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OPTIMIZING COLLAGEN TRANSPORT THROUGH TRACK-ETCHED NANOPORES

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Abstract

Polymer transport through nanopores is a potentially powerful tool for separation and organization of molecules in biotechnology applications. Our goal is to produce aligned collagen fibrils by mimicking cell-mediated collagen assembly: driving collagen monomers in solution through the aligned nanopores in track-etched membranes followed by fibrillogenesis at the pore exit. We examined type I atelo-collagen monomer transport in neutral, cold solution through polycarbonate track-etched membranes comprising 80-nm-diameter, 6- μ m-long pores at 2% areal fraction. Source concentrations of 1.0, 2.8 and 7.0 mg/ml and pressure differentials of 0, 10 and 20 inH₂O were used. Membrane surfaces were hydrophilized via covalent poly(ethylene-glycol) binding to limit solute-membrane interaction. Collagen transport through the nanopores was a non-intuitive process due to the complex behavior of this associating molecule in semi-dilute solution. Nonetheless, a modified open pore model provided reasonable predictions of transport parameters. Transport rates were concentration- and pressure-dependent, with diffusivities across the membrane in semi-dilute solution two-fold those in dilute solution, possibly via cooperative diffusion or polymer entrainment. The most significant enhancement of collagen transport was accomplished by membrane hydrophilization. The highest concentration transported (5.99 ± 2.58 mg/ml) with the highest monomer flux ($2.60 \pm 0.49 \times 10^3$ molecules s⁻¹ pore⁻¹) was observed using 2.8 mg collagen/ml, 10 inH₂O and hydrophilic membranes.

INTRODUCTION

The transport of long, linear molecules in solution through small pores has relevance for a number of processes including gel permeation chromatography [1-4], ultra filtration [5,6], protein structural characterization [7,8], gene transfer and RNA translocation [9-11] or the excretion and assembly of proteins and other molecules through cellular nanopores [12,13]. The process of polymer translocation through nanoscale pores is also a powerful tool in the development of methods to separate and perhaps organize molecules. We are interested in directing collagen self-assembly in a manner mimetic of fibroblastic cells, which are thought to secrete collagen fibrils from monomers confined in a cell membrane pore or “fibripositor” [14-17].

I. COLLAGEN

Collagen is the most abundant protein in the extracellular matrix (ECM) of vertebrates and is the main component of connective tissue [18]. There have been as many as 27 collagenous sequences (gly-x-y) identified throughout the metazoan genome [19,20]. Of

© 2008 Elsevier B.V. All rights reserved. these five types of fibril forming collagens (types I, II, III, V and XI) have been recognized,

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Symbols

a	cm	Size of a collagen monomer (Gly-x-y repeat).
A _e	cm ²	Area of the membrane that is occupied by pores.
A _k		Membrane porosity.
A _m	cm ²	Area of the membrane sandwiched between the two compartments of the diffusion chamber.
C*	mol cm ⁻³	Overlap concentration.
ΔC	mg cm ⁻³ , μM	Concentration of collagen in solution.
D ₀	cm ² s ⁻¹	Diffusivity of collagen in free solution.
d _m	cm	Diameter of the membrane sandwiched between the two compartments of the diffusion chamber.
d _p	cm	Diameter of a pore in the membrane.
f(q)		Wall correction factor.
g(q)		Wall correction factor.
h	cm	Level of fluid in a capillary directly connected to one compartment of the diffusion chamber.
J*	cm s ⁻¹	Critical solvent flux.
J _s	μmol cm ⁻² s ⁻¹	Flux of collagen through the membrane.
J _v	cm s ⁻¹	Flux of solvent through the membrane.
k _B	g cm ² s ⁻² K ⁻¹	Boltzmann's constant = 1.38×10 ⁻¹⁶ .
L	cm	Membrane thickness, corresponds to the length of a pore.
L _p	cm ³ dyne ⁻¹ s ⁻¹	Hydraulic conductance.
N		Polymerization index or number of monomers per chain.
N _A	mol ⁻¹	Avogadro's number = 6.023×10 ²³ .
P	cm/s	Permeability.
ΔP	dynes cm ⁻²	Pressure gradient across the membrane.
q		Ratio between solute radius and pore radius.
Q	cm ³ s ⁻¹	Solvent flow.
r _F	cm	Radius of gyration for the collagen molecule.
R	atm L K ⁻¹ mol ⁻¹	Ideal gas constant = 0.08206.
r _H	cm	Hydrodynamic radius of the collagen molecule.
r _p	cm	Pore radius.
S _D		Steric-hindrance factor for convective flow.
S _F		Steric-hindrance factor for diffusive flow.
T	K	Temperature.

Greek symbols

σ _o		Osmotic reflection coefficient.
μ	dyne s cm ⁻²	Viscosity.
ΔΠ	dynes cm ⁻²	Osmotic pressure gradient across the membrane.
ρ _p	pores cm ⁻²	Density of pores in the membrane's area.

the primary functional role of which is bearing and transmitting tensile mechanical loads. In order to maximize load-bearing ability, collagen fibrils must be highly-organized, typically into parallel arrays.

Structure—The molecular structure of type I collagen comprises three left-handed helical α chains whose complementary amino acid sequence results in the formation of a right-handed supramolecular triple helix stabilized by hydrogen bonds within and across the polypeptide sequence [21-23]. The overall length of the molecule has been reported as marginally less than 300 nm, its diameter as ~ 1.4 nm, its amino acid content as 3,000 and its molecular weight as 280-300 KDa [21,24-26].

Physicochemical behavior—Collagen exhibits extremely complex physicochemical behavior in solution due to its tendency to associate [27-32] in response to changes in pH, concentration, ionic strength and temperature [25]. Increasing concentration favors aggregation, such that untreated tropocollagen is highly viscous and gel-like at concentrations >5 mg/ml, but manageable up to 13 mg/ml if sonicated [28].

Diffusivity: In early studies, the collagen molecule was often modeled as a rod-like structure [24,27,31] with a length of approximately 300 nm [24,30,31,33-36], and a diameter of 1.36 nm [31]. The diffusion coefficient of the collagen molecule in free, dilute solution, D_0 , is reportedly $7.8-8.6 \times 10^{-8}$ cm²/s [30-33,36-38], and depends anomalously on concentration. Fletcher et al. [31] reported a maximum D_0 near 0.4 mg/ml and a linear decrease in D_0 with increasing concentration up to 1 mg/ml. At low concentrations (<0.4 mg/ml), the diffusion coefficient of collagen decreases [31], which may be due to the molecules forming a supramolecular structure with definite preferred molecular separation [39].

Viscosity: Intrinsic viscosities in the order of 10 dl/g have been attributed to tropocollagen monomer solutions [28], with partial aggregation resulting in higher values. For example, the intrinsic viscosities of native tropocollagen solutions is 16-20 dl/g [28] and that of a collagen dimer linked with a 10% overlap is 30 dl/g [40], which indicates that native tropocollagen solutions contain approximately 45% dimers. Since the viscosity of long molecules increases as a high power of the molecule length, it is suggested that only a very small number of long polymers can be present in collagen solutions.

The viscosity behavior of collagen has been partially explained by a flexible coil morphology suggested by the results of hydrodynamic studies. Reports on the geometry of this coil morphology include: an assumed length of 130 nm [41], a persistence length of 161 nm at acidic pH and a semi-flexible rod morphology with two loose joints at the ends and a 169-nm persistence length for the central segment at neutral pH [42], or an effective length of 280 nm, 250 nm end-to-end distances and 300 nm contour length [34]. This suggests that at acidic pH, decreased effective length as a result of molecule flexion would translate into increased diffusivity. However, a more recent investigation using direct single-molecule stretching in an optical trap puts collagen persistence length at neutral pH (7.4) at only 14.5 nm and its contour length at 309 nm, thus suggesting the molecule's behavior is that of a very flexible chain [43].

Solubility/Isoelectric Point: Native procollagen is insoluble in most buffers above pH 4.5 while sonicated tropocollagen is soluble throughout the pH range. Electrophoresis data suggest that tropocollagen will readily bind both anions and cations with a large resultant shift in the isoelectric point [28] although at neutral pH in physiological buffer collagen monomers possess a net positive charge [44].

