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PRODUCTION OF HIGHLY-ALIGNED COLLAGEN LAMELLAE BY COMBINING SHEAR FORCE AND THIN-FILM CONFINEMENT

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Abstract

Load-bearing tissues owe their mechanical strength to their highly-anisotropic collagenous structure. To date, attempts to engineer mechanically strong connective tissue have failed mainly due to the lack of the ability to reproduce native collagen organization in constructs synthesized by cultured cells *in vitro*. The ability to influence the direction of the self-assembling collagen molecules and produce highly anisotropic structures has applications ranging from *de novo* engineering of complex tissues to the production of organized scaffolds for cell culture contact guidance. In this investigation we have used the simple technique of spin coating to produce highly-aligned arrays of collagen fibrils. By a simple modification of the method we have also successfully produced orthogonal collagen lamellae. Alternating collagen lamellae are frequently seen in load-bearing tissues such as cornea, annulus fibrosus, and cortical bone. Culturing of corneal fibroblasts onto aligned collagen shows that the cells adopt the organization of the fibrils. In this investigation, we observed the reversal of fibrillar growth direction or “hook” formation similar to those seen previously in a microfluidic shear-flow chamber. Although the results of this investigation clearly show that it is possible to produce small areas (O) 1 cm² of collagen fibrils with enough alignment to guide fibroblasts, there is evidence that thin film instabilities are likely to be a significant barrier to producing organized collagen fibrils over larger areas. Successful application of this method to produce highly-controlled and organized collagenous structures will require the development of techniques to control thin film instability and will be the subject of the future work.

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Keywords

Collagen; Corneal Tissue Engineering; Extracellular Matrix; Shear Stress

INTRODUCTION

Corneal disease is a major cause of blindness, afflicting millions of individuals globally [1]. In the United States, more than 40,000 corneal transplant surgeries were performed to restore vision in 2008 alone [2]. Although improvements in transplantation methodology have resulted in a high success rate (~ 80%) [3], the increased number of refractive correction procedures, such as Laser Assisted in-Situ Keratomileusis (LASIK) and Photorefractive Keratectomy (PRK), which disqualify corneas for use in transplantation, threatens to reduce the availability of donor tissue [4]. In order to overcome the increasing deficit of donor tissue, as well as potential problems with immune rejection, corneal tissue engineering has attracted increased interest during the last few decades.

Artificial skin produced by tissue engineering methods has recently met with clinical success [5], but attempts to engineer tensile load-bearing tissues have struggled due to the complexity of manufacturing biomimetic structures comprising aligned collagen. Because it is a relatively thin acellular and avascular tissue, the cornea is an excellent candidate for a tissue engineering approach [6]. Recent efforts have successfully combined the three corneal cell lines (epithelium, endothelium and keratocytes) with a stromal scaffold [7-8]. However, in focusing on the constituent cells with little regard for the intricate arrangement and composition of the collagenous matrix of the stroma, a functional engineered tissue which can fully replace the stroma has not yet been produced.

To function at the time of implantation, an engineered cornea must protect the fragile intraocular contents of the eye, transmit light with little scattering, and form an optically refractive hemisphere. These functions are critically dependent on the highly aligned and orthogonal stacks of heterotypic type I/V collagen fibrils that comprise the collagenous matrix of the stroma [9-12]. To achieve its remarkable optical properties collagen fibrils are monodisperse in diameter (32.2 ± 1 nm in human [13]) and uniformly spaced (61.9 ± 4.5 nm center-to-center spacing in human [14]).

The challenge for the bioengineer in producing a successful biomimetic replacement cornea begins with reproducing the microstructure of the stromal lamellae. Electro-spinning [15], strong magnetic fields [16-18], microfluidic channel flow [19-20], combined fluid flow and magnetic fields [21], dip-pen nanolithography [22], and freezing and thawing [23] have been used to control the self-assembly of collagen. These processes produce relatively aligned arrays of collagen fibrils, but none are capable of generating a primary collagen matrix similar to the highly aligned, ordered structure of the native cornea. In addition, most of these techniques – with the exception of a recent investigation using a strong magnetic field [24] – are unable to produce alternating layers of fibril alignment as exists in the corneal stroma.

