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Biomedical Materials

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Polyvinyl alcohol-graft-polyethylene glycol hydrogels improve utility and biofunctionality of injectable collagen biomaterials

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Abstract

Collagen-based materials have become a staple in both research and the clinic. In wound care, collagen-based materials comprise a core gamut of biological dressings and therapeutic strategies. In research, collagen-based materials are employed in everything from 3D cultures to bioprinting. Soluble collagen is well characterized to undergo fibrillation at neutral pH and 37 °C. To remain stable, a neutralized collagen solution must be maintained at 4 °C. These physical characteristics of collagen impose limitations on its utility. In our previous work, we identified that the incorporation of a simple polyvinyl alcohol:borate hydrogel could improve the rate of collagen gel fibrillation. In this work we sought to further investigate the interactions of polyvinyl alcohol blend variants, as surfactant-like polymers, in comparison with known non-polymer surfactants. To conduct our investigations scaffold variants were created using increasing concentrations of polyvinyl alcohol, differing combinations of polymers, and non-polymer surfactants Tweens 20 and 80, and TritonX-100. Activation energy for collagen fibrillation was found to significantly decrease in the presence of polyvinyl alcohols ($p < 0.01$) at and above 0.4%w/v concentration. Further, addition of polyvinyl alcohol-graft-polyethylene glycol had the greatest enhancement (2.02 fold) on the fibrillation kinetics ($p < 0.01$), wetting properties and the stability of the collagen scaffolds post-freeze drying. Our results demonstrated that the addition of polyvinyl alcohol hydrogels to a collagen solution could stabilize collagen solution such that the solution could easily be lyophilized (at pH 7) and then reconstituted with water. Cells cultured in polyvinyl alcohol scaffolds also exhibited more organized F-actin, as well as a reduced abundance of pro-collagen and α-smooth actin. In conclusion, our results demonstrate for the first time that polyvinyl alcohol, preferably polyvinyl alcohol-graft-polyethylene glycol, directly affects the physical properties of collagen and the physiology of cells cultured within improving the utility of the combined material for both research and clinic needs.

1. Introduction

The fabrication of collagen scaffolds for medical and research purposes has been studied extensively. Characterization of collagen and its fibrillation process, both *in vivo* and *in vitro*, is well known to occur at a neutral pH through a transition state at 37 °C that involves the denaturation of collagen into a linear form that can undergo hydrogen bonding between triple-helix chains of soluble collagen [1–5]. The result of the process is an insoluble gel structure. The addition of glycosaminoglycan can further stabilize this process [6, 7]. In the body, where temperatures vary from 37 °C at the core through to 34 °C at the skin, collagen is in a constant dynamic state. Stability is achieved, in part, through hydrophobic interactions between collagen chains coupled with the solvation effects of water, hydrogen bonding and physical crosslinking. Despite its instability, collagen remains the most abundant protein in the human body, and for that matter highly conserved across the animal kingdom. There are over 28 types of collagen all of which assume a right-handed

helix resulting from a highly conserved amino acid sequence [8, 9]. In fact, even many marine species such as hydra have structural collagens that resemble that of human type IV collagen, a non-fibrillar collagen. Unlike mammalian collagen, marine collagens must undergo transition states at lower temperatures in order to form into a gel-like structure, which makes sense given a lower body temperature [9]. Nuances in the amino acid sequences, between marine and human collagen, afford marine collagen a reduction in gel transition temperature (T_g) and melting points (T_m).

The skin is the largest organ in the human body. Wound care is one of the most costly segments in healthcare, whereby the end-goal to repair the skin is universal. Current wound care innovations address a wide landscape of wounds using tissue-engineered constructs. Although many of these innovations have drastically reduced the time to heal a complex wound (e.g. burn or ulcer), the high-cost manufacture and, to a degree, limited successful outcomes hamper widespread acceptance and use. The bulk of these innovative products are pre-formed sheets of collagen (or decellularized tissue) and more recently injectable, gelable-emulsions of collagen. A gelable emulsion differs from a sheet in that when hydrated it will not form a unified sheet of collagen. One pitfall of these innovations that confounds both operations and end-user application is the limitation of 'one-size fits all', despite that there may be different sizes. One solution would be to engineer a scaffold *in situ*, and therein at the point of care the scaffold (or skin) could be combined with healing agents, cells and tailored to the etiology of the wound. The scaffold matrix, as the constant factor, would thus need to form into a structurally competent material on the patient. Ideally the material should be the same as the native tissue, which in the skin is type 1 collagen, and form a unified structure upon gelation that is mechanically and biologically stable. Aside from modification of the collagen amino acid sequence or the use of detergents, there are no known methods to alter the physical properties of collagen. Genetically altered collagens require scale-up in a bacterial culture, a costly endeavor with inherent regulatory screening. Previous work by Li demonstrated that sodium dodecyl sulfate (SDS), which is known to be cytotoxic, reduced time to collagen gelation [10]. Where both alterations in amino acid sequence and surfactants affect the thermodynamics of protein-solvent interactions, amino acid alterations also impart biological affects- some of which could be desirable, such as reducing cell-signaling interactions that result in contracture and scarring. Taken together, surfactant affects may improve collagen gelation and amino acid sequence modification of collagens may affect cell signaling. Thus one feasible approach to combine both outcomes would be to employ inert-biocompatible polymer surfactants. In fact in many ways, biological polymer surfactants already exist and serve this function, however they too add cost to manufacture and the potential for un-needed biological affects. Polyvinyl alcohol (PVA), a well known and inert biocompatible polymer, and polyethylene glycol (PEG) have been known to be useful and biocompatible surfactants [11, 12]. Variations of these polymer-surfactants have the added advantage of stabilizing the collagen structure, and altering its mechanical properties once gelled [13–16]. Toward the development of a simple cell delivery system combination of polymeric hydrogels with collagen allow for rapid collagen gelation and ultimately tissue engineering *in situ*. Our earlier work demonstrated that addition of a simple PVA-hydrogel to a crosslinked collagen-GAG scaffold could improve mechanical and physical properties [15]. Optimization of this system has now led to the fabrication a of cost-effective injectable collagen matrix with improved biological, chemical and mechanical features [17, 18]. In this work we further elucidate the role that PVA variants play in modulating the properties of collagen as benchmark, matrix protein for tissue engineered constructs. Herein we described the use of PVA and PVA-*graft*-PEG copolymers to reduce the gelation temperature of collagen fibrillogenesis *in vitro* and the benefit of such polymer combinations to stabilize neutralized, freeze-dried collagen that can be easily reconstituted for tissue engineering on a patient *in situ* at the point of care.

2. Materials and methods

2.1. Materials

Type I fibrous-bovine collagen (Advanced Biomatrix, USA), PVA 88% and 99% hydrolyzed (Alfa Aesar, USA), Kollicoat IR (PVA-*graft*-PEG) (Sigma Aldrich, Oakville, Can), sodium tetraborate decahydrate (borate) (Sigma Aldrich, Can), glutaraldehyde (25%v/v, Sigma Aldrich, Can), Dulbecco's Modified Essential Medium (10×, Life Technologies, Can), Chondroitin-6-sulfate (GAG) (Sigma Aldrich, Can), Dextran (40 000 Da, Sigma Aldrich, Can), Ascorbic acid (Sigma, Aldrich, Can), Tween20 (Sigma Aldrich, Can), Tween80 (Sigma Aldrich, Can), SDS (Sigma Aldrich, Can), (Live/Dead viability assay kit (Molecular Probes, Invitrogen, Can), Phalloidin-488 Alexa Fluor (Invitrogen, Can).

