (BCA) (Pierce, USA) by first applying a model protein (1 mg/mL bovine serum albumin (BSA)) onto the hydrogels at room temperature overnight and desorption of the proteins by incubating the hydrogels in a 3M Urea/5% sodium dodecyl sulfate (SDS) solution overnight ($n \geq 6$). The amount of protein inside the desorption solution was determined by incubating 10 μL of the solution in 200 μL of BCA reagent solution at 37 °C for 30 minutes and measuring the absorbance at 562 nm with a microplate reader. Absorbance values were converted to concentration with respect to a standard curve of known BSA concentrations.

Cell Culture

Bovine aortic smooth muscle cells and bovine arterial endothelial cells were cultured under standard tissue culture conditions (37 °C, 5% CO_2) in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. All experiments were conducted with cell between passage 10–18 for smooth muscle cells and passage 8–10 for endothelial cells.

Hydrogels were sterilized by immersion into 70% ethyl alcohol for 2 hours and following washing steps with sterile PBS, stored in culture medium in a CO_2 incubator overnight to promote protein adsorption. Alternatively, sterilized hydrogels were coated with sterile filtered rat tail collagen type I (Sigma-Aldrich, UK) at a concentration of 100 μg/cm^2.

The cells were trypsinized and the cell number was quantified by tryphan blue and then seeded onto hydrogels with a surface area of 1 cm^2, at a concentration 5×10^4 cells/cm^2 in 20 μL of medium. After 1 hour of initial attachment period, the medium was completed to 500 μL. Cell attachment and proliferation was quantified by Alamar Blue Cell proliferation assay (AbBiotech, USA) at 24 h and 7 days post seeding. Alamar Blue solution (10% in serum free RPMI 1640 medium) was applied onto the hydrogels and absorbance of the dye at 562 and 595 nm was determined after one hour of incubation in culture. Absorbance readings were converted to dye reduction% as per instructions of the provider. Dye reduction (%) is indicative of cellular metabolic activity, i.e. higher reduction signifies higher cell number. Cell morphology was assessed by staining with DAPI and observation under an epifluorescent microscope.

Results

Increasing the freeze/thaw cycle number caused significant changes in hydrogel structure. Hydrogels became stiffer and the orientation of the PVA chains which

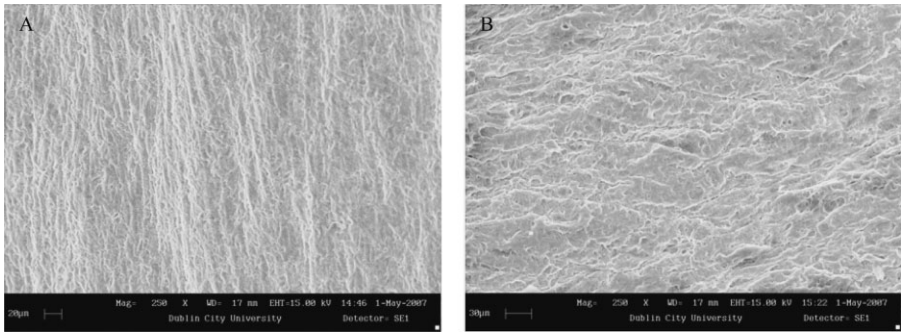

Figure 1.
SEM micrographs of 1st and 3rd cycle hydrogels before coagulation bath treatment.

can be observed with 1st cycle hydrogels was lost as the freeze/thaw cycle was repeated (Figure 1 A,B).