Collagen Self-Assembly—The assembly of collagen monomers into fibrils in solution is an entropy-driven process, where solvent molecules gain free energy (approximately ~2.00 kcal/mol at 300° K) [45,46]. *In vivo*, fibrillar collagen molecules are synthesized as soluble procollagen which is then converted to tropocollagen by enzymatic cleavage of the terminal propeptides [47]. The converted tropocollagen spontaneously assembles into D-periodic fibrils either in the ECM or in putative cell-surface “crypts” or “fibripositors” [16]. Cell-free, *in vitro* fibrillogenesis of cold, acid extracted type I collagen by neutralization and warming of collagen solution has been extensively studied [48-52], and D-periodic fibrils whose morphology depends on both the solution/environmental conditions of extraction and fibrillogenesis have been successfully assembled [49,53-56]. We are interested in producing organized D-periodic fibrils from acid-extracted or enzyme-extracted acid-soluble collagens for use as tissue engineering scaffolds. Our planned application promotes the self-assembly of collagen into fibrils at the downstream side of a track-etched nanopore in a printer-like arrangement. A critical step for the success of this application is the control of the rate of transport of collagen monomers in solution through the pores of the track-etched membrane. This study has been designed to enhance our understanding of this particular transport application. However, data from this investigation can also provide information regarding the rate of collagen assembly in cell surface crypts during collagen production by fibroblastic cells.

II. POLYMER TRANSPORT ACROSS POROUS MEMBRANES

The transport of flexible macromolecules through the nanopores of a membrane where the molecule size and the pore size are comparable is hindered with respect to transport in bulk solution. This hindrance is due to steric interactions between the solute and the pore walls that exclude the solute from certain radial positions within the pore; or to the increase in the hydrodynamic drag on the solute molecule in the confined space of the pore [57]. Thus, when analyzing collagen transport under these conditions it is important to consider that the transport coefficients are affected by factors that influence the equilibrium partitioning of solutes between the pores and the bulk solution, and therefore driving forces can not solely be expressed in terms of the concentrations of the adjacent bulk solution [58].

In general, the bulk transport of solute and solvent across membranes can be described using three phenomenological parameters that are typically independent of operating conditions such as pressure and concentration. Such parameters are the hydraulic conductance (L_p), the reflection coefficient σ_0 , and the membrane's permeability to the solute P [59]. L_p ($\text{cm}^3 \text{ dyne}^{-1} \text{ s}^{-1}$) is specific to each membrane and solvent combination. σ_0 is a dimensionless intrinsic property associated with the interaction of the solute and membrane and describes the rejection of solute by the membrane. $\sigma_0=0$ and $\sigma_0=1$ indicate no rejection and total rejection of the solute, respectively [58,60]. The estimation of L_p , σ_0 and P necessitates embracing certain assumptions regarding the shape that the macromolecular solute assumes during transport. Flexible polymer chains may deform significantly during transport, a phenomenon that dissipates energy and changes the molecule's apparent dimensions (i.e. radius and/or length) as well as the local viscosity. The dimensions usually assigned to the flexible polymer during transport analyses only represent statistical averages of the several randomly-achieved configurations [58]. One such statistical average is the hydrodynamic radius, r_H ; which assumes that the polymer chains act as rigid spheres with a radius calculated using the Stokes-Einstein equation and the diffusivity of the solute in free solution:

$$r_H = \frac{k_B T}{6D_0 \pi \eta}$$

Equation 1

k_B is Boltzmann's constant ($1.38 \times 10^{-16} \text{ g cm}^2 \text{ s}^{-2} \text{ K}^{-1}$), T is temperature, D_0 is diffusivity of the solute in free solution and η is the solvent's viscosity. The use of r_H has led to discrepancies between experimentally-observed and theoretically-predicted macromolecular solute diffusivities [5]; and in misleading results from the calculation of the hydrodynamic resistance to solute motion in pores. Theoretically, flexible polymers with radius r_H should transport through the pores faster than spheres of radius r_H due to decreased predicted hydrodynamic resistances. However, experimental results demonstrate that spheres of radius r_H transport through the nanopores faster than flexible polymers with radius r_H because the partitioning of random coils between the pores and the bulk solution is less favorable [57]. These discrepancies most likely occur because flexible polymer coils bear little resemblance to solid spheres, in particular when confined inside membrane pores. Consequently, numerous efforts to develop hydrodynamic models that more accurately describe the transport problem have been attempted [57,58,61-66]. As it turns out, the radius of gyration (r_F) may be a better parameter to characterize the size of transporting flexible polymers [5]. r_F is the radius of an isolated chain in the presence of excluded volume effects, and for a bulk dilute solution it is scaled as:

$$r_F \cong aN^\nu \quad \text{Equation 2}$$

Where a is the monomer size (cm/monomer), N is the polymerization index (monomers/chain), and $\nu=3/5$ is the excluded volume [67]. Linear flexible macromolecules in *good* solvents have a ratio of r_F/r_H in the order of 1.4 to 1.5 [68-70]. For lathyrin collagen, r_F has been experimentally determined via dynamic light scattering as $74.5 \pm 2.0 \text{ nm}$ [38].

According to Brochard and DeGennes [71], the behavior of a single monomer chain in dilute solution in a pore depends on the ratio between the radius of gyration, r_F , and the pore's diameter, d_p . When $r_F < d_p$, the solution in the pore can be treated as a conventional bulk solution. However, when $r_F > d_p$ the monomer chain must be deformed axially to enter the pore and then takes a shape comprising a succession of blobs, each of which has a diameter $\sim d_p$. However, the effective "concentration" of the monomer solution fluctuates axially through the length of the pore. Further complications in transport arise in semi-dilute solutions where chain overlap is significant [62]. The overlap concentration C^* , depends on the chain size [72]:

$$C^* = \frac{1}{8N_A r_F^3} \quad \text{Equation 3}$$

Where N_A is Avogadro's number and C^* is molar concentration. In the semi-dilute regime, where the concentration of the solution exceeds C^* , the square of the radius of gyration is proportional to the molecular weight and decreases with the concentration like C^{-x} , where x is a constant [62].

One model used to estimate the transport of flexible polymers through nanopore membranes is the "open pore model" [5,73-76]. Originally developed for rigid spherical solutes, the open pore model has also been shown to accurately predict the transport of linear macromolecules of similar weight to collagen through membranes with highly regulated cylindrical pores of dimensions in the 10^1 nm range [5]. In the open pore model, r_F is reportedly a better characteristic dimension for transport behaviour than r_H [5]. The model describes the transport parameters L_p , σ_0 and P in terms of the structural characteristics of the membrane: pore radius, pore length and membrane porosity; and in terms of the solute properties: radius and diffusivity in free solution.

Aside from random deformations, polymer chains transporting through the nanopores of a membrane may undergo flow-induced deformation when the flux of the permeate reaches a critical value that depends on the characteristics of the solution (viscosity, concentration, temperature) and those of the membrane (pore size, pore density) [58,77]. Basically, sufficiently high velocity gradients can be created at the pore entrance to cause chain elongation in the flow direction, such that the molecules arrive at the pore entrance with a transverse section that is smaller than the entrance itself [65,77]. This phenomenon has been investigated by various authors [61-63,71] under the assumptions that (a) there is no adsorption of the polymer by the walls and (b) the monomers repel each other in solution (which may not be the case for collagen monomers). Under such conditions, the critical permeate flux, J^* (cm/s) is:

$$J^* \geq \frac{k_B T A_k}{\eta r_p^2} \quad \text{for dilute solutions, } C < C^* \quad \text{Equation 4}$$

or

$$J^* \geq \frac{k_B T A_k}{\eta r_p^2} \left(\frac{C^*}{C} \right)^{\frac{15}{4}} \quad \text{for semi-dilute solutions, } C \geq C^* \quad \text{Equation 5}$$

Where A_k is the effective surface porosity, r_p is the pore radius and C is the molar concentration of solute. For dilute solutions, the value of the critical flux is independent of the concentration of the solution (Equation 4), but it decreases rapidly with the concentration in the semi-dilute regime (Equation 5) because the polymer coils become increasingly entangled with increased concentration; this increases their relaxation time constant and decreases the steady shear necessary to maintain their deformation [77].