2.2. Fabrication of collagen-polymer hybrid scaffolds

Type I fibrous-bovine collagen in 1 N HCl was combined with a collagen buffer (10× DMEM, 10× phosphate buffered saline (PBS), 10× HEPES and 1× Antibiotic, pH 7.5) and pH adjusted using 1 N NaOH as depicted in figure 1. Once neutralized Chondroitin-6-sulfate was added to scaffolds (1:6, collagen:GAG). Non-crosslinked controls were combined with either remaining hydrogel reagents, or DMEM (1×). Crosslinked Gels were mixed thoroughly with a high molecular weight dextran (40 000 Da)-glutaraldehyde mixture (0.02%v/v or as reported in table 1) and allowed to incubate on ice in the dark. Incubation times varied as per the concentration and experimental object as outlined in table 1. To optimize the crosslinker effects, gel-mixtures were

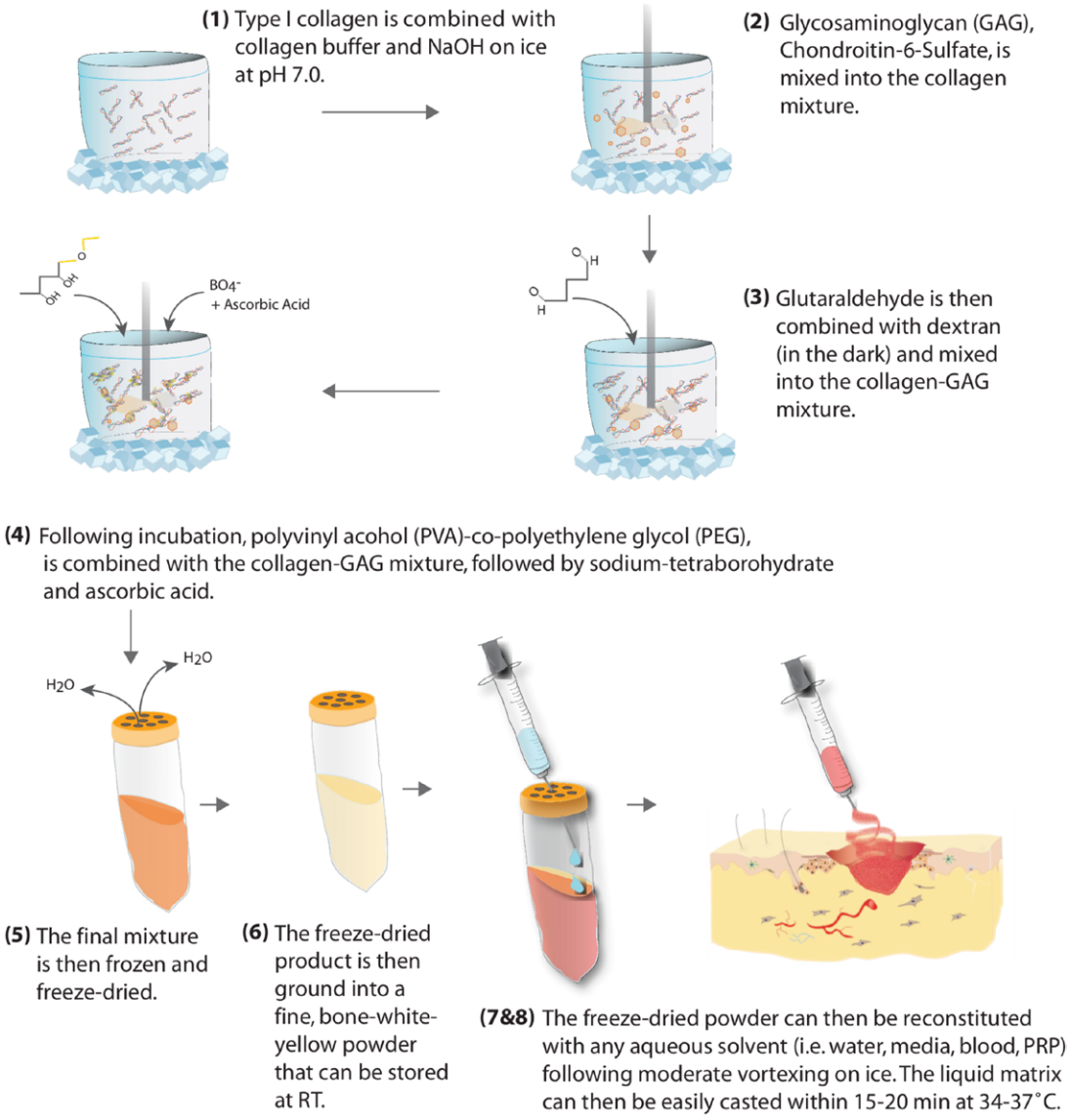


Figure 1. Process-schematic for the fabrication of the reconstitutable, collagen scaffold. Steps 1–8 describe the process by which one can manufacture the scaffold, freeze-dry, then reconstitute using water or other aqueous media and easily apply to a wound bed or cell culture container.

Table 1. Crosslinking conditions for scaffold variants depicted figure 1.

Scaffold	Stock collagen	Glutaraldehyde	Incubation time
Col	9.9 mg ml^{-1}	—	—
C1/45	9.9 mg ml^{-1}	0.013 %w/v	
C1/60	9.9 mg ml^{-1}	0.013 %w/v	
C1/70	9.9 mg ml^{-1}	0.013 %w/v	
C1/90	9.9 mg ml^{-1}	0.013 %w/v	
C2/15	9.9 mg ml^{-1}	0.032 %w/v	
C2/30	9.9 mg ml^{-1}	0.032 %w/v	
C2/60	9.9 mg ml^{-1}	0.032 %w/v	
Figure 3.2.B. 60 min	9.9 mg ml^{-1}	0.02 %w/v (reaction volume corrected to represent stock collagen of 6 mg ml^{-1})	60 min
Figure 3.2.B. 42 min	9.9 mg ml^{-1}	0.02%	42 min
Figure 3.2.B. 21 min	9.9 mg ml^{-1}	0.02%	21 min

exposed to glutaraldehyde concentrations that were either of proportion to a reaction volume (c1) or to the amount of collagen (c2) (figure 2(A)) or proportional to the incubation time (figure 2(B)). Different time periods were selected for each of the two treatment conditions such that exposure would be similar. Table 2

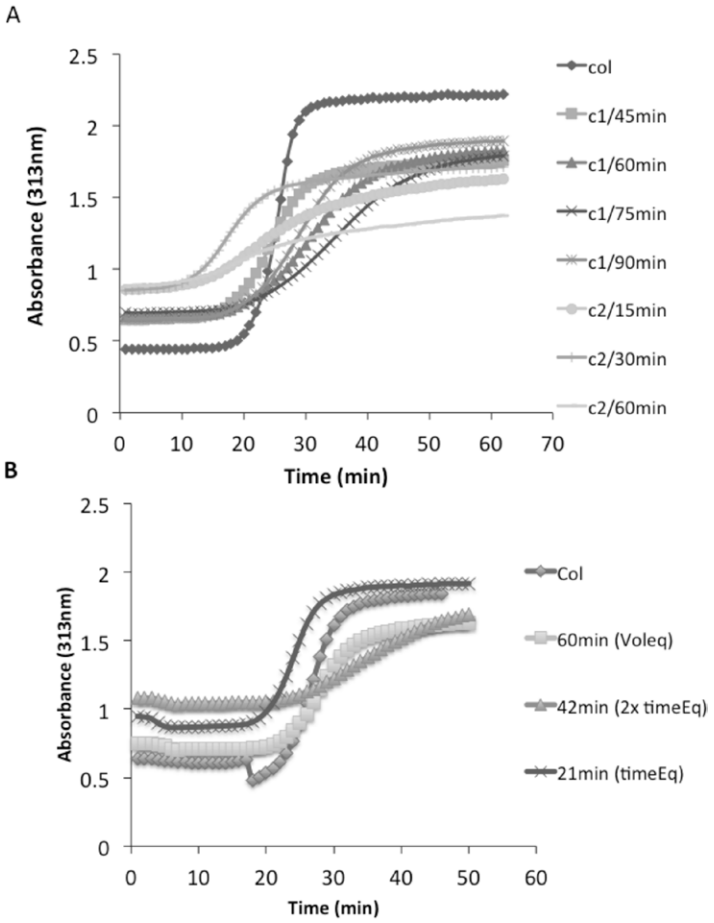


Figure 2. Effect of crosslinker concentration on fibril formation kinetics and turbidity. Collagen solutions (3 mg ml^{-1}) were fabricated either in the absence (Col) or presence of glutaraldehyde crosslinker. Collagen solutions were maintained on ice until being placed in a TECAN® spectrophotometer at 37 °C to measure turbidity as an indicator of fibril formation (gelation) at 313 nm. Panel (A) depicts collagen fibril formation kinetic profiles of three conditions described in table 1. Panel (B) depicts a third condition with collagen alone as a control (col). Here the glutaraldehyde concentration is the same as C1 in panel A (0.002%wt) and the incubation time is proportional to the reaction volume. The '60 min Voleq' solution has a reaction concentration of collagen at 1.5 mg ml^{-1}, whereas the other two solutions contain 3 mg ml^{-1} collagen.

