Electrochemically controlled growth and positioning of suspended collagen membranes

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ABSTRACT Two independently recognized *in vitro* polymer aggregation variables, electric field and pH, can be used in concert to produce suspended membranes from solutions of type I collagen monomers, without need of a supporting substrate. A collagen network film can form at the alkaline-acidic pH interface created during the normal course of water electrolysis with parallel plate electrodes, and the anchoring location can be controlled by adjusting the bulk electrolyte pH. Electrosynthesized films remain intact upon drying and rehydration and function as ion separation membranes even in sub-millimeter channels. This approach could benefit lab-on-a-chip technologies for rational placement of membranes in microfluidic devices.

Introduction

Films of collagen, the most abundant mammalian protein[1], are sought for prosthetic coatings, as support matrices for cell growth, and for permeable membranes.[2] However, many *in vitro* collagen films are substrate supported[2-5] which limits their use as membranes. Our results demonstrate that two independently recognized *in vitro* polymer aggregation variables, electric field[6-10] and pH,[1,11,12] can be used in concert to form suspended collagen network films, without the presence of a supporting substrate. This approach could benefit lab-on-a-chip technologies[13] for rational placement of ion separation membranes in microfluidic devices.

Collagen's function in the body is intimately related to its structural transformations through aggregation. Conveniently, the hierarchical collagen structures observed from *in vivo*[14,15] collagen aggregation can be replicated *in vitro*[11,14], without need of biological assistance from enzymes or growth factors. Collagen monomers can form oligomers that grow linearly and laterally and cross-link to create fibrils[15]. Alternate aggregation pathways produce different aggregate or fibril morphologies[1,15,16]. Controlled collagen aggregation requires management of electrostatics, sterics, and hydrophobicity, all of which can be affected by pH[1,14,15]. Adding an electric field places further importance on electrostatics and charge.

Collagen's surface charge is pH-dependent due to amino acid protonation at acidic pH values and deprotonation at alkaline pH values[17]. Collagen, when net charged, migrates in an electric field like any charged polymer[7,8], but beyond that, collagen's behavior in an electric field has not been widely investigated[10,17], despite increasing use of electrochemical methods to create biopolymer and biocomposite coatings[2,18]. Collagen is electrochemically inactive over a wide range of applied potentials[19]. However, a sufficiently large applied voltage to its aqueous supporting electrolyte causes alkaline conditions near the cathode, due to the hydrogen evolution reaction (HER, with E^0 = -0.83 *vs.* normal hydrogen electrode (NHE)), and acidic pH near the anode (oxygen evolution reaction, with E^0 = +1.71 *vs.* NHE), which can influence collagen migration and aggregation. Reduction of ambient dissolved oxygen also contributes to the pH change near the cathode. By exposing collagen monomers to the simultaneous pH gradient and electric field produced in an electrochemical cell, we have developed a method to control the spatial aggregation of collagen in the electrolyte, without need for a supporting substrate.

Experimental

Electrolytes, prepared using ultrapure water (Barnstead, 18.2 MΩ), contained type I collagen monomers (final concentration 0.07 - 0.30 mg/mL from 3 mg/mL Vitrogen stock solution, Inamed Biomaterials) and sufficient NaOH and HCl solutions (EMD chemicals, ACS reagent grade) to adjust the pH to the desired value (4-11). Stainless steel sheet electrodes provided an electric field of 5-10 V over 1.0-2.5 cm, yielding a current density of -0.7 to -1.2 mA/cm^2. These voltages are large relative to the potentials required for the HER, oxygen evolution and oxygen reduction reactions. Macroscopic

films were formed in a glass electrochemical cell designed and built in-house (2.5 cm x 2.5 cm x 1 cm, 3.5 mL volume), while smaller films were prepared in glass (1.1 mm ID) or polyethylene (1.57 or 0.86 mm ID) capillaries, 1 cm long. We note that, while it is possible for peroxide to form at the cathode during the hydrogen and oxygen reduction processes, we do not observe any film degradation due to the presence of such strong oxidants over the time scales of our experiments (up to several hours).

