Properties of Porcine and Recombinant Human Collagen Matrices for Optically Clear Tissue Engineering Applications

Y. Liu,[†,‡] M. Griffith,[§] M. A. Watsky,[∥] J. V. Forrester,[⊥] L. Kuffová,[⊥] D. Grant,[§] K. Merrett,[†,§] and D. J. Carlsson*[,†,#]

National Research Council Canada, 1200 Montreal Road, Ottawa, Canada ON K1A 0R6, University of Ottawa, Eye Institute, 501 Smyth Road, Ottawa, Canada ON K1H 8L6, University of Tennessee Health Center, Memphis, Tennessee, University of Aberdeen, Department of Ophthalmology, Institute of Medical Science, Aberdeen, Scotland AB25 2ZD, and University of Ottawa, Department of Ophthalmology, 451 Smyth Road, Ottawa, Canada K1H 8M5

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Porcine and recombinant human atelocollagen I solutions were cross-linked with a water soluble carbodiimide at various stoichiometries and collagen concentrations (5−20 w/w %). The resulting hydrogels were clear and, when used as cell growth matrices, allowed cell and nerve visualization in vitro and in vivo. We have previously reported that, after six months of implantation in pigs' and rabbits' corneas, these robust hydrogels allowed regeneration of host cells and nerves to give optically clear corneas with no detected loss in thickness, indicating stable engraftment. Here, the biocompatible hydrogel formulations leading to this novel in vivo performance were characterized for amine consumption, gel hydration, thermal properties, optical clarity, refractive index, nutrient diffusion, biodegradation, tensile measurements, and average pore diameters. Gels with excellent in vitro (epithelial overgrowth, neurite penetration) and in vivo performance (clarity, touch sensitivity regeneration) had 4−11 nm pores, yet had glucose and albumin diffusive coefficients similar to mammalian corneas and allowed neurite extension through the gels.

A variety of synthetic and naturally derived materials have been used to form tissue engineering (TE) scaffolds. Natural soft tissues are hydrogels; thus, synthetic and modified biopolymer hydrogels are an obvious starting point for soft tissue repair or replacement. Collagen-based biomaterials have been widely used in TE.[1−10] Collagen I is now readily available, for example, from bovine, porcine, recombinant, or other collagen sources and is an interesting starting material for TE scaffolds. Specifically, collagen contains the cell adhesion peptide motif RGD, and collagen gives robust hydrogels at modest concentrations as a result of its semirigid-rod, triple helix structure. However, an increased turnover of collagen in vivo after wounding or surgery can be expected to result from matrix metalloproteinases (including collagenase enzymes).[11] These enzymes are upregulated by loss or damage of the surface epithelium and by inflammation.[11] Consequently, any collagen-based TE material should be adequately stabilized to reduce untimely biodegradation. It is well-established that collagen biodegradation can be retarded by chemical cross-linking methods, which also provide materials with enhanced mechanical properties for implantation.[2,4,6,7,9]