Previously, we have shown that hydrodynamic shear stress is capable of producing relatively aligned arrays of collagen fibrils in a microfluidic device [25]. In that study, “hooked” fibrils formed against the flow field due to surface interactions that competed with shear-induced collagen self-assembly. Increasing the flow rate did not increase fiber alignment, and resulted in the formation of very short and curly collagen fibrils. Weissenberg number (Wi) calculations revealed that even at high shear rates (i.e. thinnest chambers) the flow in this system was not strong enough to influence the organization of the individual collagen molecules but could influence the direction of larger aggregates (e.g. dimers and trimers)

which spontaneously self-assemble under physiological conditions [25]. Thus the shear-flow technique is not suitable for assembling collagen layers with uniform fibril alignment mimetic of the cornea.

Spin-coating is a method characterized by very thin flowing films which are capable of generating both a confinement effect while also producing high shear rates. Spin-coating flows have been extensively used over the last 50 years in applications ranging from integrated circuit manufacturing [26] to studies of thin films [27]. Confinement provides an aligning force parallel to the spinning plate induced by the close proximity of the substrate surface and the free surface. These boundaries reduce the ability of flowing monomers to sample the entire three-dimensional space around them. The influence of confinement is especially strong in the thin films generated in spin-coating flows, which can approach thicknesses on the order of the length of a collagen fibril (~0.5 microns). An additional advantage to this technique is that it provides a means to produce orthogonal layers of fibril alignment [28]. In this study, we investigated whether the spin-coating method could be used to improve alignment of collagen fibrils and permit the production of alternating fibril layers which mimic the organization of collagen in the cornea.

MATERIALS AND METHODS

Experimental Apparatus

Experiments were carried out in a modified, environmentally controlled (37°C and 100% RH) spin-coater (VVS-400A-6NPP/LITE; Laurell Technologies, North Wales, PA). In order to control the temperature and humidity of the spin-coating chamber during the experiments, the device was equipped with an in-house designed heat exchanger which was controlled with a PID controlled temperature controller (CNi 32200 with accuracy of $\pm 0.5^\circ\text{C}$; OMEGA Engineering, Inc, Stamford, Connecticut).

A cold, neutralized solution of pepsin extracted collagen type I (Advanced BioMatrix, Inc., San Diego, CA) was dispensed on a rotating disc placed in the chamber. The collagen solution was neutralized by mixing with 1X PBS and 0.1M NaOH in a ratio of 8:1:1, respectively. The final concentration of collagen solution was 2.6 mg/ml). An in-line PID controlled heater/cooler was used to keep the collagen solution cold prior to dispensing on the glass (50mm diameter #1 coverslip; ProSciTech, Queensland, Australia). Following contact with the glass surface collagen molecules will spontaneously self-assemble into fibrils. Spinning rates of 750, 1000, and 3000 rpm and flow rates of 0.3, 0.5 and 1 ml/min were the experimental parameters chosen for this study which, based on the Eq. 1 and 2, result in the shear rates and thicknesses reported in Table 1. The collagen flow rate and the spinning velocity were controlled using a PHD-2000 syringe pump (Harvard Apparatus, Holliston, MA) and the software provided with the spin-coater, respectively.

To produce a single layer of radially aligned fibrils, collagen solution was injected onto the center of a 50mm diameter coverslip (#1, ProSciTech, Thuringowa, Australia) placed at the center of a rotating disc (Fig. 1A). The coverslips were ultrasonically cleaned in a solution of Micro90 and were rinsed with deionized water. To produce orthogonal sheets of fibrils, the coverslip was placed with an offset from the spinner's center of rotation (Fig. 1B-I). After coating a single layer, the coverslip was removed, dried quickly and rotated 90 degrees and another layer of collagen was deposited (Fig. 1B-II). This process was repeated to produce several orthogonal layers of collagen fibrils (Fig. 1B) [28]. It should be mentioned that because the collagen fibrils in previous layers were dried onto the glass before the addition of new neutralized collagen solution, they are expected to remain insoluble (i.e. fibrillar) and unaffected by the new deposition.

Thin Film Stabilization

Since the shear rate (and consequently the collagen alignment) is influenced by the film thickness (please see below) maintaining a stabilized and uniform film is critical for the results of this study. To investigate the stability of the thin film we attempted to use a high speed camera to digitally image the dynamics of the flow profile during the experiments (Prosilica, model CV640; black and white; frame rate, 120 fps; resolution, $9.9 \times 9.9 \mu\text{m}$ per pixel; Newburyport, MA). However, due to the environmental housing encasing the spinning disk and difficulties with condensation, it was difficult to definitively observe the film due to its extremely thin nature. We have provided images taken under optimal optical conditions and at lower speeds which show the type of dewetting we believe occurred in some of our experiments (see supplementary figure). In addition a heavy stainless steel coverslip holder was used to reduce the potential vibrations of the spinning head.