describes the composition of variants used in all other investigations which followed the same crosslinking procedure as found in the variant in table 1 (i.e. 'figure 2(B) −21 min'). Following incubation polymer hybrid gels were combined with respective amounts of PVA or PVA-PEG copolymer and gelling agent (sodium tetraborate decahydrate, 0.04–05%w/v) in a 1:4 ratio of borate molecule to hydroxyl functional group. All gel-mixes were brought to a final volume with 1× DMEM and ascorbic acid (100 uM). Gel-mixes were stored at 4 °C until casted or frozen at −80 °C for lyophilization. Select scaffolds were lyophilized (freeze-dried) for 36 h, then ground into a powder using a mortar and pestle and reconstituted to their original concentration using distilled and deionized water.

2.3. Crosslinker, PVA and copolymer effects on gelation kinetics

Gel-mixes were aliquoted (150 μl/well) into a 96 well plate on ice. Gelation kinetics were captured using a heated plate reader (Tecan, USA) with a 313 nm polarized filter set and Magellan Software™. The plate reader was first heated to the appropriate temperature (30, 32, 34, 37 °C) and measurements were captured at either 1 or 2 min intervals. Three batches (triplicate) of gel variants were used for each analysis ($n = 3$) unless otherwise reported. Turbidity is the best indicator of collagen fibrillogenesis, and more indicative of gelation when combined with translucent hydrogels such as PVA and PEG [1, 7, 10, 15]. The *time to gelation* is represented by the time at half the maximum absorbance at 313 nm. The *lag time* is the time from the start of gelation to the time at half the maximum. The slope of the curve dA/dt indicates the rate fibrillogenesis. Using the Arrhenius equation (1) the activation energy can calculated using first order kinetics of gel variants at different temperatures plotted as ln$k$ versus 1/T, where k is the rate constant, R is the ideal gas number and T is the temperature in Kelvin. The rate constant for a first order reaction can be derived from the gelation time ($t_{1/2}$) using equation (2).

$$\ln k = \ln A^{-Ea/\mathrm{RT}} \quad (1)$$

$$k = \ln[2]/t_{1/2} \quad (2)$$

Using similar principles the change in rate fibrillogenesis (dA/dt) with respect to the polymers

Table 2. Composition of scaffold variants.

Scaffold	Collagen (final)	Glutaraldehyde	Additive
Collagen (Col)	3 mg ml^{-1}	—	—
Crosslinked collagen (xCol)	3 mg ml^{-1}	0.02%w/v	—
(50/50)	3 mg ml^{-1}	0.02%w/v	0.4%w/v of a 48% PVA (99% hydrolyzed)/48% PVA (88% hydrolyzed)/2%w/v glycerol and 0.05%wt borate
K99	3 mg ml^{-1}	0.02%w/v	0.4%w/v of a 48% PVA (99% hydrolyzed)/48% Kollicoat IR®/2%w/v glycerol and 0.05%wt borate
K88	3 mg ml^{-1}	0.02%w/v	0.4%w/v of a 50% PVA (88% hydrolyzed)/48% Kollicoat IR®/2%w/v glycerol and 0.05%wt borate
PVAnb	3 mg ml^{-1}	0.02%w/v	0.4%w/v of a 48% PVA (99% hydrolyzed)/48% PVA (88% hydrolyzed)/2%w/v glycerol
TW80	3 mg ml^{-1}	0.02%w/v	0.015 mM Tween80 [43]
TritonX100	3 mg ml^{-1}	0.02%w/v	0.22mM TritonX100[a]

[a]Sigma Aldrich, MSDS.

can also be determined through the comparison of the slope of the curve in the linear region.

2.4. Mechanical strength

Gels were cast in 5-well rectangular chamber slides (400 μl each) and incubated for 24 h at 37 °C. Tensile testing was done using a KES-G1 Micro-Tensile Tester (Kato Tech, Japan), with a 1 kg load cell. Prior to loading, gels were dried of excess liquid using a KimWipe™ (Kimberly Clark, USA) and weighed. Two pieces of KimWipe™ were then used to firmly secure the gel onto the specimen holder. Gels were then stretched until breaking at a deformation rate of 0.02 cm s^{-1}. Tensile strength (engineering stress) was calculated by dividing breaking load (g) with sample width (mm) and area density (g.sq.m) of the polymerized gels, thereby to obtain the elastic modulus (E). For statistical purposes, three batches of gel were evaluated.

2.5. Scaffold architecture using scanning electron microscopy (SEM)

Collagen scaffolds (100 μl) were cast in 96 well plates for 24 h followed by fixation in 4% Formalin solution for 24 h at 4 °C. After fixation the gels were dehydrated twice for 12 h in 70% ethanol and then frozen at −80 °C prior to lyophilization. SEM samples were first gold coated prior to loading inside the vacuum of a Hitachi S4700 SEM (Hitachi, Japan).

2.6. Contact angle

Gel-mixtures were formulated as described and then cast into thin films on glass slides at 37 °C and then allowed to dry in a laminar flow hood for 24 h. Using a contact angle tool (KSV Instruments), contact angle of a water droplet on the surface of the thin film was calculated using Attention Theta Software V4.1.

2.7. Cell viability

Viability was assessed using Live/Dead toxicity assay. Cells (primary or cell line) were cultured for in scaffolds for 24 h. After 24 h, scaffolds containing cells were washed 3 times with 1× PBS (pH 7.0) and a mixture of ethidium-homodimer and calcein-AM according to manufacture's instructions. After 30 min scaffolds were washed 3 times with 1× PBS and visualized using a Zeiss Axiovert 200 M fluorescence microscope and Axiovision software. Cell counts were obtained using Image J software (NIH, USA).

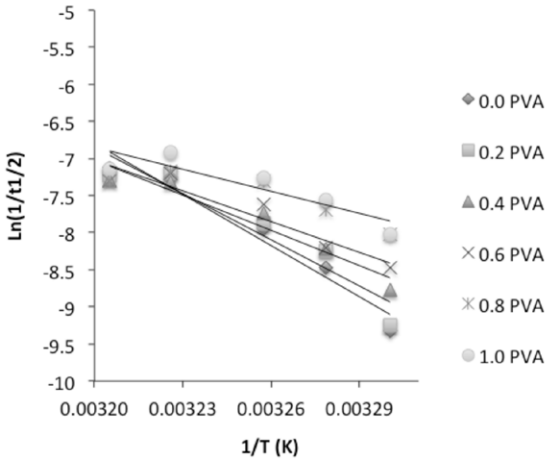
2.8. Cell adhesion and migration

Cell attachment and spreading was evaluated using phalloidin-Alexa-fluor 488 for staining of actin in fibroblasts cultured within collagen scaffolds 24 and 48 h after casting. Briefly, gels were created and combined with cells as per the assessment of cell viability (above). Evaluation of cellular migration consisted of creating 4 mm punch biopsies in the center of the gels and then filling the hole with acellular gel. Images were captured over a 10 d period, and migrating cells were designated as those that crossed into the new gel from the margin of the old. Images were captured using a Zeiss Axiovert 200 M fluorescence microscope and Axiovision software.