Water-soluble carbodiimides (WSCs) are a family of protein cross-linking reagents which promote the formation of zero-length cross-links by facilitating the aqueous-phase reaction between collagen's amine and carboxylic acid side-groups to form covalent, amide bonds.[1,2,6−8] In contrast to other cross-linkers such as glutaraldehyde and diisocyanates, WSCs do not themselves become incorporated as part of the final cross-links in these hydrogels. Hence, there is no possibility of toxic substance release into tissues from subsequent cross-link break down.[2] In addition, all unreacted reagents and byproducts from the WSC reaction are water-soluble and thus can be removed easily after gel formation. Although many WSC cross-linked collagen materials have been previously reported, most of them were formed from very dilute collagen solutions (lower than 1 w/v %), resulting in either very soft gels or insoluble collagen suspensions giving very opaque sponges or highly fibrous materials. Optically clear matrices can be beneficial for cell biology studies of the in vitro and in vivo behavior of cell lines, greatly simplifying characterization by microscopy, even in thick samples such as ocular implants. In recent work[12] on corneal performance of a WSC cross-linked type I porcine collagen implant (9 w/w % collagen), we have shown that in vitro (using established cell lines) and in vivo (rabbit and pig lamellar keratoplasty) these sterile matrices have excellent biocompatibility. Briefly, our porcine collagen matrices allowed epithelial overgrowth, followed by normal stratification in vitro. Furthermore, porcine implants recruited in vivo epithelial and stromal cells from each host animal, leading to integration with the host tissue. In pigs, these implants promoted growth of functional neurites as shown by restoration of touch sensitivity. Porcine implants in pigs and rabbits (24 animals in total) retained their original thicknesses and maintained high optical clarity for over 6 months for these ongoing, in vivo tests as shown by slit lamp, confocal microscopy, and topography measurements. Here, we report the preparation of these clear collagen matrices and some of the properties critical for cell overgrowth and infiltration. To our knowledge, this work is the first reported use of WSC chemistry to cross-link collagen solutions at high concentrations

* To whom correspondence should be addressed. Tel: +1+613-990-3644. E-mail: dave.carlsson@nrc-cnrc.gc.ca.
† National Research Council Canada.
‡ Current address: CooperVision, Pleasanton, CA 94588-4520.
§ University of Ottawa, Eye Institute.
∥ University of Tennessee Health Center.
⊥ University of Aberdeen.
University of Ottawa, Department of Ophthalmology.

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(5−20 w/w %), including recombinant human collagen (rhc), to produce transparent and robust hydrogels suitable for in vitro and in vivo use in cell biology and TE.

Materials and Methods

Materials. The WSC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and albumin fluorescein isothiocyanate conjugate (FTTC-albumin) were purchased from Sigma-Aldrich (Oakville, Ontario). Acid freeze-dried, type I porcine atelocollagen powder was from Nippon Ham or Koken (both in Tokyo, Japan) and type I rhc in acid solution (0.3 w/w %) was from FibroGen, Inc. (South San Francisco, CA), produced by transfected yeast cells (a strain of Pichia pastoris).[5,8] Koken specify that their collagen is 95% collagen I and 5% collagen III. PBS (phosphate buffer saline, pH = 7.2) was prepared from the dry powder (Invitrogen Canada, Inc., Burlington, Ontario). MilliQ deionized water (Millipore, Billerica, MD) was used throughout. All other reagents and solvents were of analytical grade and used as received. Both porcine collagens gave very similar chemical and physical performance and are not differentiated here.

Methods. *Collagen Hydrogel Preparation.* The acid freeze-dried collagen powders were dissolved in water to give 5−20 w/w % concentrations. Solution required stirring for 24 h or longer at 4 °C with a powerful magnetic stirrer, especially for 20 w/w % solutions. Each resulting acidic collagen solution was loaded into a plastic syringe and suspended air bubbles removed by centrifugation at 4 °C to give clear, bubble-free viscous liquids, ready for dispensing and for mixing with the cross-linking reagents. Only acid freeze-dried collagen powders were found to dissolve completely at 4 °C at up to 20 w/w % to give clear solutions. Neutral freeze-dried collagen samples were all found to give opalescent solutions, possibly because of spontaneous reaction of collagen's free amine groups with its acid groups during freeze-drying. Under acidic conditions, these amine groups are protonated, preventing the cross-linking reaction.

The very dilute, acidic rhc solution was concentrated to 10 w/w % by vacuum evaporation at 4 °C, then loaded into syringes as for the porcine collagen samples.