Differential Interference Contrast (DIC) Microscopy

The long range organization of the collagen fibrils was investigated using DIC microscopy [29]. An inverted microscope (TE2000U; Nikon) equipped with a DIC objective (60x-1.4NA; Nikon) was used for the light microscopy imaging. To prevent salt precipitation after collagen was dispensed, the coverslips were rinsed with deionized water by running water over the spinning glass through the dispensing needle. A minimum of four images 20 mm from the coverslip center and 90° apart were captured to investigate the uniformity of collagen organization across the coverslip (Fig. 1C). These locations were chosen based on the theoretical calculations which indicated that the film thickness remains almost constant (it only changes for 0.1%) from this location to the edge of the glass (i.e. 25 mm). In addition, the use of an oil immersion objective precluded imaging locations closer than 3mm to the edge of the glass.

Scanning Electron Microscopy (SEM)

SEM was used to investigate the ultra-structure of the collagen fibrils. Following DIC imaging, the specimens were coated with platinum-palladium (80-20%) by thermal evaporation in a Denton DV 502 vacuum evaporator (Denton Vacuum Inc., Cherry Hill, NJ) and examined with a Hitachi S4800 SEM.

Quantification of Fibrillar Alignment

Fibrillar orientation and alignment in DIC images was assessed with a Fourier transform method described elsewhere [25, 30]. Briefly, images were imported into Matlab, a square region (1040×1040 pixels) from the center of the image was extracted, and fibril edges in the DIC images were identified with a Canny edge detector to produce a binary edge-segmented image. The edge-segmented image was filtered with a Welch window, and transformed into the Fourier domain. The power spectrum was band pass filtered with cutoff frequencies of 16 and 260 (as used in [30]). The remaining annular region was converted to polar coordinates and divided into 1° intervals to obtain number-averaged-line intensities, which were then rotated 90° degrees to give the fibril orientation distribution, $I(\theta)$, in the spatial domain. To assess the strength of alignment in the images, $I(\theta)$, was converted to a second rank orientation tensor (see [30]) and the eigenvalues and eigenvectors were extracted to give the magnitudes and principal directions of orientation, respectively. To measure alignment strength of the distribution we calculated the anisotropy index as $\alpha = 1 - \lambda_1/\lambda_2$, where λ_1 and λ_2 are the minor and major eigenvalues, respectively. As a result, a completely aligned network has $\alpha = 1$ and an isotropic network has $\alpha = 0$.

RESULTS

DIC Imaging

Spin-coated collagen monomers self-assembled into locally aligned fibrils in a manner dependent on shear rate and fluid film thickness (Fig. 2, Fig. 3.). Fibril organization was influenced strongly by the shear rate, and fibril density increased with flow rate. The collagen fibrils were best organized at 1000 rpm rotational speed which corresponds to the shear rates (τ) ranging from ~ 400 – 600 1/S and the anisotropy indices reaching as high as 0.85. At 750 rpm ($\tau < 400$ 1/S), although there was a local organization discernable at high magnifications, fibrils did not show any general organization across the image (Fig. 3A and B). In addition, clumps of collagen fibrils were frequently observed on the surface. The rotation speeds of more than 1000 rpm ($\tau > 1000$ 1/S) resulted in the self-assembly of the collagen molecules into aggregates (Fig. 3C and D) which appeared in some areas of the sample in the form of a dense coating with no apparent fibrillar structures (Fig. 3C), or orientation (Fig. 3D). Furthermore, at high spinning rates no collagen self-assembly or aggregation was seen over large areas of the coverslips which could be due to the excessive flow rates and shear rates at these conditions which reduces the residence time of the molecules in the fluid film and sweeps away larger fibrillar structures (please see below).

DIC imaging at fixed radius across the coverslip also revealed that collagen self-assembly was not homogenous (Fig. 2, 4 and 5). While in some areas fibrils were completely aligned in the radial direction (Fig. 2 A1, B1, and C1), fibrils in other regions appeared sparse and less organized (Fig. 2 A2, B2, and C2). Figure 5 clearly demonstrates the boundary between an area of organized collagen fibrils and an area with a negligible coating of disorganized collagen fibrils.

SEM Imaging

The SEM images of the collagen fibrils confirmed the observations previously made by DIC in which the spin-coating method produces locally aligned collagen fibrils (Fig. 6A and B). In addition, the SEM imaging of the collagen fibrils produced using the offset arrangement revealed the possibility of the production of the orthogonal layers of collagen fibrils. Figure 6C and D represent two orthogonally aligned layers of collagen fibrils produced using the offset method.