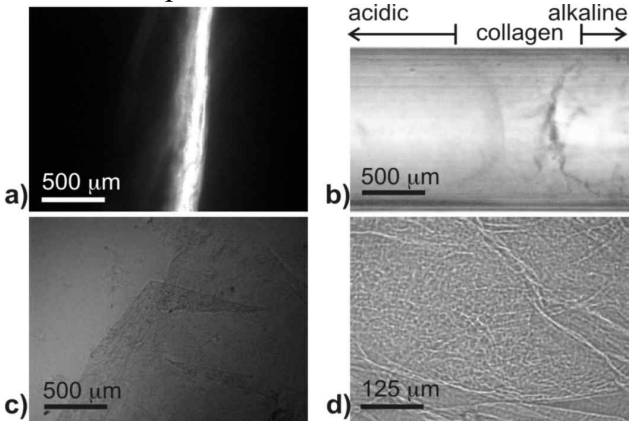
Optical microscopy investigations employed a Leica DM2500 with polarized light capability. Atomic force microscopy studies with an Asylum Research MFP-3D used silicon cantilevers (Au back coating, force constant ~ 0.3 N/m, Mikromasch). Raman scattering spectroscopy data were obtained with a Jobin Yvon Horiba LabRAM in the confocal configuration (532 nm excitation).

Within minutes of exposing a pH-adjusted electrolyte of collagen monomers (0.3 mg/mL) to an electric field (3-20 V/cm), a collagen film – visible by eye – forms in the electrolyte, parallel to the anode and cathode, and with its edges anchored to the container walls, whether glass or polyethylene. Videos and representative current versus time data taken during the film formation process are available as Supporting Information.

Results and Discussion

Our electrosynthesis method for forming collagen films controls three essential stages of collagen aggregation: localization, organization, and association. The coexistence of the electric field and pH gradient enables a spatially localized region of high collagen concentration through a serendipitous combination of pH-dependent surface charge and the resulting electromobility it induces. When the electric field is first applied, collagen migrates away from the anode[10] to build up a critical mass of collagen near the middle of the cell. As the collagen migrates closer to the pH region that matches its isoelectric point, it approaches net neutral charge and is less affected by the electric field. These changes in surface charge distribution impact charge association and polymer alignment[6,10].

Fig. 1a and Fig. 1b show electrosynthesized suspended collagen films, one with centimeter-scale dimensions (2.5 cm^2) prepared in an open cell and another with sub-millimeter dimensions (0.01 cm^2)



prepared in a glass capillary. Macroscopic collagen films can be removed while wet, air-dried, and rehydrated to recover their pliability. The films are brittle when dry but are flexible when partially wetted. They are robust enough to be handled with tweezers. Optical micrographs of a dried, partially folded film portray the sheet-like nature of the film (Fig. 1c), and higher magnifications (Fig. 1d) highlight the complex microstructure of the collagen sheets. Atomic Force Microscopy (AFM) studies show that air-drying reduces film thickness from ~100 μm to 0.6±0.2 μm. AFM images presented in the Supplemental Information show continuation of the mesh-like structure on the micrometer length scale.

Figure 1. Electrosynthesized collagen network films can be macroscopic and removable (a) or synthesized in smaller channels (b). The macroscopic film in (a) is shown during its later stages of growth, viewed edge-on with *in situ* polarized light microscopy. The edge-on view of the sub-millimeter film in (b) depicts the early stages of formation relative to the acidic and alkaline regions of the surrounding electrolyte. Air-dried collagen films, viewed with crossed polarizers, are sheet-like (c); higher magnifications (d) highlight their complex network structure.