2.9. Procollagen and alpha-smooth muscle actin (α-sma)

Fibroblasts and myo-fibroblasts were differentiated using double staining methods that identified pro-collagen and α-sma in cells. Cells expressing both pro-collagen and α-sma are said to have a myofibroblast phenotype, and whether or not the phenotype is permanent or transient, the purpose of this study is to determine if the scaffold variants impart (direct or indirect) any differentiating affect on cultured cells. Scaffold variants (250 μl) comprising 2.5 × 10^4 ml^{-1} human primary fibroblasts (Passages 4–5) were created and topped with 200 μl of complete media (1× DMEM with 10% FBS and 1× antibiotic) as described above. Scaffold media was refreshed every 2 d and scaffolds were maintained for 4 d (exactly 102 h) before fixation to allow for spreading and any initiation of proliferation that could transition to a change in phenotype. On the fifth day, scaffolds were washed 3 times with 1× PBS (pH 7.5) and then fixed, individually, in an excess of

A



B

[PVA]	Ea (J/mol)		R^2	P value
0	2602.86	±424.88	0.93	*
0.2	2524.42	±487.56	0.90	
0.4	2025.82	±240.48	0.96	**
0.6	1504.28	±280.20	0.91	0.00035*/0.00612**
0.8	760.95	±237.50	0.78	<0.0001*/**
1	1276.45	±399.88	0.78	0.00024*/0.001**

Figure 3. Arrhenius plot for collagen fibrillation as a function of PVA-borate hydrogel concentration. Uncrosslinked collagen scaffold variants were prepared as described in the materials and methods, containing 0–1.0%w/v PVA (50%wt 99% hydrolyzed and 50%wt 88% hydrolyzed). Using equation (1) a reduction in the activation energy for collagen fibrillogenesis is observed at 313 nm as the concentration of PVA is increased.

10% Formalin for 1 h, following which the formalin was exchanged for 70% ethanol. Scaffolds were then washed 3 times in 1× TBS-Triton 100 × (0.03%) and blocked for 1 h with blocking buffer (1× TBS with 5% Goat Serum and 2% Serum Albumin). Scaffolds were then incubated with both primary monoclonal antibodies for procollagen (human anti-rabbit; 1:2; hybridoma supernatant) and α-sma (mouse anti-goat; 1:500; Abcam, USA) for 8 h at 4 °C. Following washing of the primary, scaffolds were incubated with corresponding secondary antibody-conjugated-fluorophores (Alexa488 and Alexa568) for 1 h, and afterward washed and left for visualization in 1× TBS. Hoescht 33258 was used as a nuclear stain (Fisher Scientific, Can). Non-primary controls were used to optimize fluorescence. Scaffold images were captured as described above.

2.10. Statistics

The number of repeats represents different batches of gels. Experimental results were evaluated using analysis of variance (ANOVA) with a post-hoc Tukey Test. Error calculations for linear regression represent the mean standard error of the fit for a given R^2 value of triplicate samples. Statistical significance was estimated with an alpha value of 0.05 ($p < 0.05$). Measurements were reported as means ± standard deviation.

Table 3. Effect of PVA-borate gels on activation energy of collagen fibrillation.

(PVA)	E_a (J mol^{-1})		R^2	P value
0	2602.86	±424.88	0.93	[a]
0.2	2524.42	±487.56	0.90	
0.4	2025.82	±240.48	0.96	[b]
0.6	1504.28	±280.20	0.91	0.000 35[a]/0.006 12[b]
0.8	760.95	±237.50	0.78	<0.0001[a]/<0.0001[b]
1	1276.45	±399.88	0.78	0.000 24[a]/0.001[b]

3. Results

3.1. Effect of crosslinker concentration, PVA and time on fibril formation

In order to optimize the formulation of the gel with respect to changes in stock collagen concentration we sought to first investigate the effect of both glutaraldehyde concentration and crosslinking incubation time on the change in fibril formation rate. First, when a change in glutaraldehyde concentration was proportional to a change in stock collagen concentration (where we previously utilized 6 mg ml^{-1} [15]) the crosslinking reaction would result in gel solidification on ice. As such, it was found that increasing the concentration of would actually

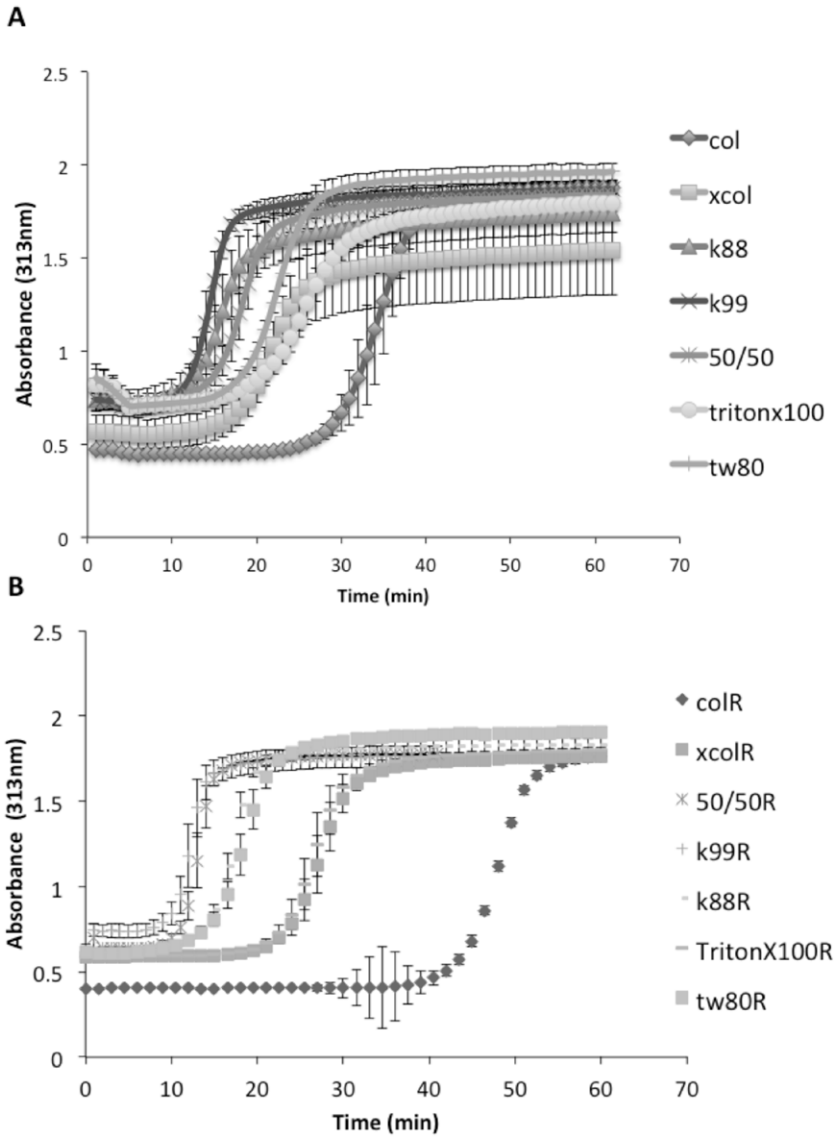


Figure 4. Effect of PVA variants on collagen fibrillogenesis pre- and post lyophilization/reconstitution. Collagen solutions of (3 mg ml^{-1}) alone (col) or crosslinked and combined with hydrogels or surfactants as follows: (xcol) crosslinked without any additives, (50/50) 50% PVA88/ 50% PVA99, (K99) 50% Kollicoat® / 50% PVA99, (K88) 50% Kollicoat®/ 50% PVA88, (TritonX100) TritonX-100, (tw80) Tween 80. Panel (A) depicts the shift in $t_{1/2max}$ for collagen fibrillogenesis with the addition of crosslinker, surfactant and PVA-hydrogel combination. Panel (B) depicts the fibrillogenesis of lyophilized and reconstituted collagen solutions matched to panel (A) and denoting the shifts in $t_{1/2max}$, with the exception of k99, k88 and 50/50.