The transport of flexible polymer chains across membrane pores also depends on the concentration. At low concentrations the effective radius of the molecule hinders the diffusion for molecular radius comparable to the radius of the pore, and the diffusion problem follows first order exponential kinetics [66]. At increasing concentrations, chains that would normally be blocked diffuse through the membrane, although the diffusion kinetics is not exponential and depends highly on concentration [66]. In the semi-dilute range, theory and experiments have shown that the diffusion coefficient increases with the concentration [77] such that its effective diffusion coefficient may be two orders of magnitude larger than in the dilute concentration range, even for molecular radius comparable to the pore radius [66]. While increased diffusivity may be partially explained by cooperative diffusion, the partitioning of the solute between the bulk solution and the pores also plays an important role [66]. Since the critical solute flux decreases rapidly with the concentration in the semi-dilute regime, it can be assumed that the molecule deforms more easily (or more precisely remains deformed more readily). Therefore, even though the rate of solvent flow to the membrane may decrease at higher concentrations due to viscosity effects, the diffusion of polymer through the membrane increases [77].

Another characteristic of flexible chain polymer transport is its dependence on the applied pressure [65]. While globular proteins tend to gel and block the pores at the surface of the membrane with increases in pressure, flexible chain polymers transport more readily through the pores as the pressure increases. Polotsky and Cherkasov [65] suggested that the transport of flexible polymers through membrane nanopores is governed by two mechanisms: operating pressure and the molecular mass of the polymer. Low pressures and low molecular masses tend to allow the molecules to retain shapes similar to globular

proteins which closely adhere to the stagnant film model of concentration polarization. Increased pressure (which results in an increased volume flux) leads to an increase in solute concentration near the membrane surface. Increased pressure *and* molecular mass changes the physics of the problem, and the transport of flexible polymers through nanopores is dominated by polymer chain uncoiling. Thus, for high molecular masses (a fixed quantity in our case), increased pressures of operation should result in transport behavior which reflects the mechanics of polymer chain uncoiling at the membrane's pores.

The osmotic flow of dilute solutions of non-interacting molecules in porous membranes can be described by classical transport and irreversible thermodynamics [59], with the solvent flux across a membrane, J_v (cm/s):

$$J_v = L_p [\Delta P_\infty - \sigma_0 \Delta \Pi_\infty] \quad \text{Equation 6}$$

Where the subscript ∞ indicates bulk solution conditions on each side of the membrane, ΔP is the hydrostatic pressure gradient across the membrane, and $\Delta \Pi$ equals the hydrostatic pressure difference when $J_v = 0$ and depends on the concentration gradient across the membrane. For an ideal solution $\Delta \Pi$ is equal to the product $RT\Delta C_\infty$, where R is the ideal gas constant and T is the temperature.

The equation for the flux of solute through the membrane (J_s , moles $\text{cm}^{-2} \text{s}^{-1}$) is:

$$J_s = J_v \hat{C} [1 - \sigma_0] + \frac{D_m \Delta C}{L} \quad \text{Equation 7}$$

Where $\hat{C}$ indicates the average molar concentration in the bulk solution or in the membrane, D_m (cm^2/s) is the diffusion coefficient of collagen in the membrane, L (cm) is the thickness of the membrane and ΔC is the concentration gradient across the membrane. Although ΔC has molar units when used in Equation 7, we will refer to it throughout this manuscript in units of mg/ml, for simplicity (and consistency with the collagen solution-related literature).

This investigation examined the transport of collagen I in solution through 80 nm diameter right-circular cylindrical pores in a polycarbonate track-etched membrane. The pores were 6- μm -long and distributed to cover a 2% areal fraction of the membrane surface. Collagen transport rates were investigated at concentration gradients of 1.0, 2.8 and 7.0 mg/ml and at pressure differentials of 0, 10 and 20 in H_2O . Additionally, some of the membranes were pretreated by coating with poly (ethylene-glycol) which should increase their hydrophilicity and therefore limit solute/pore wall interaction. The resulting fluxes of collagen and solvent were compared against transport rates determined using experimental parameters and literature models that describe flexible polymer transport through nanoscale channels [5,78].

MATERIALS AND METHODS

Open Pore Model

When considering the transport of long linear molecules in confining pores, the standard membrane transport equations do not apply without modification. A critical measure of the influence of the solute interaction with the pore is the ratio of the radius of the collagen monomer to that of the pore, q [5]. q was calculated for both the radius of gyration r_F (q_F) and the hydrodynamic radius r_H (q_H) and used to estimate the steric-hindrance factors for convective flow and for diffusive flow (S_F and S_D , respectively) whose derivation [5] employs irreversible thermodynamics [59] and the open pore model relationships [74,76]. S_F

and S_D were used to estimate diffusivity through the membrane (D_m), permeability (P), and reflection coefficient (σ_0), as follows:

$$D_m = S_D f(q) D_0 \quad \text{Equation 8}$$

$$P = D_m \frac{A_k}{L} \quad \text{Equation 9}$$

$$\sigma_0 = 1 - S_F g(q) \quad \text{Equation 10}$$

Where $f(q)$ and $g(q)$ are wall correction factors for diffusive and convective flow [5].

Collagen Solution

PureCol™ (Inamed, Irvine, CA) was the source of collagen in all experiments. It contained 3 mg/ml of pepsin-solubilized bovine dermal collagen dissolved in 0.01 N HCl (pH~ 2). The sterile solution was 99.9% collagen, of which 97% and 3% correspond to collagen types I and III, respectively. According to the product insert and as verified by others [79], PureCol™ has high monomer content. Chilled PureCol™ solution was neutralized by mixing 8:1 with 10X phosphate buffered saline (PBS) (Fisher Bioreagents, Fairlawn, NJ), and bringing to pH 7.0±0.2 with 0.1 M NaOH. The concentration of the neutralized collagen solution was then adjusted to desired range. For solution concentrations <3.0 mg/ml, 1X PBS was added. For solution concentrations >3.0 mg/ml, chilled PureCol™ was dialyzed prior to neutralization against a large volume of 40% poly(ethylene glycol) at 4°C for 8 to 20 hours. The resulting solution (>3 mg/ml) was neutralized and brought to desired concentration by addition of 1X PBS. Collagen solutions thus prepared were kept cold (4°C) at all times to prevent assembly of collagen fibers [50,51].

Rheometry

The rheometry of 2.8 and 7.0 mg/ml collagen solutions at 4°C was investigated in a 2-degree- angle cone viscometer (TA instruments AR2000, Newcastle, DE) fitted with a 40 mm aluminum plate. Solutions that were stored at 4°C for >1 and <3 days after preparation were used in these measurements, to investigate whether aggregation-induced changes in viscosity occurred during storage (and prior to diffusion experiments). Aggregation would increase the solutions' viscosities. Briefly, 650 µl of solution were placed in the viscometer and shear stress was applied in a continuous linear ramp (0.006 to 0.6 Pa), in order to verify the absence of a yield stress within this range. After the absence of a yield stress was verified, a stepped ramp within the same shear stress range (0.006 to 0.6 Pa) was applied, and the sample was allowed to equilibrate at each shear stress before acquiring data.

Diffusion Chamber

The diffusion apparatus comprised a low-volume NaviCyte® single-channel vertical diffusion chamber (Harvard Apparatus, Holliston MA) (Figure 1). The chamber had two separate compartments joined only by a 9-mm diameter channel (d_m). The entire area of this channel was covered by a 6-µm (±10%) thick (L) polycarbonate membrane (Sterlitech, Kent, WA) track-etched with 80(+0%, -20%)-nm-diameter pores (d_p) at a density (ρ_p) of 4×10^8 pores/cm² (±15%). This pore density resulted in an effective area of the membrane (A_e), defined as the area of the membrane that is occupied by pores, of 1.3×10^{-2} cm² (±15%), which corresponds to 2% of the entire membrane area. The pore diameter $d_p=80$ nm was

chosen because it was roughly twice as large as the diameter of an individual collagen fiber in the human cornea (30 nm). Our ultimate goal is the assembly and transport of individual fibers of cornea-like collagen through the pores of the track-etched membrane.