Raman scattering spectroscopy can indicate the degree and type of monomer association in polymeric films[4,20]. Fig. 2 compares the Raman spectra for our collagen films with those for precursor monomers and for fibrils prepared by a standard dialysis method[14]. Based on Raman data from the amide I (1630-1670 cm^{-1}), amide III (1240-1270 cm^{-1}) and C-N stretch (1095 cm^{-1}) regions[4], along with supporting AFM studies shown in the Supporting Information, we conclude that our electrosynthesized collagen films are heterogeneous, containing a relatively high amount of monomeric collagen.

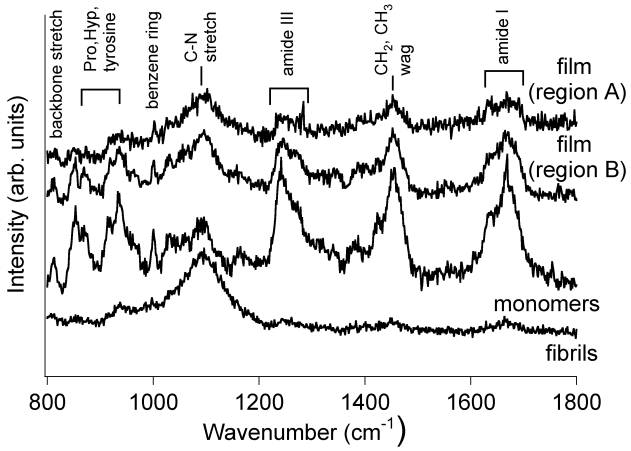


Figure 2. Raman spectra indicate heterogeneous aggregation in the air-dried electrosynthesized collagen film (two regions, A and B) when compared with spectra for air-dried monomeric and fibrillar collagen. The C-N stretch (~1100 cm^{-1}, strongest intensity for fibrillar collagen), amide I (1630-1670 cm^{-1}), and amide III (1240-1270 cm^{-1}) regions are most sensitive to conformational changes.

The aggregational heterogeneity evident in the Raman data suggests a time-dependent conversion from non-specific monomer aggregates to collagen fibrils during electrosynthesis. Early in film formation, it is possible to partially or wholly dissolve the film and reform it at a different location in the electrochemical cell simply by reversing the polarity of the applied electric field. However, films formed for longer times show incomplete or minimal dissolution upon field reversal, suggesting that more robust and massive fibrils[21] exist after prolonged exposure to electric field and pH gradients.

Despite the mixture of monomeric and fibrillar collagen, film formation and function is quite reliable when three key experimental parameters are controlled

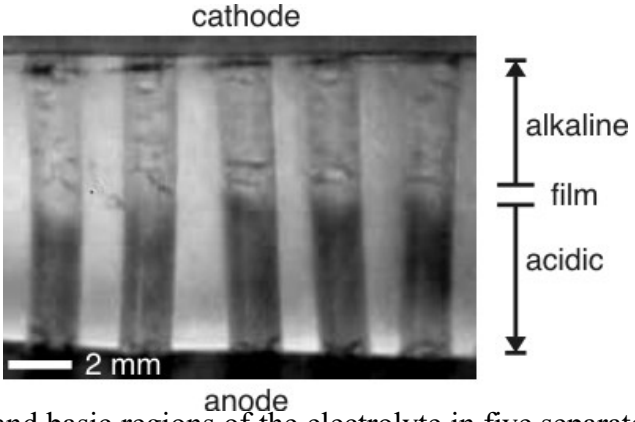
First, there exists a collagen concentration threshold below which no film-like aggregation occurs, dependent on the volume of electrolyte relative to the surface area of the desired collagen film. Surface area to volume ratios near 1 cm^2 : 1 mL, consistent with the conditions used to produce the films shown in Fig. 1, required collagen concentrations of 0.10-0.30 mg/mL.

Second, a sufficiently large electric field is essential for spatially controlled aggregation. Earlier studies that employed pH gradients in the absence of an electric field[12] report collagen aggregation but no evidence of film formation in the electrolyte. Other conditions[10] demonstrate electromigration of positively charged collagen away from an anode for smaller electric fields (< 1 V/cm), but show no film-

like aggregation. The larger electric fields used in this study (3 – 20 V/cm) cause faster migration and more rapid development of a distinct acidic-alkaline pH interface.