decrease the gelation rate, rather than increase it as might be predicted (figure 2(A) (C1)). Furthermore higher concentrations of crosslinker resulted in a higher initial absorbance at 313 nm. Interestingly, this pattern continued within the reaction-volume matched samples, which contained less glutaraldehyde crosslinker (C2). Collagen alone, without crosslinker exhibited the lowest initial absorbance and highest final absorbance, together with the fastest fibril formation as shown in figure 2(A). Again $t_{1/2max}$ decreased, slightly, with the duration of incubation within the volume-matched reaction groups (figure 2(A)). It was apparent that a higher glutaraldehyde concentration in the reaction also corresponded to a reduction in $t_{1/2max}$ that was proportional to incubation time. This reduction in gelation time would ultimately correspond to both a reduction in the formulation time and time for gel solidification as a working mixture. Interestingly, when the incubation time was adjusted for the stock concentration of collagen used in the reaction vessel (figure 2(B)) the reaction kinetics could be controlled. This result demonstrated that the optimal final concentration of crosslinker is 0.02%w/v and that crosslinking incubation time should be adjusted in proportion to the stock concentration (reaction vessel concentration) of collagen.

The effect of PVA-hydrogel addition to the gel-mixtures demonstrated an increase in rate of fibril formation when the concentration of PVA is increased from 0 to 1.0%w/v, which corresponded to a significant reduction in activation energy (figure 3). As shown in table 3 the addition of 1%w/v PVA resulted in more than 50% reduction in the required activation energy for fibril formation. The slight increase in gelation rate was also associated with a decrease in overall gelation time, however this change was not significant. Toward the

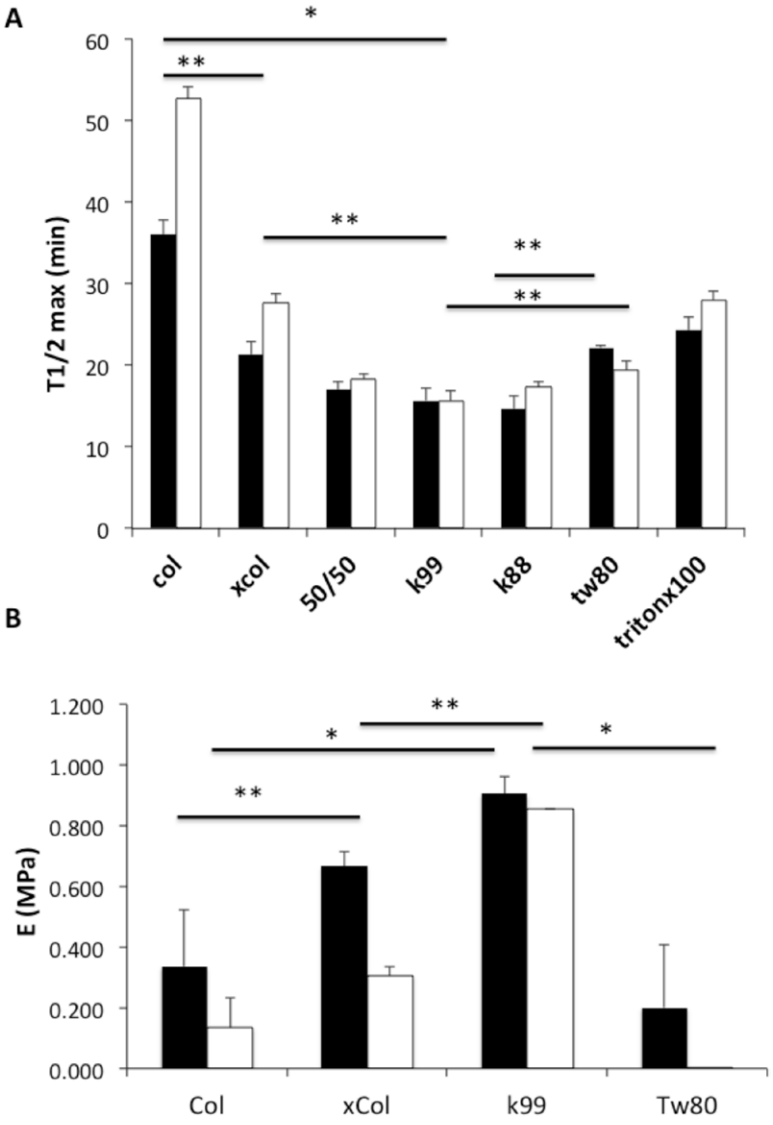


Figure 5. Quantification of collagen solution gelation kinetics and mechanical properties pre- and post-lyophilization (freeze drying). Panel (A) quantification of scaffold gelation time (fibrillation) as indicated by $t_{1/2max}$. Scaffolds were prepared as described in materials and methods. Panel (B) uniaxial mechanical strength (elastic modulus, E) of scaffold variants. Bar grouping with (*) denotes a statistical significance of $p < 0.001$ and (**) $p < 0.05$.

formulation of a scaffold that could cast within a wound bed, or in the working range of 30 °C–37 °C for cell delivery and transplantation, gel-mixtures containing different PVA-hydrogel concentrations were explored. The results demonstrated that the PVA-borate hydrogels could in fact alter the fibril formation kinetics of Type I bovine collagen, in order to permit gelation at 30 °C. Where typical inverted test-tube tests may demonstrate gelation at these lower temperatures, evaluation of turbidity at 313 nm wavelengths remove the possibility of the PVA-hydrogel system to present artifact.

3.2. PVA-hydrogel addition preserves gelation and mechanical properties of gel-mixtures following lyophilization

As an attempt to increase the stability of the gel-mixture (for transport and storage purposes), mixtures were lyophilized, ground into a powder and then reconstituted. Lyophilized mixtures were reconstituted with deionized and distilled water, without the need for pH neutralization. Interestingly, the PVA-hydrogel systems that were created using a PVA-PEG co-polymer (Kollicoat®) were most easily reconstituted. Collagen scaffold variants that were not crosslinked and did not contain PVA exhibited significantly longer gelation times when compared with the matched non-lyophilized scaffold mixture (52.6 min ±1.52 versus 36 min ±1.73) (figures 4 and 5). The crosslinked-only (xcol) scaffolds were able to form fibrils, yet at a significantly longer gelation time than prior to lyophilization. Liquid mixtures of scaffold variants that contained PVA-hydrogels exhibited initial absorbances that were slightly higher than prior to lyophilization, yet formed fibrils within a statistically similar amount of time (figures 4 and 5). The Kollicoat® samples, most notably K99, were among the fastest to