DISCUSSION

Previously we have shown that using microfluidic channels, it is possible to generate relatively aligned layers of collagen fibrils [25]. The method is not however capable of producing highly-aligned arrays of fibrils. In the previous work, we used scaling arguments to show that the flexibility of the collagen monomers and the associated rotational Brownian motion could not be strongly influenced by the shearing flow close to the substrate surface. Thus, the alignment of polymerizing fibrils could not be fully controlled by the flow and fibril formation exhibited upstream growth instabilities (termed “hooks”), which were more prevalent at very high shear rates [25]. In this investigation we tested the effect of adding confinement to thin shearing films on the alignment of collagen molecules self-assembled under shearing stress. We use scaling laws of statistical physics to gain a better understanding of the detailed dynamics of collagen self-assembly under these conditions.

In its simplest form, the spin-coating fluid is undergoing quasi-solid body rotation on the disk and the only velocity components are rotational (small) and radial (large). The fluid behavior is therefore controlled by the balance between the centrifugal and viscous forces [31]. For the situation where there is a steady flow of fluid being deposited onto a spinning disk the solution requires solution of the full Navier-Stokes equations. However, Leneweit et

al. [32] outlines work by Rauscher et al. [33] which generates a simple solution to the steady flow problem. To first order, the solutions are:

$$h = \left(\frac{3}{2\pi} \frac{Q\nu}{\omega^2 r^2} \right)^{1/3} \quad (1)$$

$$u = \frac{r\omega^2 h^2}{\nu} \left(\frac{z}{h} - \frac{1}{2} \left[\frac{z}{h} \right]^2 \right) \quad (2)$$

where u is the radial velocity as a function of z (height) at a position r radially, h is the thickness of the film, ω the rotation rate of the disk, ν is the kinematic viscosity of the solution, and Q the volume flow rate [34]. The shear rate is therefore calculated as:

$$\dot{\gamma} = \frac{r\omega^2}{\nu} \left(1 - \frac{z}{h} \right) \quad (3)$$

The above solutions therefore allow the layer profile, velocity gradient, and shear rate to be calculated for relevant flow conditions. Figure 7 presents the representative data for flow conditions for the 1000 rpm spinning rate and the flow rates used in this investigation. The film thickness only decreases 10% from $r = 2.0$ cm to $r = 2.5$ cm. Thus, with the very simple procedure of spin-coating, a thin, virtually flat layer can be generated that will confine the growing collagen film and provide a wide range of shear rates near the substrate surface.

Since both hydrodynamic shear stress and physical confinement strongly influence how collagen molecules organize into fibrils, we examine the effect of these parameters on the assembly process.

Hydrodynamic Shear Stress

The shear stress is an aligning force in the flow direction that competes with the molecule's Brownian motion. The dimensionless Weissenberg number (Wi) is used in polymer physics as a measure of the balance of shear rate and relaxation time for polymer chain in a flow field. It is given by:

$$Wi = \dot{\gamma} \varepsilon \quad (4)$$

where ε is the longest relaxation time of the polymer.

At small Wi numbers ($Wi \ll 1$), a molecule relaxation dominates the deformation/extension forces caused by the flow. When $Wi \sim 1$, the molecule is not able to relax and can be deformed significantly. Therefore, at $Wi > 1$ the flow is strong enough to deform and stretch the collagen molecules. To calculate the Wi we used the equation developed for the longest relaxation time from the Rouse model [35-36]:

$$\varepsilon = \frac{2\mu L^2 \ell_p}{3\pi K_B T \ln(L/d)} \quad (5)$$

where ℓ_p is the persistence length, K_B is the Boltzmann constant, T is the temperature, and L and d are the molecule length and diameter, respectively. The detailed mathematical derivation of Weissenberg number (Wi) and relaxation time are presented in the supplementary information.

The calculated Wi for the experimental parameters used in this investigation are presented in Table 1. Based on these data, Wi only exceeds one for shear rates above 1300 1/S (which corresponds to the spinning rates of more than 3000 rpm). This indicates that for all the shear rates below this value – including 400-600, which resulted in the highest alignment of the fibrils – the flow is not strong enough to influence the orientation of a *single* molecule and therefore no fibrillar alignment should be expected. However, the fact that we observed organization indicates that collagen molecules undergoes self-assembly via larger aggregates (dimers and trimers) which dramatically enhances the effects of both shear rate and confinement (please see below).