To cross-link collagen, an aliquot of a collagen solution (5−20 w/w %, 0.5−1.0 mL) was loaded into a syringe mixing system free of air bubbles.[4,9] The pH of the collagen solution was adjusted to 5 ± 0.5 (optimum for EDC chemistry) by injection of microliter quantities of 1.0 M aqueous NaOH, followed by thorough mixing under ice water to reduce heating resulting from the energy of mixing. Calculated volumes of aqueous EDC and NHS solutions (both at 10 w/v %, EDC/NHS ratio 2:1 in all reactions) were added from a second syringe through a septum in the syringe mixing system and mixed thoroughly with the collagen solution, then cooled in an ice/water bath. This homogeneous solution was immediately dispensed into polypropylene lens molds (12 mm diameter; 80, 350, 440, or 500 μm spacing) and cured at 100% humidity (at 21 °C for 24 h and then at 37 °C for 24 h). Most cross-linked, cornea-shaped hydrogel samples were removed from the molds after soaking in PBS (pH = 7.2) for 2 h or longer before prying the molds apart. The gels were washed for 4 h in 3 batches of PBS at 4 °C. These fully hydrated hydrogels were then stored in chloroform-saturated PBS to maintain sterility. Storage of hydrogels in chloroform-saturated PBS had no detectable effect on clarity or tensile properties, even after 12 month storage. For comparison purposes, some collagen gels were prepared without addition of a chemical cross-linker, relying on collagen's spontaneous self-gelation propensity (fibrilogenesis) upon incubation of solutions at 37 °C under close to neutral pH conditions.[9]

The EDC-to-collagen stoichiometry is reported in this paper as an [EDC/$-NH_2$] factor, which is the ratio of gram equivalents of EDC to gram equivalents of collagen's primary, ϵ-amine groups (from lysine and hydroxylysine residues) in each gel formulation. The gram equivalents of free amine groups in a collagen sample were calculated by assuming that on average 34−35 amine groups are present per 1000 amino acid residues, both theoretically[13] and from $-NH_2$ measurements (see below).

Mechanical Testing. Tensile properties of the hydrogels were measured on an Instron Tensile Testing Machine (model 1123, cross-head speed 10 mm/min) by suture pull out (Ethilon, nylon 10/0 sutures, 28-μm-diameter black monofilaments from Johnson and Johnson, Markham, Canada) as described previously.[9] This tensile test avoided jaw breaks produced in the fully hydrated hydrogels during direct clamping and drawing. Tensile measurements were performed in triplicate on each gel formulation. Although the failure mode in suture pull out testing is a complex mix of tensile and tearing failure, this method simulates conditions often found during surgical implantation of corneal matrices.

Amine Reaction Quantification. The amine content in gels was measured by the trinitrobenzenesulfonic acid (TNBS) method.[14] Briefly, each collagen hydrogel cross-linked by EDC/NHS at different [EDC/$-NH_2$] levels was put into a separate vial with 4% $NaHCO_3$ and 0.5% TNBS and heated for 4 h at 40 °C, followed by hydrolytic breakdown of the collagen with 6 M HCl at 60 °C. The absorbance of the clear hydrolysate at 345 nm was measured (Hewlett-Packard 8453 spectrophotometer) against a blank, treated in a similar manner but containing no protein. The $-NH_2$ content was calculated from the literature molar absorptivity of 1.46×10^4 cm^1 mole^{-1} for TNBS−lysine.[14] Each reacted amine percentage was obtained from

$$100 \times (1 - [-NH_2 \text{ content post-cross-linking}]/[-NH_2 \text{ content pre-cross-linking}])$$

Hydrogel Hydration Levels. Some hydrogel implants were removed carefully from their molds without presoaking in PBS, immediately weighed, and then placed in PBS to equilibrate to constant weight. Post-gelation increase in water uptake (%) is defined as

$$[(\text{gel wt at equilibrium hydration}) - (\text{gel wt direct from mold})] \times 100/(\text{gel wt direct from mould})$$