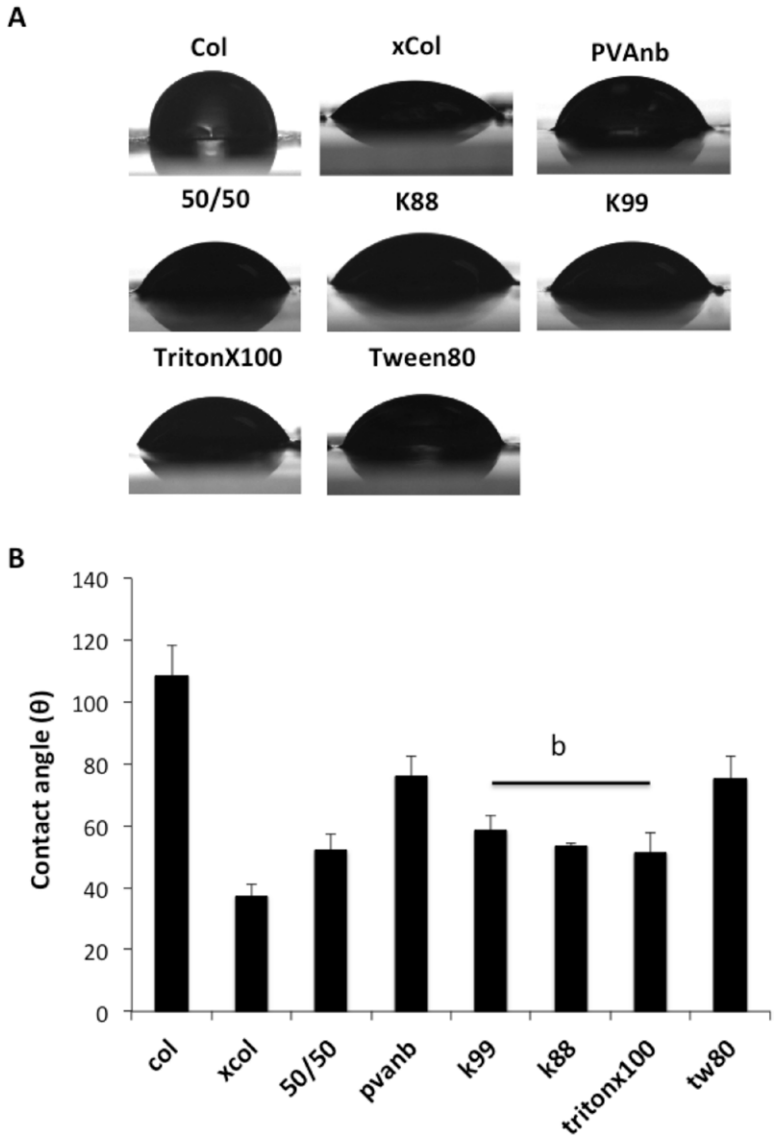


Figure 6. Contact angle measurements of dry gelled collagen films.

gel (15.7 min ± 1.16) and exhibited the smallest change turbidity (from $t = 0$ to t_{max} absorbance) suggesting that that the PVA-PEG must have had a protective effect on the collagen structure in powder form, and again when reconstituted. As shown in figures 4 and 5 PVA-hydrogel scaffolds exhibited a significant reduction in gelation time when compared with col, xcol, Tw80 and Tritonx100 variants. The greatest reduction in gelation time was observed in scaffolds that contained the PVA-PEG hydrogels (2.02 fold). In comparison to previous studies that examined the effect of surfactants on collagen gelation kinetics [10, 19] Tween 20, Tween 80 and Triton-x100 were combined within gel mixtures at their respective central micelle concentration's (CMC). (Note: the effect of Tween 20 was comparable to Tritonx100 and therefore was omitted for figure clarity). As shown in figures 4 and 5 all surfactants increased the gelation rate (lowering $t_{1/2max}$) of both pre- and post-lyophilized samples, but to a significantly lesser extent than a majority of the PVA's.

3.3. PVA-PEG and PVA-hydrogel systems exhibit a surfactant like effect on collagen gel-mixtures

A primary role of surfactants is to improve the hydrophilicity of a hydrophobic surface. The addition of Tween 20, 80 and Triton-X100 to the collagen gel mixtures significantly reduced the aqueous contact angle of a water droplet sitting on top of a casted, thin-film of a gel mixture from 108° (Collagen:GAG) to 51, 75° for Triton X100 and Tween 80 respectively (figure 6). Additionally, there were differences in the contact angle depending upon the type of PVA that was used. Interestingly, when omitting borate (PVAnb) from the system the contact angle increased significantly from (52° to 72°). The crosslinked only gel-mixtures demonstrated the lowest contact angle suggesting the most hydrophilic surface formed by all gel mixtures (37°). PVA99, PVAnb and Tw80 were relatively similar at 75°, 76° and 75° respectively. Whereas Kollicoat® 99 and 88 blends were the next most hydrophilic with contact angles at 58° and 53° respectively. As would be

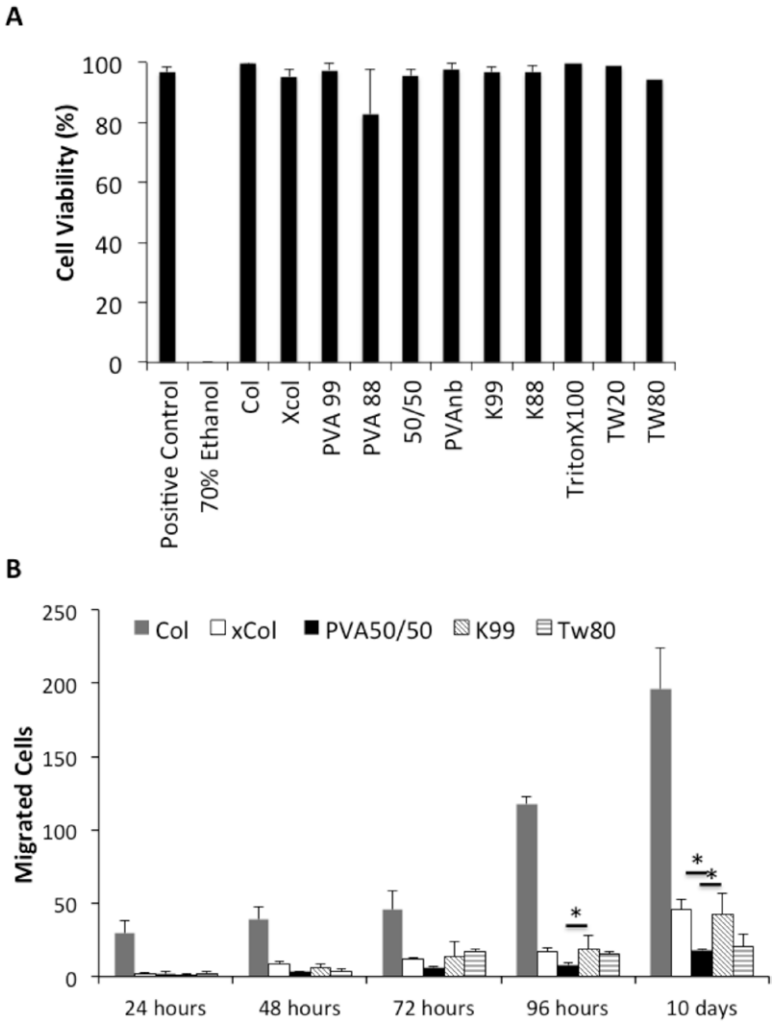


Figure 7. Cell viability and migration in collagen scaffold variants. (A) viability of primary fibroblasts cultured in collagen scaffold variants following 24 h. Live/Dead ratios were utilized to calculate the percent of viable cells, using 70% ethanol as a dead control. (B) 4 mm punches were made in scaffolds and then refilled with an acellular scaffold variant. Cells were counted as they migrated from the old scaffold into the new scaffold over a period of 10 d. Cell numbers represent the total number of cells counted per scaffold variant at the denoted time point.

expected the more hydrophobic PVA's had a greater surfactant-like effect and therefore a greater reduction in contact angle compared to similar molecular weight PVAs.

The effect of additive on surface hydrophilicity/hydrophobicity (surface wettability) was investigated through contact angle calculations. Variants were prepared as described in the materials and methods. Panel (A) photomicrograph images of a single water drop on the collagen scaffold surface. Panel (B) calculated contact angle of scaffold variants. Statistical significance of $p < 0.05$ was found between all treatments, with the exception of K88, K99, TritonX100 as denoted by the bar 'b'. Statistical significance between the 'b' group and all other treatments was observed.