Hydrodynamic Confinement

Confinement of the molecules and aggregates between a solid surface and free surface restricts their motion to a plane where the influence of the flow is high. The effect of the confinement on the alignment of the semiflexible molecules (such as collagen) is particularly significant if the molecules are tethered. Therefore, confining the molecules in a tight vertical space close to the substrate surface should reduce the volume available to assembling moieties and therefore, increase the chance of assembled fibril alignment in the direction of the flow. Bakajin et. al. showed that [37] confinement results in the enhanced alignment of DNA molecules in the hydrodynamic flow. Figure 8 is a schematic of the two possible scenarios available to a tethered collagen molecule or aggregate in a spin coating flow. In the first case, the half film thickness above the tethered molecules is larger than the radius of gyration of a molecule (see below). In this case, the free end of the molecule freely diffuses in a volume of a hemisphere with a radius equal to the contour length of a collagen molecule ($L=300$ nm). Therefore, the molecular confinement is not directly influencing the alignment of the molecule. In the second case, the half film thickness is less than the radius of gyration. Therefore, the fluid free surface limits the volume available to the free end of the tethered molecule to a quarter of a sphere with a radius equal to the film thickness. In this condition, the fluid free surface strongly influences the alignment of the molecules and the resulting fibrils.

Previous investigations showed that in order for the confinement to be effective on the conformation of the molecules, the thickness of the thin film should be on the order of $2R_g$, where R_g is the radius of gyration of a collagen molecule. R_g for a semi flexible chain is scaled as [38]:

$$R_g \cong dN^\theta \quad (6)$$

where, d is the monomer size (diameter), N is the polymerization index (i.e. number of monomers in a polymer) and θ is the Flory's coefficient which is 3/5 for a good solvent. In the case of a collagen molecule, d is the persistence length of a monomer (ℓ_p) and N is the ratio of the contour length to the persistence length (i.e. flexibility index). Inserting these numbers ($\ell_p=15$ nm and $N=20$) into Eq. 6, the calculated radius of gyration for a collagen molecule is 90 nm which is in good agreement with the R_g determined from the dynamic light scattering of a lathyrin collagen (74.5 ± 2.0 nm [39]). Therefore, R_g of collagen molecules is significantly smaller than the film thicknesses produced with spin-coating, which indicates that confinement will have negligible effect on the organization of the collagen fibrils.

Therefore, for the flow rates below 2000 rpm, neither the flow nor the confinement are strong enough to influence the alignment of the collagen fibrils. However, as both the DIC and SEM images show, the spin-coated collagen molecules self-assembled into organized array of collagen fibrils. Since these experiments are performed under conditions conducive to polymerization, the collagen molecules are prone to spontaneously self-assembly. Self-

assembly of the molecules into the larger aggregates (e.g. dimers, trimers, and etc.) increases the length and diameter of the chains, which consequently increases significantly the effects of the flow and confinement. As an example, depending the condition of the spin coating (i.e. flow rate and spinning velocity) the Wi for a collagen dimer with the length of 570nm [40] could be as much as one order of magnitude larger than that for a single monomer (Table 2). Similarly, the radius of gyration for a collagen dimer is considerably larger than the monomer, which would makes the effect of the confinement more significant (Table 2). As an example, a collagen dimer has a diameter of 2nm and length of 570nm [40] which using the Eq. 6 would result in R_g of 133nm which is almost 50% larger than that of a collagen monomer. Therefore, spontaneous self-assembly of the monomers in the solution must play an important role in organizing the collagen molecules in the spin-coating process and generating aligned collagen fibrils.

Interestingly, collagen fibrils formed using the spin-coating method exhibited some of the features previously observed in the collagen fibrils self-assembled in microfluidic chambers. In this study, fibrils also grew against the flow field (“hooks”, Fig. 2A and B), which suggests that this feature is an inherent property of shear-influenced collagen self-assembly [25].

Monomer Accessibility

Since the goal of the present study was to investigate the self-assembly of the collagen molecules on the solid surface it is necessary for the molecules to have enough time to diffuse to the surface before being swept away by the flow. The Peclet number (Pe) is the dimensionless number that is the ratio of the convection to diffusion in the flow. Therefore, for $Pe > 1$ the flow is too strong and the residence time of the most molecules do not have enough residence time in the flow to diffuse to the surface (diffusion limited) and for $Pe < 1$ the diffusion dominates the convection. For the case of thin films on rotating discs the Peclet number is calculated according to [41]:

$$Pe \cong \frac{\tau a^3}{K_B T} \quad (7)$$

where, τ is the shear stress, a is size of the molecule (radius of gyration is this case, Eq. 6), $K_B T$ is the thermal energy.