Polycarbonate membranes were pre-wetted in deionized water, and sealed in the channel between the two compartments, which were held together via a C ring. Visual inspection suggested the absence of leaks and consistency of flow rates across experiments confirmed leak-free operation of the chamber.

Membrane Surface Modification

Because the solute may interact with the pores in the membrane via hydrophobic association, in some experiments the membranes' surfaces were modified to increase their hydrophilicity and to reduce polymer/pore interaction by the method of Murthy et al. [80]. Poly(ethylene glycol) (PEG) attachment onto the membrane surface was expected to prevent protein (collagen) adsorption due to the well-documented biological non-adhesiveness of PEG [81,82]. Additionally, a membrane with increased hydrophilicity would potentially reduce bubble formation at the pores [83,84]. These membranes will be referred to as PEG-modified membranes. To generate PEG-modified membranes, the carbodiimide coupling reaction [85] was used to bind a reactive intermediate to the carboxylic acid group in the polycarbonate molecule. This reactive intermediate attached to the primary amine group of amine-terminated PEG of 10-KDa (Nektar, Huntsville, AL). PEG-modified membranes were kept in storage buffer at 4°C for up to four weeks prior to use in collagen transport experiments.

General Transport Experiment

At the start of all collagen transport experiments, one of the chamber's compartments ("collagen side") was filled with chilled collagen solution of concentration C (mg collagen/ml). The second compartment ("saline side") was filled with PBS ($C=0$ mg collagen/ml). The gradient in the concentration of collagen between the two compartments at the start of the experiments was thus ΔC and equal to the concentration of collagen in the collagen side. Three ΔC were investigated (1.0, 2.8 and 7.0 mg/ml). An ice bath was used to chill the chamber and prevent the self-assembly of collagen into fibrils. The contents of each half-chamber were continuously mixed by either recirculation or one-way flow, but no intense mixing to dramatically reduce the size of the unstirred boundary layers [86] was performed. The reasoning behind this approach was an attempt to simulate the conditions under which collagen will be assembled in our printer-like setup where the ability to mix the solutions on either side of the membrane will be impaired. After transport occurred for a period of time t (short enough to ensure a negligible change in the ΔC across the membrane) the contents of each one of the chamber's compartments were collected separately and stored at 4°C for analysis. The concentration of collagen in stored samples was determined via a high sensitivity (5 µg/ml) Sirius-red dye assay (SIRCOL, Biocolor, Ireland). A new membrane was used for each experiment and recovered membranes were gently rinsed with deionized water and stored for scanning electron microscopy.

Determination of effective osmotic pressure and reflection coefficient

Collagen activity—Before the potential gradients could be assigned, it was first necessary to determine the activity of the collagen solution. To find the collagen activity, both sides of the chamber were filled as described in the general transport experiment above; except that the membrane used to separate the chambers was a low molecular weight cut-off membrane (3,500 MWCO; Pierce, Rockford, IL). The low MWCO membrane was used to set the reflection coefficient in Equation 6 equal to 1.0. Each side was filled to the same level within the capillaries, h (in), and the experiment started. Transport of solvent due to osmotic

flow was allowed to proceed undisturbed until the levels of fluid on both capillaries reached a stationary level (defined as no change in h over a 5-minute period). At that point, J_v in Equation 6 becomes zero. The difference in the levels of fluid in the capillaries, Δh , was recorded at the end of the experiment and translated into a pressure head. This pressure head was considered equivalent to the difference in the osmotic pressure of the contents of the chambers $\Delta \Pi$. This procedure was carried out using the three concentration gradients described above.

Reflection Coefficient—Once the activity of the collagen was known, it was then possible to determine the reflection coefficient of the track-etched membrane. To find the reflection coefficient, the previous experiment was repeated using the track-etched 80-nm-pore polycarbonate membranes, and the pressure head required to stop the volume flux, ΔP ($J_v = 0$) was recorded. The reflection coefficient of the track-etched membrane was determined by a linear regression of ΔP vs $\Delta \Pi$ according to Equation 6.

Determination of collagen diffusivity through the membrane

To determine the experimental diffusion coefficient D_m (cm^2/s) of collagen through the membrane, transport experiments were carried out with a pressure head imposed on the collagen side of the membrane. This pressure head was of the precise magnitude required to eliminate the osmotic pressure gradient between the two sides of the chamber and thus prevent the osmotic flow of solvent across the membrane. In this situation the diffusivity can be determined from a regression of J_s vs. ΔC , according to Equation 7.

Pressure driven collagen transport

To both overcome the osmotically induced flow and to enhance the flux of solution and collagen monomer across the membrane pores, one of two hydrostatic pressures (10 or 20 in H_2O) was applied on the collagen side of the chamber. The hydrostatic pressure gradient is referred to as ΔP , where ΔP is the gravity-induced pressure on the collagen side minus that on the saline side. The term ΔP is not the overall pressure difference between the two chambers as it does not take into account the osmotic pressure difference that exists within the two chambers. The fluid levels were maintained in both chambers throughout the duration of the experiment. Figure 2 illustrates the setup of the pressure gradient experiments. On the collagen side, the fluid level was maintained via an L-shaped capillary and the constant injection of collagen solution at a slow flow rate ($\sim 2 \times 10^{-2}$ ml/min) using a syringe pump (PHD2000, Harvard Apparatus, Holliston, MA). On the saline side, the air opening had a relatively large diameter and the changes in fluid level were such that ΔP was maintained to within 5% of its intended value. To maintain the concentration gradient ΔC through the duration of the experiment, the contents of the saline side of the chamber were constantly circulating through a peristaltic pump (Watson-Marlow* Sci-Q 401U/D, England) at 0.1 ml/min. The collagen solution was delivered from a gastight syringe to ensure bubble-free conditions and all fluid lines were kept chilled in order to prevent collagen self-assembly into fibrils. All other procedures were similar to the general transport experiment description above.

Determination of hydraulic conductance

In order to determine the hydraulic conductance of the membrane with intact pores (i.e. unclogged pores), the chamber was setup with fresh untreated or PEG-modified membranes and both sides were filled with deionized water at 4°C . Capillary tubes were attached to the air-lift openings of each one of the chamber compartments. Pressure heads varying from 2 to 15 in H_2O were applied on one arbitrary side of the chamber, and the resulting change in fluid level (Δh) was recorded at 5 s intervals. Curves of water flow (Q , cm^3/s) vs. ΔP were

generated, and the hydraulic conductance L_p of the membrane was calculated according to the equation [87]:

$$L_p = \frac{Q}{\Delta P A_m} \quad \text{Equation 11}$$

The value of the experimental hydraulic conductance calculated using Equation 11 was compared to that calculated using Hagen-Poiseuille flow through the cylindrical pores of a membrane:

$$L_p = \frac{Q}{A_m \Delta P} = \frac{r_p^2 A_k}{8 \mu L} \quad \text{Equation 12}$$

Where μ is the viscosity of water at 4°C (0.016 dyne s cm⁻²) and A_m is the membrane's area. Equation 12 yields a hydraulic conductance of 4.19×10⁻⁹ cm³ dyne⁻¹ s⁻¹.

In addition, the hydraulic conductance was measured for both untreated and PEG-modified membranes using degassed deionized water. The use of degassed water was motivated by the need to determine if transport of solvent through the membranes' pores was being hindered by the presence of nanobubbles at the interface of the pore surface and the liquid [83,84].

Scanning Electron Microscopy

Two of each unused untreated and unused PEG-modified membranes were coated with platinum in a vacuum evaporator (DV-502, Denton Vacuum, Moorestown, NJ) and SEM was performed in a Hitachi S4800 field emission scanning electron microscope (Hitachi, Ontario, Canada). SEM images of these membranes were qualitatively analyzed to determine if PEG-modification caused clogging of the pores. Similar qualitative observations were carried out with used membranes from all experimental groups.

Statistics

Unless otherwise indicated, all results presented correspond to the average ± standard error of the results of n independent experiments. Sample populations were compared using Student's t test after verification of equal or unequal variances. Statistical significance was defined as $p < 0.05$.