3.4. Cell viability and cell migration

Cells cultured in gel-mixture variants were cultured for 24 h within scaffolds prior to staining. All scaffold variants were found to be non-toxic *in vitro* (figure 7(A)). Similar fibroblast populated collagen lattices were created to evaluate the migration. A punch biopsy was taken from the center of the scaffold and then filled with a matched acellular gel. There was no significant difference in cellular migration among all the scaffolds, with exception of the uncrosslinked, collagen:GAG scaffolds which exhibited the highest rate of cellular migration over a ten day period (figure 7(B)). On day 10, significantly more migrated cells were found in the K99 and xcol variants compared to PVA50/50 (figure 7(B)).

3.5. Fibroblast morphology and scaffold architecture

Figure 8 depicts the morphology of fibroblasts cultured within five different scaffold variants. As found in our earlier work, cells cultured within an uncrosslinked collagen scaffold exhibit extensive spreading of lamellopodia and filopodia extensions, which is evident in panel (A) of stress fiber formation (filamentous actin) with phalloidin-488 stain [20, 21]. Notable parallel fiber arrangement and reduced dendritic appearance, consistent with fewer filopodia, was observed fibroblasts cultured within a scaffold

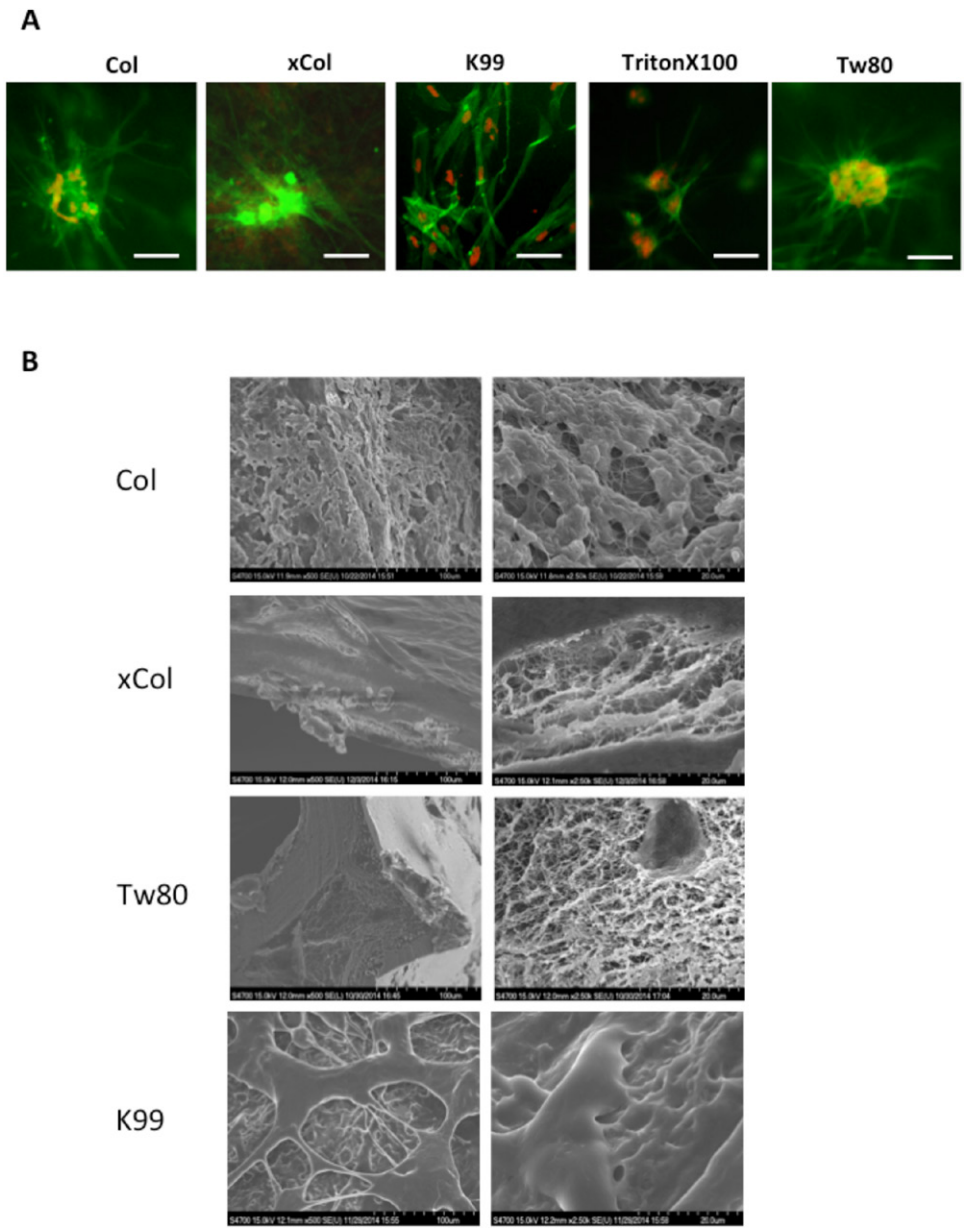


Figure 8. Fibroblast morphology and scaffold architecture in collagen variants. Panel (A) depicts F-actin staining (green) of primary fibroblasts cultured in thin 100 μm scaffold variants. Nuclear stain (red) and scale bar (white) 100 μm. (B) Scanning electron micrographs of freeze-dried collagen scaffold variants: (col) collagen, (xcol) crosslinked collagen scaffold without additives, (Tw80) crosslinked collagen scaffold with tween 80 additive, and (K99) crosslinked collagen scaffold with kollicoat/PVA99 additive. Left panel scale bar: 100 μm and right panel scale bar: 20 μm).

variant that contained a 50%wt Kollicoat®/ 50%wt PVA99 hydrogel (0.4%wt). Surfactant containing scaffolds exhibited a dendritic-like appearance with narrow filopodia-like extensions, and smaller cell bodies that are suggestive of cell stress and possible poor adhesion (figure 8, Panel A) even though the cells remain viable (figure 7(A)). Supplementary figure S1 (stacks.iop.org/BMM/11/035013/mmedia) depicts that the fibroblast phenotype is generally conserved when cultured in the scaffolds. It is well documented that extracellular matrix type and mechanics, *in vitro* culture surfaces included, can prompt trans-differentiation of fibroblasts toward a myo-fibroblast phenotype exhibiting production of both collagen and alpha smooth muscle actin (α-sma) [22]. Without conclusive markers, apart from morphology, pro-collagen expression is a consistent defining feature of fibroblasts, however under normal conditions (non-repair/inflammatory) collagen expression should be minimal. Wound healing environments and inflammation can prompt fibroblast (and perhaps other precursor cells) to exhibit a contractile phenotype, defined as a myo-fibroblast, which is found in high-abundance in scar tissue. Notably, figure S1 indicate that PVA-PEG scaffolds (K99; containing PVA-PEG hydrogels) differ from all other scaffold variants and suggestive of a lower abundance of both pro-collagen and α-sma positive cells. A more quiescent fibroblast phenotype is consistent with two of our other previous works whereby there was a reduction in cell proliferation, gel contraction and cellularity (*in vivo*) [15, 23]. Further investigation on the molecular