The overall (or equilibrium) degree of hydration of the hydrogel implants is defined as that measured after demolding under PBS followed by gel equilibration in PBS for at least 24 h at 4 °C and 2 h at room temperature prior to testing. Hydrogels were removed from the PBS, their surfaces gently tamped dry and then immediately weighed. These samples were then dried at room temperature under vacuum for 24 h or longer to constant weight. The degree of overall hydration was calculated from

$$(\text{wet weight} - \text{dry weight})/(\text{dry weight})$$

To measure the rehydration ratio of fully dried samples, weighed fully hydrated implants were vacuum-dried at room temperature to constant weight, followed by re-equilibration in PBS. The rehydration ratio (%) is defined as

$$(\text{wt of gel after rehydration}) \times 100/(\text{wt of gel at equilibrium hydration})$$

Thermal Analysis. Collagen solutions and hydrogels were examined by differential scanning calorimetry (DSC, TA Instruments DSC 2920) after calibration with indium standards. Each preweighed sample of solution or fully hydrated hydrogel (about 15 mg) was placed in an aluminum DSC sample pan, and then, the pan was hermetically sealed. Sealed pans were heated at 2 °C/min from 20 to 80 °C. The denaturing temperature (T_d) at the maximum of the endothermic peak and enthalpy of denaturing (ΔH_d) were measured.

Optical Properties. As described previously,[9] transmission and backscattering measurements were made in vitro at 21 °C both for white light (quartz-halogen lamp source) and for narrow spectral regions

(centered at 450, 500, 550, 600, and 650 nm) for hydrogels and rabbit or human corneas. The latter were from the Eye Bank of Canada, Toronto, and all five were healthy corneas (center thickness 550 ± 5 μm) from Caucasians, stored for ≤7 days at 4 °C. Because of the dependence of scatter intensity and asymmetry upon the size of the scattering centers, absolute calibration is extremely difficult, and our reported data can only be used for intersample comparison.

Refractive indexes of solutions and hydrogels (fully hydrated in PBS) were measured on samples placed between the prisms of an Abbé refractometer (Bellingham and Stanley, U.K.) at room temperature, using sodium D-line illumination.

Glucose and Albumin Permeability. Diffusion permeability studies were carried out at 35 °C (the human cornea's normal, physiological temperature) using two-compartment diffusion chambers with either air flow or mechanical stirring as described previously.[12] Briefly, each hydrogel (440 μm in thickness) was used as the membrane between the permeate chamber and the PBS-filled (receptor) chamber. The permeate chamber was filled with either a glucose solution or a bovine, fluorescein isothiocyanate (FITC)-labeled albumin solution. The receptor chamber was sampled at 15 min intervals followed by spectrophotometric analysis using either a glucose assay kit[15] or a fluorophotometric analysis procedure for FITC-labeled albumin (495 nm excitation, 519 nm emission).[12]

Hydraulic Permeability. Flow of PBS through each hydrogel under pressure was measured as reported previously.[9] Average pore sizes were derived from the hydraulic permeation coefficient (K_s) and the specific water content of each gel as reported previously for neurite extension studies.[16]

Statistics. All samples were measured for $N \geq 3$. Errors are expressed as ± standard deviations.

Biocompatibility. Some in vitro and in vivo procedures have been described previously.[12] In brief, for in vitro testing, immortalized human corneal epithelial cells were seeded at ~8800 cells/ cm^2 on top of hydrogel pieces ($N = 4$, each 0.57 cm^2). Time to complete colonization of the hydrogel surface by epithelial cells in KSFM and then SHEM media was evaluated. Neurite growth from dorsal root ganglia was visualized on and in gels by immunofluorescence.

In vitro biodegradation of hydrogels and fresh human cornea material was followed gravimetrically, using the approach of Angele at al.[7] Collagen hydrogels cross-linked with EDC and human cornea samples were immersed in DMEM solution augmented to 5 mM in Ca^{2+} ions for 15 h, then each sample transferred to a fresh DMEM/ Ca^{2+} solution containing 5.0 units/mL of collagenase Type I (from clostridium histolyticum, Gibco-Invitrogen, Burlington, Ontario). During incubation at 37 °C, samples ($n = 4$ for each condition) were sequentially removed, treated in EDTA to block enzyme activity, extracted by ×3 in water to remove salts and nutrients, and then vacuum-dried to constant weight. Biodegradation was followed gravimetrically up to ~50% decrease in sample dry weight, at which point all samples were too weak for further handling.