Moreover, coagulation bath treatment had affected the water content and surface hydrophilicity of the hydrogels (Table 1). Initially, the hydrogels had water contents close to each other and even though the coagulation bath caused a decrease in both 1st and 3rd cycle hydrogels; the decrease was more prominent for 3rd cycle hydrogels. On the other hand, initially the 1st cycle hydrogels were less hydrophilic compared to the 3rd cycle ones. However following immersion into the coagulation bath; both groups showed quite similar surface hydrophilicities, which may indicate an increase in concentration of PVA on the surface as a thin coating; since PVA is the more hydrophilic component of the composite. Increasing freeze/thaw cycles had a significant effect on the strength of the hydrogels (Table 1). 3rd cycle hydrogels had a higher ultimate strength and also they had lower elasticity (Elongation at break: 129.09% and 92.84% respectively for 1st and 3rd cycle hydrogels). However, after further crosslinking with the coagulation bath the difference between the strength of 1st and 3rd cycle hydrogels was less significant which might be an indication of an upper limit of degree of crosslinking via coagulation that may cause a leveling of mechanical properties.

The adsorption of proteins to the surface of the scaffolds is an essential step in successful population of them.[11] One of the main reasons for the inability of PVA to promote cell attachment is the low level of protein adsorption on its surface.[8] In contrast, it has been shown that chitosan has good protein adsorption capabilities. Of course, other parameters such as surface topography and roughness would also influence hydrogels capability to retain proteins.[12] BCA assay showed that 3rd cycle hydrogels had retained significantly more protein on their surfaces. For 1st cycle hydrogels, 48.16% of the protein applied was adsorped whereas 56.52% of the protein was still on the surface for 3rd cycle hydrogels.

Protein adsorption results were very consistent with cell attachment results, where 3rd cycle hydrogels showed a higher

Table 1.
Water content and water contact angle measurements of hydrogels before (BCB) and after (ACB) coagulation bath treatment.

	1st cycle		3rd cycle	
	BCB	ACB	BCB	ACB
Water Content (%)	83.67 ± 1.69	82.31 ± 1.05	86.34 ± 1.00	68.04 ± 1.36
Contact Angle (°)	31.76 ± 4.54	15.46 ± 2.06	20.09 ± 4.45	15.14 ± 2.01
Ultimate Strength (MPa)	0.05 ± 0.01	0.44 ± 0.12	0.30 ± 0.08	0.44 ± 0.08

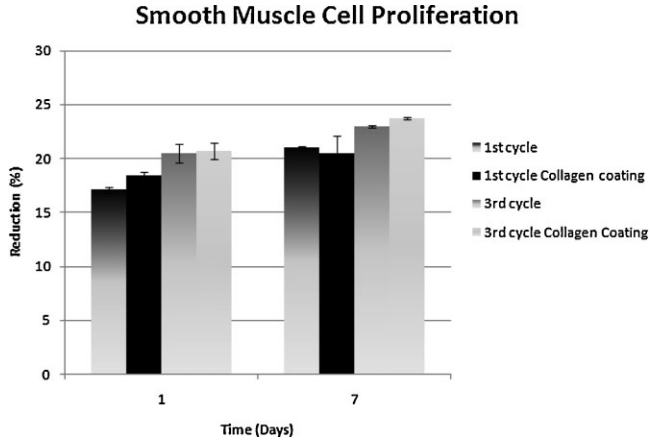

Figure 2.
Cell attachment and proliferation on PVA/chitosan hydrogels determined by Alamar Blue Proliferation Test. Increase in percentage reduction of Alamar Blue signifies a higher cell number.

initial cell attachment (Figure 2) compared to tissue culture polystyrene (TCP) for which the percentage reduction was $24.17\% \pm 1.07$ and 41.37 ± 5.45 for day 1 and day 7 respectively. Both hydrogels had inferior cell attachment but aside from the fact that TCP is an optimized surface for cell attachment, available surface area on TCP was higher and also some of the cells on plates might have migrated towards TCP, since the whole base of the plate was not covered by the hydrogels.

Vascular smooth muscle cells proliferated slowly but steadily on hydrogels, and collagen coating improved the cell attachment and proliferation slightly (Figure 3). In addition to that, collagen coating caused a more even distribution of cells compared to bare hydrogels; in which cells attached to the surface as patches; which implies chitosan-rich and chitosan-poor surface locations (Figure 3).