Since the shear stress in the spin coating is a function of the distance from the center of rotation (r the Fig. 8) and the distance from the solid surface at which the molecule travels (Z in the Fig. 8) the Pe can only be calculated locally. The calculated local Peclet numbers (at $r = 25$ mm) for all the conditions tested in this investigation has been provided in the table 1. As the numbers demonstrate, the Pe significantly increases as the spinning rate increases. For example, for the spin rates of 750 rpm and 1000 rpm the Pe is less than unity or around unity and for the spinning rate of 3000 rpm the Pe is significantly higher. Therefore, the Pe calculations can explain, in part, the lower fibrillar densities observed at higher spin rates (i.e. shear rates).

Thin film stability

Asymmetric self-assembly of the collagen molecules across the coverslips was the biggest deficiency of the spin-coating method. Despite our efforts to prevent or reduce the film instability, mainly through reducing the spinning disc vibration, such destructive effects still persisted through this study. Spin-coated collagen molecules/aggregates are confined in a thin fluid film adjacent to the spinning disc. Therefore, film stability plays a critical role in the uniform coating of the coverslips and global organization of the self-assembled collagen

fibrils. Film instability, that might be the result of the unstable thin films or unstable rotating disc, would result in uneven film thicknesses and therefore, asymmetric shear rates.

As discussed in the previous sections the alignment of the spin-coated collagen fibrils are highly dependent on both the hydrodynamic shear stress and hydrodynamic confinement. However, both of these factors are a function of thin film thickness and therefore, the stability of the thin film plays a critical role in the outcome of the spin coating process. The generation of a stable thin film on a solid substrate (either an inclined wall or a rotating disc) is a highly complex phenomenon and has been the subject of the extensive experimental and theoretical research [42-45].

Although one would expect that due to their reduced degree of freedom the thin films would be a simpler system to study compared to the bulk of fluid, this has been proven not to be the case [46]. In fact, due to their reduced dimensions, thin films are highly sensitive to several influencing factors that are negligible in the bulk. Contamination in the solution, fluctuations in temperature and density, defects in the solid surface are among the major parameters influencing the dewetting process and hence, the final morphology of thin films [46]. Two distinct mechanisms have been identified to influence dewetting [47]: 1) dewetting due to material heterogeneity such as dust and defects and 2) dewetting due to the thermal fluctuations at the free surface of the thin film (spinodal dewetting). For the case of the present study both of these factors may have been contributed to the formation of heterogeneous collagen patterns across the coverslips. Collagen monomer extraction is a relatively crude process. It involves several steps of tissue digestion with enzymes such as pepsin followed by dialysis and purification steps. These methods are mainly performed to remove larger pieces of tissue remnants of pepsin digestion and not smaller impurities. Furthermore, glass coverslips were used as the solid substrates in this investigation. Glass coverslips were used because they allow DIC light microscopy and therefore, provide the possibility of fast and large scale investigation of fibrillar assemblies (compared to other methods such as SEM or AFM). Due to their production method, glass slides are prone to macroscopic superficial defects that are mainly formed during conformation step and are caused by the contact of the glass with the rollers of the conveyors and to the thermal process associated with this operation [48]. These defects are not detectable with naked eye could potentially cause dramatic instability of the thin films. Therefore, to prevent the dewetting caused by the solid surface defects perhaps more pure and highly controlled solid surfaces such as the silicon wafers used in the nano-technology industry such as the ones used by Leneweit et al. [32] should be used.

Spinodal dewetting, which is caused by a non-uniform evaporation of the thin film and results in a spontaneous rupture also plays a critical role in the heterogeneous collagen organization observed in this investigation. It is well documented that collagen self-assembly is highly dependent on collagen concentration [49] such that a two fold increase in collagen concentration increases the rate of self-assembly up to three times. In spite of our attempts to control humidity, non-uniform evaporation of the thin film would result in localized changes in the concentration of the collagen molecules in the thin film and therefore, influence the density of the collagen fibrils, diameter of the fibrils and possibly, the fibrillar organization. Indeed the non-uniform collagen density patterns observed in the fig. 5 could be explained by the spinodal dewetting patterns. In an elegant investigation Thiele et. al. [50] demonstrated that poor control of the humidity during the spin coating has significant effects on the patterns in film instabilities and the final film coating.