RESULTS

Characterization of Collagen Solutions

The radius of gyration for the collagen monomer calculated using the scaled Equation 2, with $a = 8.4 \times 10^{-8}$ cm/monomer and $N = 2.98 \times 10^{-5}$ cm [88] was $r_F = 2.85 \times 10^{-6}$ cm, which is approximately 38% of that reported by Kubota et al. [38]. The hydrodynamic radius r_H of the collagen monomer calculated using Equation 1 and the viscosity of water at 4°C was $r_H = 2.42 \times 10^{-6}$ cm. The overlap concentration C^* calculated with Equation 3 was 9.0×10^{-9} mol/cm³ or 2.70 mg/ml. Since all pressure-gradient experiments were thus carried out within the semi-dilute range where the concentration in the collagen side of the membrane was above C^* , the critical fluxes over the membrane's effective area were calculated using Equation 5. The results were $J^* = 2.62 \times 10^{-3}$ cm/s for $\Delta C = 2.8$ mg/ml and 8.44×10^{-5} cm/s for $\Delta C = 7.0$ mg/ml. Rheological measurements demonstrated no significant differences indicative of monomer aggregation in solutions that were stored for 1-3 days. As observed in

Figure 3, there was no yield stress between 0.006 to 0.6 Pa for either collagen concentration, and shear thinning was observed in 7.0 mg/ml solutions in the 0-1 Pa shear stress range. The results indicated that 7.0 mg/ml solutions were 3.4 ± 0.3 -fold more viscous than 2.8 mg/ml solutions (0.120 ± 0.004 Pa vs. 0.035 ± 0.002 Pa, respectively).

Open pore model

The results for the transport parameters calculated using the open pore model equations [5], Equations 8-10 with $r_F = 2.85 \times 10^{-6}$ cm or $r_H = 2.42 \times 10^{-6}$ cm are presented in Table 1.

Hydraulic Conductance

The experimental values of the hydraulic conductance determined using Equation 11 were $L_p = (6.22 \pm 0.34) \times 10^{-9}$ cm³ dyne⁻¹ s⁻¹ ($R^2 = 0.94$) for unmodified membranes and $L_p = (3.29 \pm 0.25) \times 10^{-9}$ cm³ dyne⁻¹ s⁻¹ ($R^2 = 0.77$) for PEG-modified membranes. The modification of membranes by addition of PEG to the surface thus resulted in a significant (53±7)% reduction in the hydraulic conductance, possibly because of a reduction in the average pore size.

For experiments with degassed water, the results indicated hydraulic conductance values of $(6.67 \pm 0.44) \times 10^{-9}$ ($R^2 = 0.93$) and $(5.37 \pm 0.19) \times 10^{-9}$ ($R^2 = 0.96$) cm³ dyne⁻¹ s⁻¹ for untreated and PEG-modified membranes, respectively. In experiments with regular deionized water, nanobubbles may have reduced the hydraulic conductance value to (61±7)% of the value for degassed water in PEG-modified membranes ($p < 0.05$). The discrepancy between hydraulic conductance values for regular and degassed water indicates some hindrance of solvent flux possibly due to the presence of nanobubbles at the liquid-solid interface.

Osmotic Flow Parameters

A gradient in solution activity between the saline and collagen sides of the chamber was observed and reported as a gradient in osmotic pressure ($\Delta\pi$). The gradient, which caused the osmotic flow of saline into the collagen solution, was a non-linear increasing function of the concentration of the collagen solution. $\Delta\pi$ (dynes/cm²) was 934 ± 75 , $19,616 \pm 1,220$ and $26,154 \pm 3,140$ for $\Delta C = 1.0$, 2.8 and 7.0 mg collagen/ml, respectively (Figure 4).

Because $\Delta\pi$ increased with concentration from 2.8 to 7.0 mg/ml collagen solutions, the effective pressure differential across the membrane in pressure-driven transport experiments varied. The actual pressure differential is equal to the hydrostatic pressure gradient (10 or 20 in H₂O) minus the osmotic pressure gradient. Thus, the effective pressure differential for 2.8 mg/ml solutions was 1.72×10^4 and 4.21×10^4 dynes/cm² for 10 and 20 in H₂O hydrostatic pressure differentials, respectively. In contrast, for 7.0 mg/ml solutions the actual pressure differential was 0.97×10^4 and 3.46×10^4 dynes/cm² for 10 and 20 in H₂O hydrostatic pressure differentials, respectively. Consequently, the pressure differential in 7.0 mg/ml solutions at 10 and 20 in H₂O fluid head was, respectively, ~56% and ~82% of that in 2.8 mg/ml solutions. This must be kept in mind when analyzing the effects of pressure as a driving force in the transport experiments.

The regression of the values of pressure difference at zero solvent flow obtained using a track-etched polycarbonate 80-nm pore membrane vs. those obtained using the low molecular weight cut-off membrane yielded a reflection coefficient $\sigma_0 = 0.52 \pm 0.08$ for untreated membranes (Figure 5).

Diffusivity

The measured diffusivity of collagen through untreated polycarbonate membranes was $D_m = (1.04 \pm 0.08) \times 10^{-9}$ cm²/s, or approximately 1% of the reported diffusivity of collagen in

bulk dilute solution: $7.8\text{--}8.5 \times 10^{-8} \text{ cm}^2/\text{s}$ [36–38]. When D_m was calculated for each value of ΔC using Equation 7 with $J_v=0$, the results were $(4.00 \pm 1.15) \times 10^{-10}$, $(9.24 \pm 3.14) \times 10^{-10}$ and $(1.08 \pm 0.06) \times 10^{-9} \text{ cm}^2/\text{s}$ for $\Delta C=1.0$, 2.8 and 7.0 mg/ml, respectively. When compared to D_0 [37], collagen moved through each individual pore at $0.5 \pm 0.1\%$, $1.1 \pm 0.4\%$ and 1.3% of the diffusivity in free solution for $\Delta C=1.0$, 2.8 and 7.0 mg/ml, respectively.

Comparison to open pore model

For the highly-controlled experiments (where each parameter was isolated) using unmodified membranes, the obtained transport parameters (σ_0 , L_p and D_m) were compared to those calculated via the open pore model [5] (see Table 1). The comparison was based on the ratio between the open pore model value and the experimental value. The results are presented in Table 2. The reflection coefficient and hydraulic conductance predicted by the open pore model were lower than the experimentally-derived values. The diffusivity of collagen through the membrane predicted by the open pore model was comparable to the observed experimental value, in particular when the scaled radius of gyration was used in the pore model calculations. The hydrodynamic radius of the collagen monomer, however, was a better estimate for the molecule's size when calculating the reflection coefficient (based on closeness between the open pore model and the experimental values).

Fluxes of collagen and solvent in response to concentration and pressure gradients

Unmodified membrane—As seen in **Figure 6a**, increasing the concentration gradient had no significant effect on the flux of collagen on experiments with $\Delta P=10$ in H_2O , but resulted in a 7.6-fold increase in collagen flux ($p<0.05$) for experiments with $\Delta P=20$ in H_2O . Increasing the pressure gradient did not enhance the flux of collagen for $\Delta C=2.8$ mg/ml, but for $\Delta C=7.0$ mg/ml it caused a 13-fold increase ($p<0.05$). **Figure 6b** illustrates how the flux of solvent at $\Delta C=7.0$ mg/ml was significantly increased (2.1-fold) by increasing the pressure gradient. This effect was not observed at $\Delta C=2.8$ mg/ml.

PEG modified membranes—With PEG-modified membranes, the transport of collagen was not improved by increasing ΔP on experiments carried out with either concentration gradient. However, increasing ΔC caused a decrease in collagen flux by a factor of 0.23 ± 0.12 at 10 in H_2O ($p=0.001$). This effect was not observed at $\Delta P=20$ in H_2O . For PEG-modified membrane experiments with $\Delta C=7.0$ mg/ml, the increase in solvent flux that resulted from an increased pressure gradient (20 in H_2O vs. 10 in H_2O) was 2.2-fold ($p<0.001$); this difference was not observed for $\Delta C=2.8$ mg/ml. Solvent flux was not significantly affected by concentration gradients in PEG-modified membranes.