Third, bulk electrolyte pH influences the spatial location of film formation, offering interesting possibilities for membrane positioning in microfluidic channels. Measurements with pH microelectrodes and universal pH indicator show that the region where the film forms has pH ~6, regardless of the electrolyte's initial bulk pH. Extreme bulk pH values (< 4 or > 11) resulted in fragile or incomplete films, even at sufficiently high collagen concentrations. For example, acidic bulk electrolytes (pH = 2) show limited OH^- diffusion away from the working electrode (< 50 mm) even after hours of HER. As a result, the optimal pH region for film formation is so close to the cathode surface that hydrogen bubbles from the HER physically disrupt film formation. Fig. 3 is a representative image that shows the reproducibility of film positioning.

Figure 3. Collagen film positioning is quite reproducible. This representative image shows the films that

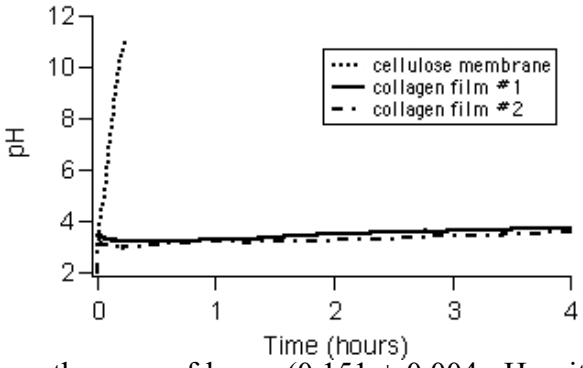


form interfaces between acidic and basic regions of the electrolyte in five separate capillaries, each with an initial bulk electrolyte pH = 8. The visual demarcation between acidic (dark grey, near the cathode) and alkaline (light grey, near the anode) regions of the electrolytes was enhanced in this experiment by adding universal pH indicator.

These anchored films can be reliably positioned in small channels where manually inserting membranes would be extremely difficult, as the sub-millimter capillaries shown in Figures 1 and 3 demonstrate. Thus, there is promise for using electrosynthesis to place collagen membranes in

microfluidic devices[13] with either parallel plate or more complicated electrode geometries. Fig. 4 shows that the functionality of such membranes could include maintaining pH gradients or other ion separation. The possibility of size selectivity for larger particles is also intriguing, and experiments (described in more detail in the Supporting Information) have shown that colloidal silica spheres (400-500 nm diameter) do not diffuse readily through these collagen membranes. Thus, suspended electrosynthesized collagen films offer exciting possibilities for lab-on-a-chip technologies.

Figure 4. Representative pH versus time data shows that electrosynthesized collagen films can



maintain pH gradients well over the span of hours (0.151 ± 0.004 pH units/hour, starting with pH 3 on one side of the membrane and pH 11 on the other side). For reference, comparable plots are shown for commercial (cellulose) dialysis membranes (Spectra/Por Biotech Regenerated Cellulose, 8000 MWCO), which show a change of 1.0 ± 0.5 pH units/minute).

ACKNOWLEDGMENT Thanks to the Department of Biochemistry for the use of their Raman spectroscopy facilities, A. Yethiraj and N. Li for supplying colloids, and R. Kumar for helpful discussions. We thank NSERC (Canada), the Canada Foundation for Innovation, and the Canada Summer Jobs program (HRB) for funding support.

SUPPORTING INFORMATION Schematic diagram and videos of collagen film formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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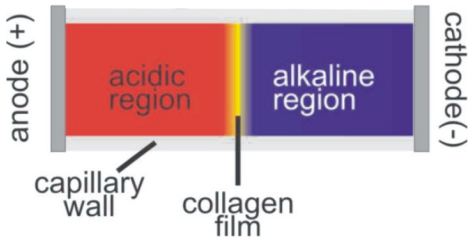

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