CONCLUSION

The goal of the present investigation was to study the effect of confinement in addition to the hydrodynamic forces (i.e. shear stress). Spin-coating is a method capable of producing both a confining thin layer of fluid next to a surface as well as a shear stress in the radial direction. Confinement restricts the Brownian motion of the free end of the tethered fibrils and monomer aggregates in the direction of the flow. Therefore, using confinement, smaller shear rates would be required to align collagen fibrils and produce organized arrays. This effect is advantageous because, as has been shown previously [25], high shear rates disrupt normal fibrillar growth and results in the formation of very short and curved collagen fibrils.

The results of the present investigation reveal that spin-coating is capable of producing highly-aligned layers of collagen fibrils. In addition, the possibility of using an offset configuration enables us to produce multi-lamellar collagenous constructs with alternating alignments. The success and reliability of the method however is greatly dependent to the balance of the rotating disc and the stability of the fluid film. An unstable fluid film results in the asymmetric assembly of the fibrils across the coverslip. The thin film stability caused by material heterogeneity can be greatly reduced. Purifying the collagen solution, performing the experiments in a dust free environment (such as a clean room), and using a higher quality glass slides with minimum surface defects (such as the ones used previously [32]) are among the steps that can be taken to increase the film stability. Spinodal dewetting can be also influenced by the control of the temperature and humidity of the spin coating conditions as well as reducing the surface tension of the solution (e.g. using surfactants).

This investigation also indicates that fibrillar growth against the flow field (creating “hooks”) is an inherent property of *any* shear-influenced collagen self-assembly method (e.g. microfluid chambers, spin-coating, and drainage methods). Although the “hooks” reduce the overall calculated strength of the alignment, their presence does not prevent cellular alignment [51].

In summary, this study was designed and performed with two applications in mind: 1) generation of organized arrays or lamellae of the fibrils to be used as a cell-free, *de novo* approach to corneal tissue engineering and 2) production of organized scaffolds (or templates) to guide cell organization in a classical approach to corneal tissue engineering. Considering that a fully functional corneal replacement requires highly-aligned (nearly perfect) arrays of monodisperse diameter collagen fibrils (to facilitate forward scattering) we concluded that spin coating of collagen is not a viable approach due to persistent hook formation. However the method does provide a simple way of producing scaffolds with sufficient alignment to produce an aligned or organized cell culture. Addressing the factors which contribute to the generation of thin film instabilities should permit the rapid production of organized collagen arrays which persist over large length scales.

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Figure 1.

Schematic of the spin-coating experiments (A) and the offset disc method to produce orthogonal layer (B). (A) Cold, neutralized solution of the collagen molecules were continuously dispensed on the surface of the rotating disc. The molecules self-assemble into collagen fibrils (or aggregates) immediately following contact with the surface. (B) In order to produce orthogonal layers of fibrils, the coverslip was placed with an offset from the spinner's center of rotation (I) side view). The collagen solution was dispensed at the center of rotation. (II) Top view with arrows showing fibril direction relative to the injection port. (III) After coating a single layer, the coverslip was rotated 90 degrees and another layer of collagen was coated. (IV) By implementing this process four times (at 90 degree intervals), orthogonal fibril layers are produced at the center of the coverslip. (C) DIC imaging was performed in at least four spots located 20 mm from the center of deposition and 90° apart.

Figure 2. DIC composite image of a single layer of collagen fibrils self-assembled using spin-coating at 1000 rpm and different flow rates. Although this set of parameters produced aligned collagen fibrils (A1, B1, and C1), the organization was not homogeneous across the disc (A2, B2, and C2). Note the formation of fibril “hooks” pointed out by red arrow heads. White arrows indicate the approximate direction of the flow. Bars are 10 microns.

Figure 3.

DIC composite image of the collagen fibrils self-assembled using spin-coating at 750 rpm (A and B) and 3000 rpm. The flow rate for both conditions was 1 ml/min (C and D). (A, B) Spin-coated collagen molecules at low ω self-assembled into loosely aligned arrays of collagen fibrils. Occasionally masses of randomly organized collagen fibrils were observed on the glass (arrow heads in A). (C, D) at high spinning rates, the molecules predominantly formed aggregates with no discernible fibrillar structure, and in some cases no discernable orientation (D), which could be due to the excessive shear rate. In addition, no collagen self-assembly was observed on large areas of the coverslips. Bars are 10 microns.

Figure 4.

Representative orientation distribution ($I(\theta)$) results of FTM corresponding to the DIC image presented in the Figure 2. By convention, the peaks of the distributions were centered at 0 degree. Please note the significant differences between the fibrillar alignment and anisotropy indices α across the coverslips.