Interestingly, the modification of the membrane surface through the incorporation of PEG was the parameter that most strongly influenced the transport of both collagen and solvent through the membrane. At $\Delta C=2.8$ mg/ml, the flux of collagen was one order of magnitude greater: 24-fold ($p<0.001$ and $p=0.07$ at 10 in H_2O and 20 in H_2O , respectively) after PEG-modification of the membrane. However, at $\Delta C=7.0$ mg/ml, PEG-modification of the membrane was not effective at significantly increasing collagen flux for experiments carried out at either pressure gradient. Solvent flux was increased in PEG-modified (vs. untreated) membranes for the combinations $\Delta C=2.8$ mg/ml + $\Delta P=20$ in H_2O (2.3-fold; $p<0.05$), $\Delta C=7.0$ mg/ml + $\Delta P=10$ in H_2O (1.9-fold; $p<0.05$), and $\Delta C=7.0$ mg/ml + $\Delta P=20$ in H_2O (2-fold; $p<0.01$).

The values of J^* (critical flux of solvent through the pore) were compared with all the experimentally observed *solvent fluxes per unit of membrane's effective area* (J_v/A_k). The results are presented in Table 3. It becomes evident that in all cases except for unmodified membranes at $\Delta C=2.8$ mg/ml and $\Delta P=10$ in H_2O , the experiments were carried out with

solvent flowing through the pores at a rate that was higher than the estimated critical solvent flux and therefore polymer deformation/entrainment should have been facilitated.

Equation 6 and Equation 7 were used to predict the fluxes of collagen and solvent based on: (a) the open pore model values for the reflection coefficient, hydraulic conductance and effective diffusivity of collagen through the membrane (Table 1), or (b) the experimentally derived values for the same parameters with D_m independent of collagen concentration and equal to $(1.04 \pm 0.08) \times 10^{-9} \text{ cm}^2/\text{s}$. The predicted J_s and J_v were then compared to the experimentally observed values ($J_{s,predicted}/J_{s,observed}$ and $J_{v,predicted}/J_{v,observed}$). The results indicated that the open pore model transport parameters (**Figure 7a** and **c**) predicted the transport of solute and solvent better than the experimentally derived parameters (**Figure 7b** and **d**). Nonetheless, for the most part the use of Equation 6 and Equation 7 overestimated the transport of collagen and solvent across untreated track-etched membranes. Interestingly, PEG-modification of the membrane surfaces brings the experimental fluxes to values that are closer to the theoretical predictions compared with untreated membranes.

Membrane characterization

The qualitative comparison of digital SEM images from unused membranes revealed that the PEG-modification of the membranes' surfaces did not cause a striking change in appearance of individual pores or in the apparent number of open pores (**Figure 8**). Although the SEM images in **Figure 8** are representative, they do not provide accurate enough information to draw statistical conclusions regarding specific pore size differences, because the processing of samples for SEM deposits a fairly thick layer of platinum on the dried sample. SEM images from used membranes (both with and without PEG-modification) suggested that some of the pores may have been clogged during the transport experiments (not shown).

DISCUSSION

A study to investigate and optimize the transport of monomeric atelo-type I collagen in neutral, cold solution through the nanoscale pores of a polymer track-etched membrane was carried out as part of the development of a specific biomedical device application. In the proposed application, collagen monomers must be transported through the pores of the membrane before assembling into fibrils at the membrane's exit due to a steep temperature gradient. The problem also has relevance to the transport of collagen from secretory vesicles along surface crypts or "fibripositors" during collagen fibril assembly *in vivo*. Although the results presented here are specific to collagen, they may relate to the general transport of long, linear molecules in solution through small pores, a process that is important for several biotechnology applications.

Collagen aggregation/viscosity

The interpretation of the results is complicated by the complex physical chemistry of collagen in solution, in particular by its marked tendency towards aggregation with changes in concentration, temperature and pH [27-32]. Because neutral pH promotes aggregation, using a more acidic solution would have helped ensure that the collagen remained monomeric [25,27]; however, our final application requires neutral pH. One of the results of the present study that suggests monomer aggregation is the departure of the expected behavior of the osmotic pressure as a power function of solution concentration [61] between 2.8 and 7.0 mg/ml. We thus acknowledge the possibility that in spite of preventive measures, there was some association of the collagen in all of our working solutions (and in particular at 7.0 mg/ml), however: (i) our collagen source (PureCol™) is 100% monomeric [79] or has "high monomer content" (according to product insert), and (ii) collagen tends to

remain monomeric in saline solution at the temperature and pH conditions used in the study (4°C, pH=7.0) [44]. Even though fibril assembly intermediates can form in collagen solutions stored at low temperature (0°C); the kinetic energy necessary for the collision of these intermediates and their subsequent conversion into productive fibril nuclei may not be available at temperatures below 7°C [89] (such as the ones used in the present study). In addition, we did not observe significant increases in the viscosity of collagen solutions with time in storage (>3 days) at neutral pH and 4°C, and this observation suggests the absence of significant aggregation. The viscosity of 2.8 mg/ml solutions also suggests absence of monomer aggregation, since during the lag phase of collagen gelation, solution viscosities remain below 0.1 dynes s cm⁻² [89]. However, the viscosity of 7.0 mg/ml solutions (>0.1 dynes s cm⁻²) may be indicative of initiation of monomer association. Therefore, with the caveat that some association could have occurred, our analysis assumes that the working solutions contained mostly monomeric collagen.

Also complicating the investigation was the marked concentration-dependent viscosity. At low concentration (1.0 mg/ml) solutions appeared patently Newtonian with viscosity similar to that of water, while at higher concentration (2.8 and 7.0 mg/ml) solutions did not flow with ease and bubbles dissipated with difficulty. This problematic behavior was markedly more pronounced in 7.0 mg/ml solutions (vs. 2.8 mg/ml), which suggested the possibility that the solution sustained a yield stress; however this possibility was disproven via rheological measurements in the shear stress range 0.006 to 0.6 Pa (Figure 3). In agreement with the orders of magnitude of the viscosities found in our rheological measurements, viscosities on the order of 0.02, 0.07 and 0.75 dyne s cm⁻² have been reported for collagen solutions of 1.0, 2.8 and 7.0 mg/ml, respectively [90]. Consequently, a 1.0 mg/ml collagen solution is approximately twice as viscous as water, a 2.8 mg/ml solution may be 2- to 7-fold more viscous than water, and more strikingly, a 7-fold increase in collagen concentration (from 1.0 to 7.0 mg/ml) results in a 1-order of magnitude increase in viscosity. Although the possibility of such a high viscosity interfering with the transport of collagen across the membrane was anticipated, it was of interest to the final application to investigate the merit of using high concentration gradients across the membrane to increase collagen transport.

Critical overlap concentration

Another complicating factor was the critical overlap concentration (for collagen it is $C^*=2.7$ mg/ml). All of the transport experiments carried out using pressure-concentration gradient combinations were conducted in the semi-dilute range of concentration (2.8 and 7.0 mg/ml). Chains in semi-dilute solutions no longer behave as independent units; instead, neighboring chains affect each other's rotational and linear transport behavior. A potentially useful phenomenon of overlap is the fact that chains in semi-dilute solutions can diffuse across membrane pores at a rate that increases with the concentration [66].