To examine direct suturability of our implants under surgical conditions, collagen matrices were implanted by penetrating keratoplasty (PK) on live mice ($N = 3$). On each mouse, a 1.5-mm-diameter, full-thickness cornea center was removed (~100 μm in thickness) and replaced with a 2-mm-diameter implant (80 μm thickness). The implant was secured with one continuous running suture (11-0 Ethilon, black monofilament, polyamide suture) which pierced both the implant and the adjacent corneal rim. Sutures were left in the transplanted eye for the whole observation period (120 days).

Results and Discussion

In comparison to bovine collagen, porcine collagen implants are expected to be less prone to causing health problems (allergic responses, prion-transmitted diseases, etc.). Furthermore, the use of rhc implants will reduce immunological or allergic reactions and completely prevent the transmission of animal-related infectious agents (especially viral and prion-based).[8] Because of the expected biodegradation in vivo of natural collagen I following surgery, cross-linking is necessary to allow a useful implant lifetime or to allow time for tissue regeneration by recruitment of cells and neurite extension from the surrounding host tissue. WSCs are very effective collagen cross-linkers. This cross-linking procedure involves the activation of the carboxylic acid groups in collagen by EDC to give an *O*-acylisourea derivative, but to minimize side reactions, *N*-hydroxysuccinimide (NHS) is often used as a coreactant.[1] NHS converts the initial *O*-acylisourea reaction product into an activated oxysuccinimide ester. This ester intermediate in turn reacts smoothly and rapidly with primary amine groups in proteins at pH ≈ 5 in aqueous solutions to form the final, stable amide bond, with elimination of *N*-hydroxysuccinimide.[18] In atelocollagen I, the concentrations of carboxylic acid and ϵ-NH_2 groups are 75 and 35 groups/ 1000 amino acid repeats, respectively, assuming complete cleavage of pro- and telopeptide end groups during collagen purification.[13] Chemical identification of EDC-generated, amide cross-links in collagen is very difficult, because only a small number of these new links may be produced in a matrix already highly populated with amide links of the biopolymer's backbone. However, cross-linking by EDC can be indirectly indicated by gelation and effects on the degree of hydration, by the increase in tensile strength and aggregate stiffness, by biodegradation resistance, by denaturing resistance from DSC, and by consumption of collagen's pendant amine (ϵ-NH_2) groups.

The natural cornea is a collagen I hydrogel (constituting ~15% of the fully hydrated weight) cross-linked through glucosaminoglycans (GAGs), which also help control the hydration level of the gel.[19] Vision deterioration or loss from corneal damage afflicts many millions of people worldwide and is currently treated by lamellar or penetrating keratoplasty allografts of fresh, human donor corneal tissue.[20] However, there is a world shortage of acceptable donor corneas, exacerbated by the storage life of the viable living tissue being only 1 to 4 weeks depending on the storage procedure.[21] Consequently, there is an urgent need to develop practical, long-term-storable alternatives to the use of human donor tissue, especially when the risk of disease transmission is taken into account. Furthermore, rejection of donor corneal tissue is quite high, reaching 64% at 5 years after implantation, and innervation is very slow.[22,23] The cornea is the main refractive element of the eye, and as such has several key properties and criteria essential for any artificial replacement. These criteria include high optical clarity (high light transmission and low scatter) and appropriate refractive power (controlled curvature and refractive index), adequate biocompatibility, and toughness to withstand surgical procedures and in-use wear and tear.[9,10]

Although this article is focused on the characterization of the gels, some brief examples of performance in vitro and in vivo suturability are included in addition to the detail already published.[12]