Figure 5. DIC images of collagen fibrils representing the asymmetric assembly of the collagen fibrils on the spinning disc. The images detail the sharp boundary between a region of a highly organized fibrils and a region with almost no collagen fibrils. Scale bars are 10 microns.

Figure 6.

SEM images showing the alignment of collagen fibrils using the spin-coating method. (A, B) Collagen fibrils aligned in the radial direction on the coverslip (arrows). The inset in A is showing the organization of the individual collagen fibrils at higher magnification. (C, D) Production of alternating layers of collagen fibrils using an offset disc. (C) Two orthogonal layers of collagen fibrils with 90° angle between lamellae. (D) Low magnification SEM image of two layer run. A scratch was intentionally made to provide better insight regarding the lamellar structure of the spin-coated construct. The smooth surface in the middle of the image is the coverslip on which the collagen was deposited. Note that collagen film clearly has two distinct alignment directions. The angle between lamellae is not 90° because of image location on the disk, which was close to the edge of the coverslip [28].

Figure 7. Estimated film thickness (i.e., velocity profile, solid symbols) and shear rate (open symbols) for the case of 1000 rpm and various flow rates. Note that the film thickness changes minimally from 2.0 to 2.5 cm.

Figure 8.

Figure 8 is a schematic of the two possible scenarios possible to a tethered collagen molecule in a spin coating flow. In the case shown on left, the half film thickness above the tethered molecules is larger than the radius of gyration of a molecule. In this case, the free end of the molecule freely diffuses in a volume of a hemisphere with a radius equal to the contour length of a collagen molecule ($L=300$ nm). Therefore, the molecular confinement is not directly influencing the alignment of the molecule. In the case shown on right, the half film thickness is less than the radius of gyration. Therefore, the fluid free surface limits the volume available to the free end of the tethered molecule to a quarter of a sphere with a radius equal to the film thickness. In this condition, the fluid free surface strongly influences the alignment of the molecules and the resulting fibrils.

Table 1

Wall shear rate (τ), film thicknesses (δ), Weissenberg numbers (Wi) for single monomer case, and Peclet numbers (Pe) calculated based on the experimental parameters used in this investigation. The calculations are performed for a 2.5 cm from the center of rotation.

Spin rate (ω , rpm)	750	1000	3000
Flow rate (Q, ml/min)			
0.1	$\tau=181$ 1/s $\delta=41.6$ μm $Wi=0.14$ $Pe=0.6$	$\tau=266$ 1/s $\delta=34.3$ μm $Wi=0.21$ $Pe=0.9$	$\tau=1150$ 1/s $\delta=16.5$ μm $Wi=0.90$ $Pe=3.9$
0.5	$\tau=310$ 1/s $\delta=71.1$ μm $Wi=0.24$ $Pe=1.0$	$\tau=455$ 1/s $\delta=58.7$ μm $Wi=0.36$ $Pe=1.5$	$\tau=1970$ 1/s $\delta=28.2$ μm $Wi=1.54$ $Pe=6.8$
1	$\tau=391$ 1/s $\delta=89.6$ μm $Wi=0.31$ $Pe=1.3$	$\tau=574$ 1/s $\delta=74$ μm $Wi=0.45$ $Pe=2.0$	$\tau=2480$ 1/s $\delta=35.6$ μm $Wi=1.94$ $Pe=8.5$

The calculated Relaxation times (ϵ) and Weissenberg numbers (Wi) for a collagen molecule and collagen aggregates with lengths up to 1.53 μ m. Both Wi and ϵ significantly increase for longer collagen aggregates.

Table 2

Aggregate's Length (nm)	Relaxation Time (ϵ)	Wi (at 750 rpm)			Wi (at 1000 rpm)			Wi (at 3000 rpm)		
		Q=1 (ml/min)	Q=0.5 (ml/min)	Q=0.1 (ml/min)	Q=1 (ml/min)	Q=0.5 (ml/min)	Q=0.1 (ml/min)	Q=1 (ml/min)	Q=0.5 (ml/min)	Q=0.1 (ml/min)
300	0.0008									
600	0.0025	0.31	0.24	0.14	0.45	0.36	0.21	1.94	1.54	0.90
900	0.0052	0.99	0.79	0.46	1.46	1.16	0.68	6.31	5.01	2.93
1200	0.0086	2.01	1.60	0.94	2.96	2.35	1.37	12.79	10.15	5.94
1500	0.0127	3.35	2.66	1.55	4.91	3.90	2.28	21.25	16.86	9.86