Critical solvent flux

In addition, in contrast to dilute solutions in which the critical solvent flux is independent of the concentration, in semi-dilute solutions J^* decreases rapidly with the concentration. In our experiments, J^* was 2.62×10^{-3} cm/s for $\Delta C=2.8$ mg/ml and 8.44×10^{-5} cm/s for $\Delta C=7.0$ mg/ml solutions. For fluxes of solvent above J^* , the chains elongate in the flow direction near the pore entrance (which induces an extensional flow regime), facilitating their passing through the membrane's pores [61-63,65,77]. The fluxes of solvent J_v (Figure 6) were estimated to exceed the critical solvent flux J^* for all experimental conditions except for $\Delta C=2.8$ mg/ml and $\Delta P=10$ in H₂O across untreated membranes (Table 3, top two rows). With the solvent flux below its critical value, low transport of collagen across the membrane was consequently and non-surprisingly observed (Figure 6a). According to polymer

transport theory [61-63,65,77], when $J_v < J^*$, monomers cannot elongate and retain their larger randomly coiled diameter which is on the same order as the pore diameter in our experiments. For experiments using source concentration gradients of 7.0 mg/ml, the flux of solvent was consistently 2-3 orders of magnitude larger than the critical solvent flux (Table 3). However, this dramatic difference between observed and critical solvent flux did not lead to an equally dramatic (nor consistent) increase in solute flux across the membrane (**Figure 6a**). For example, even though J_v was 137-fold of J^* for PEG-modified membranes at $\Delta P=20$ in H_2O and $\Delta C=7.0$ mg/ml, the flux of solute through the membrane was uncharacteristically low. It is possible that collagen chains were concentrated at the liquid-membrane interface by the large solvent flux (i.e. formation of a high-concentration boundary layer, or concentration polarization [65,77]). Furthermore, it is documented that increasing the concentration of collagen in a confined space (such as the one that would be generated by a viscous solution being “blown” against a membrane by pressure driven flow) results in spontaneous liquid crystalline assemblies that could give rise to monomer associations [91]. This could render transport through the pores more difficult due to increased effective monomer size.

Radius of Monomer

Another consideration is the comparison between the chain's radius of gyration r_F and the pore's diameter d_p . Although r_F for the collagen molecule (2.85×10^{-6} cm) was below d_p (8×10^{-6} cm), both dimensions were within the same order of magnitude. We suggest that this similarity in magnitude, together with the use of semi-dilute solutions may explain why the results of the experiments suggest a departure from the bulk solution behavior expected for $r_F < d_p$ [62]. The solutions used in this study cannot be described as “bulk, dilute” but rather as semi-dilute; where (i) r_F decreases as a power function of the concentration [62] and (ii) single chains overlap and coil into a string of blobs. Within each blob, excluded volume effects are important; the more blobs, the more free energy in the chains [62,63] which would result in higher diffusion rates. All of the above mentioned factors become dominant over the size difference between the radius of gyration and the pore size [66].

Transport Parameters: Experiments vs. Theory

Three important membrane-solution interaction parameters were experimentally derived: hydraulic conductance L_p , reflection coefficient σ_0 and diffusivity of collagen through the membrane D_m . L_p , D_m and σ_0 all showed a noteworthy departure from their theoretically calculated values [5].

Hydraulic conductance—The average of the hydraulic conductance determined using Equation 12 was between 0.6- and 1.3-fold the experimentally derived values for L_p depending on whether regular or degassed water and untreated or PEG-modified membranes were used. The differences between theoretical and experimental values for L_p may be explained by the tolerances on the membrane's physical parameters. With -20%, $\pm 15\%$ and $\pm 10\%$ tolerance for the pore diameter, membrane areal fraction and membrane thickness, respectively, the hydraulic conductance determined using Equation 12 may be as high as 5.36×10^{-9} or as low as 2.09×10^{-9} $\text{cm}^3 \text{ dynes}^{-1} \text{ s}^{-1}$. These maximum and minimum theoretical values for L_p correspond, respectively, to 1.3- and 0.5-fold the average of 4.19×10^{-9} $\text{cm}^3 \text{ dynes}^{-1} \text{ s}^{-1}$. Thus we consider our experimental findings for L_p to be consistent with theory within a reasonable error.

Reflection Coefficient—The reflection coefficient predicted by the open pore model was closer to its experimentally determined value (0.52 ± 0.08) when the hydrodynamic radius was used in the calculation. The radius of gyration is thought to decrease with increasing concentration in the semi-dilute range, thus approaching the hydrodynamic radius. Because

the available value for the radius of gyration in our experimental system is a scaled quantity (not a precise one), the hydrodynamic radius may constitute a better statistical approximation of the collagen molecule's conformation during transport.

Diffusivity—The experimental diffusivity of collagen through the membrane (1.04 ± 0.08) $\times 10^{-9}$ cm²/s was better estimated by using the *scaled* radius of gyration in the open pore model. Collagen diffused through the untreated membranes with an average diffusivity (calculated by linear regression of solute flux vs. concentration gradient) that was approximately 1% of the diffusivity of collagen in free dilute solution [30-33,36-38]. Since the diffusivity of collagen and/or other high molecular weight chains in solution depends on their concentration due to viscous effects, cooperative diffusion and/or polymer chain deformation [31,66,77], we also analyzed concentration-specific diffusivities. For semi-dilute solutions, the expected increase in diffusivity with concentration from 2.8 to 7.0 mg/ml was observed; and furthermore, a 100-150% increase in diffusivity between dilute (1.0 mg/ml) and semi-dilute (2.8 and 7.0 mg/ml) solutions was observed. Although this study includes only one data point in the dilute concentration range (1.0 mg/ml), it is noteworthy to cite Fletcher et al., who reported a maximum diffusivity of collagen in the dilute range at 0.4 mg/ml and a linear decrease in diffusivity up to 1.0 mg/ml [31]. Because to the best of the authors' knowledge collagen diffusivity data available in the literature were determined for the most part using concentrations <1.5 mg/ml [24,29,31,32,34,45], a comprehensive comparison to our results for 2.8-7.0 mg/ml is not possible. However, Obrink [30] reported a diffusion coefficient of collagen in 3.2 mg/ml solutions in the order of 6.0×10^{-8} cm²/s, as well as a decrease in the diffusion coefficient with concentration of the solutions between 1.0 and 3.2 mg/ml. Nonetheless, we suggest that at concentrations in the dilute range (<1.0 mg/ml) the size of the collagen molecule may control the diffusion; with hindered diffusion for molecule effective radius (~20 nm) similar to pore diameter (80 nm) [66]. At increasing concentrations (>1.0 mg/ml), however, the concentration may become the dominant factor, in particular once the semi-dilute range of concentration is reached [66,77]. Therefore, although it was not surprising that for 2.8 and 7.0 mg/ml solutions the diffusion coefficients were 2-to 3-fold that for 1.0 mg/ml solutions, this difference was not as high as the two-orders-of-magnitude differences in diffusivities between the dilute and semi-dilute concentration ranges reported by Guillot et al. [66]. We believe that this may be because our only dilute concentration case (1.0 mg/ml) may actually show some association and behave as a semi-dilute solution, given the complex physicochemical properties of collagen in solution. In particular, we suggest that when working with solutions of collagen, the viscosity and association becomes an important factor in the diffusion and may hinder the movement of molecules. Regardless, the increase in diffusivity with concentration in the semi-dilute range is likely to be due to the partitioning of the solute between the bulk solution and the pores, i.e. collagen chains were entangled outside the pores but stretched inside the pores [66]. One can argue that molecules deform more easily as the concentration increases in the semi-dilute range because the critical flux decreases rapidly with the concentration.

Estimation of fluxes—The phenomenological expressions used to predict solute and solvent flux through a membrane (Equations 6 and 7) tended towards overestimation of the transport of collagen and saline through the nanopores of our untreated track-etched membranes. This is possibly because these expressions assume dilute solutions of non-interacting molecules, as opposed to the case of a semi-dilute solution of a highly associating molecule transporting through a small pore. Furthermore, the high affinity to protein adsorption of hydrophobic membranes such as the ones used in this study may contribute to the initiation of collagen transport through the membrane, and this contribution may have been increased at the higher collagen concentrations. However, this affinity may

have eventually led to clogging of the pores and impaired transport, further explaining the large deviation observed between theoretical and experimental fluxes in the untreated membranes. The coating of the membranes' surfaces with PEG brought the observed solute and solvent fluxes closer to their predicted values, likely because it diminished the interactions between the solute and the membrane's surface.