Differences in cell attachment between 1st and 3rd cycle hydrogels might have other reasons aside from protein adsorption and surface roughness. Substrate stiffness has been shown to be quite influential on cell behaviour[13] and stiffness differences between 1st and 3rd cycle hydrogels might have a direct effect on the observed

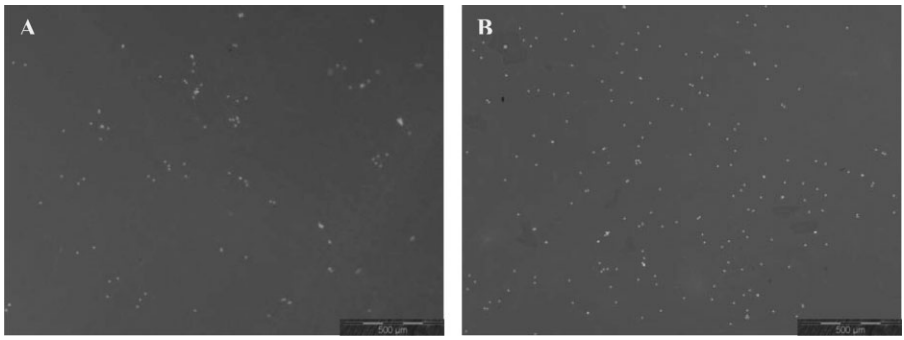

Figure 3.
Fluoresence micrographs of DAPI stained smooth muscle cells on 3rd cycle. A) Hydrogel with no coating, B) Hydrogel with collagen coating.

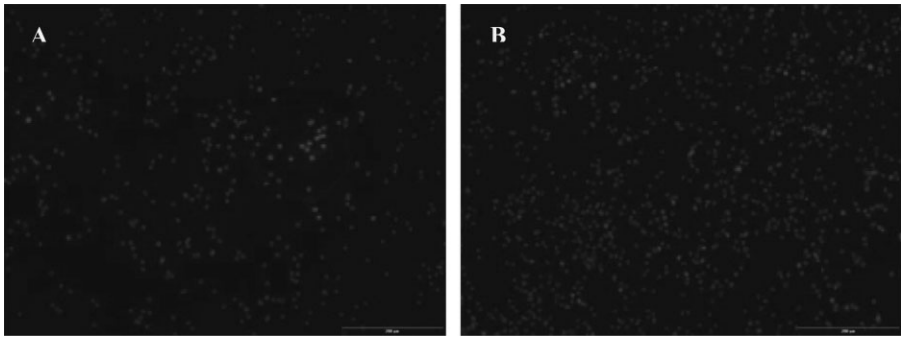

Figure 4.
Bovine Vascular Endothelial Cells on A) 1st and B) 3rd cycle PVA/chitosan hydrogels after 10 days of incubation. Image analysis showed that the number of cells on 3rd cycle hydrogels was significantly higher.

behaviour. However, mechanical differences between 1st and 3rd cycle precoagulation bath hydrogels are essentially eliminated by the coagulation bath treatment. Thus, the difference observed might be mainly due to the difference in surface topography. A similar difference in attachment and proliferation on 1st and 3rd cycle hydrogels was also observed with bovine aortic endothelial cells (Figure 4), which further justifies the advantage of using several freeze/thaw cycles. Optimization of surface properties would allow finer tuning of cell behaviour on the hydrogels.

Conclusion

The superior physical characteristics of PVA hydrogels compensate for their limited cell attachment properties. Addition of chitosan into the structure is a viable option for improving cell attachment without compromising the physical properties of the hydrogels. Moreover, optimization of several fabrication parameters such as; initial cell seeding number, type of surface coating and its density, number of freeze/thaw cycles and concentration of coagulation bath can provide further improvements. Our future efforts will focus on elucidation of the interactions between these paramaters.

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