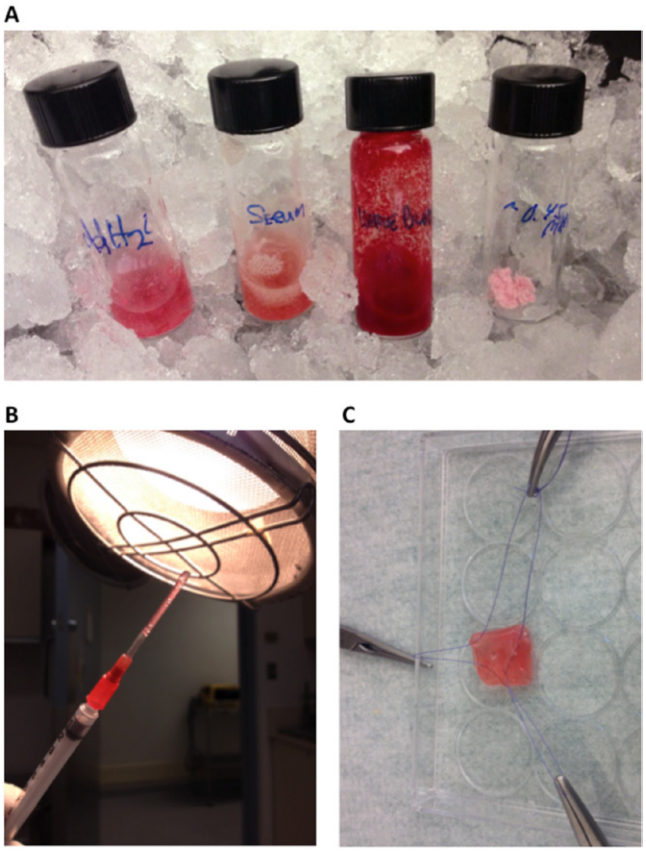


Figure 9. Utility of lyophilized (freeze-dried) collagen scaffold. (A) Photomicrograph of K99 scaffolds reconstituted (left to right) with distilled and deionized water, serum, whole blood, and non-reconstituted powder. (B) Reconstituted K99 scaffold contained within a 16 G BD® IV catheter. Panel (C) depicts the ability to suture a gelled, reconstituted K99 scaffold.

level is warranted, but beyond the scope of this study, to elucidate what cell-material interactions are at play. Scanning electron microscope cross-sectional images (Panel B) recapitulated our findings in the previous work, whereby larger, more irregular pores between collagen fibers are evident in the non-crosslinked scaffold (col) compared to other crosslinked scaffold variants. Interestingly surfactant treatment of collagen scaffolds with Tween80 (Tw80) resulted in a dense network of collagen fibers, with a smaller pore and a more tortuous void-path through the scaffold than any of the other scaffolds. The K99 scaffold demonstrated a similar film like appearance to what we have observed previously, where by the thin surface coatings of the fibers are evident by the lack of electron-beam penetration at high (2500×) magnification.

4. Discussion

Scaffold fabrication is core facet of tissue engineering. Modalities moving toward cell transplantation have prompted the demand for soft-materials that can conform to surrounding tissues, and exhibit tissue specific mechanical properties (strength, viscosity, elasticity, etc) and physical characteristics (pore size, surface chemistry, gel transition temperature) [15, 17, 24–27]. Synthetic biocompatible polymers or modified biomaterials are often chosen for the creation of injectable scaffolds and hydrogels. Although type I collagen can also be used as an injectable material, it's gelation temperature (and time) retard its utility as an *in situ* forming scaffold. To circumvent this issue, it can be chemically modified through crosslinking in order to create a more viscous material that would also avoid rapid degradation because of the chemical crosslinks. Excellagen® and Integra® Flowable are two currently marketed injectable products for use as a dermal filler and wound healing modality respectively [28–30]. Both Excellagen and Integra® Flowable are new to the market, yet as soft materials are unable to form intact solid scaffolds (gels) *in situ*. Furthermore, most collagen scaffold preparation for tissue engineering research and clinical, first requires neutralization of a collagen solution prior to use. The utility of a reconstitutable collagen scaffold, as opposed to a gel-slurry, is that aside from forming *in situ* it would also have similar mechanical properties to preformed solid scaffolds, such as holding sutures or serving as an interface between an implant and tissue. Examples of clinical utility are shown in figure 9.

As shown in figure 3 the addition of PVA to a crosslinked collagen:GAG gel-mixture significantly reduced the activation required to form fibrils and an intact scaffold, without actually modifying the collagen itself. PVA-PEG (Kollicoat®) is a biocompatible synthetic biopolymer that is an accepted pharmaceutical

component under most jurisdictions worldwide. In the paint and plastics industry, PVA is added as a surfactant and in the presence of borates (tackifying agent) the polymer chains can be ionically crosslinked, facilitating extensive hydrogen bonding [15, 31–33]. Freeze-thaw cycling of the polymer, without crosslinker has been shown previously to produce robust cryogels [34]. *In vitro*, biocompatibility data (figure 7) demonstrated that none of the PVA variants examined were toxic to cells. Previous reports describe the lack of cell adhesion to PVA coated surfaces, and the differences in cell morphology among the variants may be due to the coatings that PVA make on the collagen fibers and fibrils. Although, higher concentrations may prove to be toxic they are outside the workable range, as at concentrations exceeding 1.0%w/v the gel mixtures become too viscous to ensure homogeneity. Previous reports have demonstrated that surfactants could alter the kinetics of collagen gel formation [10, 19]. Further other previous reports, such as Yunoki *et al*, have demonstrated the utility of pre-crosslinking collagen using agents such as genipin, achieving reductions in gelation temperature but with time points in excess of 30 min [35]. On the other hand, it was postulated in the surfactant studies that the enhancement of fibrillogenesis was due to the surfactant de-stabilization of the collagen which is advantageous over pre-crosslinking methods that are limited by the extent of crosslinking due to premature gelation. The observed effects of the surfactants are similar to what was achieved using our PVA variants, and as such we further investigated the incorporation of biocompatible nonionic surfactants Tween 20, Tween 80 and Triton-x100 at their CMC. Interestingly, the surfactants did reduce the overall time to gelation without compromising cell viability, but had no overall improvement in gelation rate, mechanical strength and change in activation energy. Of the PVA variants, the best gel-mixture consisted of a combination (volume) of 48% PVA 99 and 48% Kollicoat® and 2% glycerol. The second best variant overall was the Kollicoat® mixture containing PVA 88 over PVA 99. As expected the surfactants and more hydrophobic PVA's provided the greatest improvement in surface wettability, with the exception of the crosslinked only gel-mixtures, which were unexpectedly the most hydrophilic figure 5. The improved wettability of the scaffold surfaces compared to the uncrosslinked collagen scaffold could, in part, be related to the observed improvements in cellular morphology and migration [36–40]. Furthermore the changes in surface wettability are indicative of the coatings that the polymer variants are forming on the collagen scaffold architecture. As with pore sizes and mechanical stiffness, hydrophilic and hydrophobic effects have been shown to influence cell physiology *in vitro*. Optimization of the surface characteristics has been shown to reduce protein deposition and aggregation and alter cell attachment and expression of adhesion molecules. Given the amphipathic nature of PVA and to a greater extent, PVA-PEG, it is possible that the surface modification of the collagen scaffold provides adequate adhesion sites, as is achieved *in vivo* through proteoglycan interactions with collagens. For example heparin sulfates and other glycosaminoglycans have also been known to change the surface of collagen scaffolds, but unlike PVA they are also implicated in signaling cascades through receptor-ligand interactions [6, 41, 42].

5. Conclusion

Type I collagen is one of the most widely used biomaterials in tissue engineering but its use has primarily been limited to the fabrication of pre-formed solid materials and weak injectable gels rather than used as a material that could form solid structures *in vivo*. Herein we investigated the use of polymer variants of PVA that have surfactant-like effects and have shown that collagen could be destabilized in order to form a gel with reduced activation energy while maintaining its mechanical properties. Taking our approach one step further we were able to demonstrate that PVA could stabilize lyophilized collagen gel-mixtures (in powder form) in order to improve the handling and storage. With great advancements being made in bioprinting, tissue-bioreactors, and cell transplantation there is a large unmet need to *in situ* forming scaffolds of biological origin. As collagen is the most abundant protein in the body, by weight, we are hopeful that the use of our hybrid (PVA-PEG)-collagen blends could offer a simple, cost effective solution for tissue repair and regeneration.

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