Membrane modification—PEG-modification of the membranes was carried out with the goal of increasing their hydrophilicity and reducing the adhesion of collagen to the pores which could result in clogging. Treated membranes appeared to have small pores on SEM (Figure 8), likely due to the surface PEG coating. This would have been a concern if the PEG-modified membranes showed a markedly lower collagen transport rates, however, the opposite was true. Thus, if there was a significant effect of the reduction of pore size, it was overrun by the beneficial effect of the PEG coating. Furthermore, there was an unavoidable manufacturer's variation in the pore size (+0%, -20%) and this variation equally affected untreated and treated membranes. Therefore statistical treatment of both sets of experiments should reject the effect of the variation.

When measuring the hydraulic conductance of the membranes used in this study, results demonstrated lower conductance in PEG-modified (vs. untreated) membranes. However, increased hydraulic conductance with PEG-modification was expected because by increasing the hydrophilicity of the surface, the presence of nanobubbles at the liquid-membrane interface would have been reduced [83]. In experiments carried out using degassed deionized water, the hydraulic conductance increased for PEG-modified membranes with respect to regular deionized water. This result suggests the existence of some hindrance of solvent flux which may be attributed to nanobubble formation at the entrance or within the membrane pores. Such nanobubbles have been observed at liquid solid interfaces [83] and evidence of their effects on solid-state membranes with nanoscale pores has been presented [84]. Nanobubbles can have a dramatic impact on hydrophobic surfaces, because much of the membrane surface is not in direct contact with the water and this reduces transport across the membrane. Thus it would be desirable to exert some control over the formation of nanobubbles to enhance the efficiency of membrane transport. The results of this study suggested that even if the PEG-modification of the surfaces partially controlled the formation of nanobubbles, it had no significant effect on increasing the hydraulic conductance possibly because it may have caused a slight reduction in the pore size.

Nonetheless, the incorporation of PEG onto the membranes' surfaces not only resulted in the most improvement in the transport of collagen through the membrane, but also in a transport behavior that more closely approximated the advanced transport models used here. Unexpectedly, working with higher concentration gradients (7.0 mg/ml vs. 2.8 mg/ml) did not significantly improve the transport of solute through the membrane, except for the case of PEG-modified membranes with $\Delta P = 20$ in H₂O. It is likely that the increased viscosity and (possibly) association in the 7.0 mg/ml solutions renders solute transport more difficult in spite of the increased concentration gradient and the decreased critical solvent flux. For varying pressure gradients, significant improvement in collagen transport with respect to pressure was observed only for 7.0 mg/ml solutions in untreated membranes. That improvement was accompanied by a significant increase in solvent transport, also observed in untreated membranes at $\Delta C = 2.8$ mg/ml. In contrast, for PEG-modified membranes, pressure increased solvent flux at 7.0 mg/ml but not at 2.8 mg/ml. The PEG-modification of membranes served to increase the transport of collagen by one order of magnitude in 2.8 mg/ml solutions (regardless of pressure) but not for 7.0 mg/ml. As mentioned before, high viscosity at higher concentrations may have induced concentration polarization [65,77] and

the association of collagen monomers thus interfering with transport through the membranes.

CONCLUSIONS

The lack of suitable models to predict the transport of collagen through nanoporous membranes motivated this study; for our collagen printing application to be successful the availability of empirical data was a necessity. Indeed it was found that the equations used to predict osmotic flow through porous membranes (Equation 6 and 7), even when modified to account for linear molecules in small pores [5], appeared to overestimate the flux of collagen through untreated membranes by as much as one order of magnitude, and the flux of solvent by a factor 2. However, PEG-modification of the membranes resulted in fluxes of collagen and solvent that were more accurately described by these equations. In particular, PEG-modification of the membranes resulted in solvent fluxes that were above predicted values for all concentration and pressure gradients, and in collagen fluxes that were above predicted values for source concentrations of 2.8 mg/ml.

Though this experiment was designed for a particular application, there are some general findings of interest. First, there was an observable, significant and not readily explainable effect of degassing on the transport of saline solution through the pores (particularly for the PEG modified membranes). This raises very important concerns for transport engineers who use hydrophobic membranes with gas containing fluids. It also raises the interesting possibility that membrane transport (perhaps even in living cells) can be modulated by inhibiting or enhancing the formation of channel blocking nanobubbles (perhaps via hydrophilicity modulation). Second, the coating of the polycarbonate membranes with PEG greatly enhanced the transport rate of collagen and resulted in transport behaviour that can be more accurately represented by advanced transport models. This is possibly due to a reduction in the interaction between the pore and the collagen molecule. Finally, the data clearly demonstrate a potential “sweet spot” in the concentration-pressure gradient space where the concentration of transported collagen and the rate of its transport may be optimized.

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Figure 1.
(a) Schematic representation of the vertical diffusion chamber. (b) Photograph of the vertical diffusion chamber setup.

Figure 2.

Schematic of the experimental setup used in pressure gradient experiments. A fluid head of Δh (in) was applied on the collagen side of the diffusion chamber by means of an L-shaped capillary tube and maintained through constant injection of collagen solution via a syringe pump. On the saline side of the diffusion chamber, a peristaltic pump helped recirculate the fluid and maintain a uniform concentration of collagen.

Figure 3. Rheometry of 2.8 (blank symbols) and 7.0 (filled symbols) mg/ml collagen solutions at 4°C. Shear stress was increased in a stepped ramp up to 6 Pa in a 2-degree-angle cone viscometer. Data are representative of a single run for each solution. The solutions used to generate the data in this graph were stored for three days at neutral pH and 4°C prior to rheological measurements.

Figure 4. Gradient in solution activity across the membrane when one side of the chamber was filled with 1X saline and the other side with a solution of collagen of concentration ΔC . The chambers were separated by a low molecular weight cut-off membrane. Data represent average $\pm$ standard error of four independent experiments.

Figure 5.

Linear regression of the pressure difference between the two chambers at equilibrium (zero solvent flow) when the chambers were separated by a low molecular weight cut-off membrane ($\Delta \Pi$) and an 80-nm-pore track-etched polycarbonate membrane (ΔP). Data are the average $\pm$ standard error of a minimum of three independent experiments.

Figure 6. Observed fluxes of collagen (a) and solvent (b) through the track-etched membrane for various experimental conditions. Data are the average $\pm$ standard error of 4 independent experiments.

(c)

Figure 7.

Comparison between the predicted and the observed values for the fluxes of: (a) and (b) collagen, J_s , and (c) and (d) solvent, J_v . Predicted fluxes were calculated using Equations 6 & 7 with transport parameters obtained via the open pore model (a and c) or determined experimentally (b and d). A ratio of 1.0 between the predicted and the observed value would indicate perfect agreement and is represented by the dotted line. Data are presented $\pm$ standard error.

Figure 8. Scanning electron microscopy images of unused polycarbonate track-etched membranes before (a) and after (b) modification of the surfaces by addition of 10-KDa poly(ethylene glycol). Bar is 1 μm .

Table 1

Transport parameters estimated using the open pore model equations [5]

	D_m (cm ² /s)	σ_0	P (cm/s)
for $q_F = 0.59$	1.39×10^{-9}	0.69	0.47×10^{-7}
for $q_H = 0.50$	3.51×10^{-9}	0.57	1.18×10^{-7}

Table 2

Comparison between the values for the transport parameters derived from the open pore model equations and those derived from experimental observations. Results are presented $\pm$ standard error

Parameter	Open pore model/experimental		
	$\sigma_{0, open\ pore}/\sigma_{0, experimental}$	$L_{p, open\ pore}/L_{p, experimental}$	$D_{m, open\ pore}/D_{m, experimental}$
for $r=r_F$	0.75 ± 0.12	0.67 ± 0.20	1.34 ± 0.11
for $r=r_H$	0.91 ± 0.14	0.67 ± 0.20	3.39 ± 0.27

Table 3Comparison between the critical (J^*) and the observed (J_v) flux of solvent

Membrane Treatment	ΔP (in H_2O)	ΔC (mg/ml)	$(J_v/A_k)/J^*$
None	10	2.8	0.78 ± 0.59
	20	2.8	1.67 ± 1.10
	10	7.0	32.33 ± 28.98
	20	7.0	67.89 ± 42.94
PEG-modified	10	2.8	2.19 ± 1.74
	20	2.8	3.75 ± 2.80
	10	7.0	61.10 ± 4.58
	20	7.0	137.17 ± 86.64