Biocompatibility. Biocompatibility is a critical area for any material for TE. It encompasses a complex array of interacting parameters, including cell compatibility, biodegradation resistance, nontoxicity, nonimmunogenicity, and noninflammatory properties. Selection of a TE material is always an optimization of these parameters, together with mechanical properties. Figure 1A illustrates in vitro, surface colonization by epithelial cells (confluence, that is, complete colonization of the whole surface, occurred in 4−5 days). Epithelial stratification is not shown here, but was previously confirmed in vivo by histology on cross-sections.[12] Figure 1B shows neurite extension over a gel,

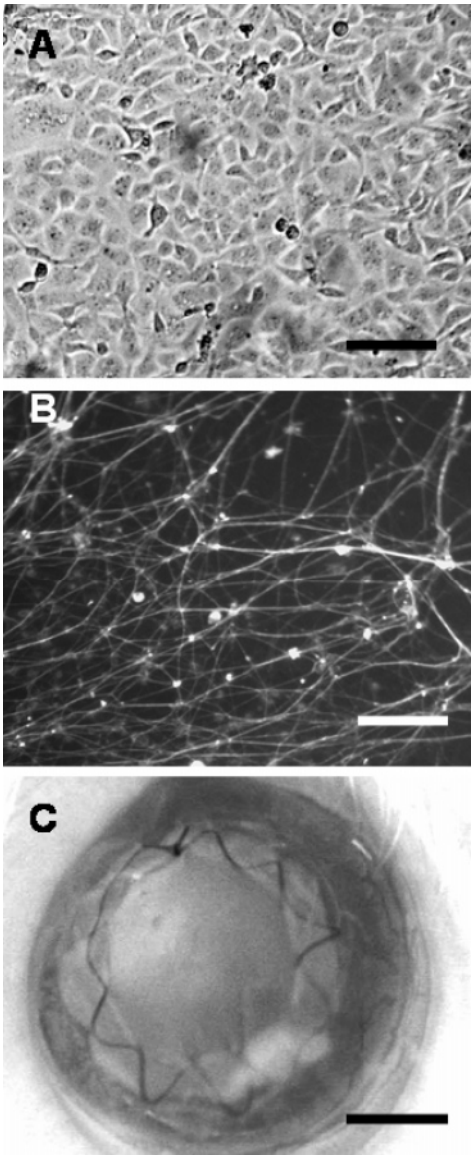

Figure 1. Biocompatibility of collagen−EDC polymers. (A) In vitro corneal epithelial cell attachment and growth to confluence over 5 days (bar = 100 μm) on 9% collagen gel, [EDC/−NH_2] = 0.5 equiv/equiv. (B) In vitro nerve outgrowth from a dorsal root ganglion over the hydrogel surface (bar = 100 μm) on 9% collagen gel, [EDC/−NH_2] = 0.5 equiv/equiv. (C) Mouse cornea immediately after PK operation and surgical suturing of a hydrogel implant (9 w/w % collagen, [EDC/-NH_2] = 0.5 equiv/equiv, 2 mm diameter, 80 μm thickness). Continuous running 11-0 suture, 10 bites. Bar = 1 mm.

and Figure 1C indicates successful surgical suturing of a 80 μm hydrogel implant during PK on a representative mouse cornea. We have previously reported[12] successful in vivo, deep, lamellar keratoplasty on miniature pigs and on rabbits by suturing an implant in place with a Zirm retention bridge overlay of sutures.[17] However, in this procedure, sutures overlayed the implant but pierced only the surrounding cornea. This prior in vivo work[12] on pigs and rabbits provided the rational for the present detailed study to evaluate the underlying properties which lead to the novel in vivo performance. One formulation (9 w/w % collagen and an [EDC/−NH_2] value of 0.5 equiv/equiv) was selected for all in vivo studies on pigs, rabbits, and mice based on the strength and adequate elongation of the resulting implant matrix (Figures 2 and 3).

Our previously reported[12] in vivo and cell biology performance of our collagen−EDC matrices showed that new nerve

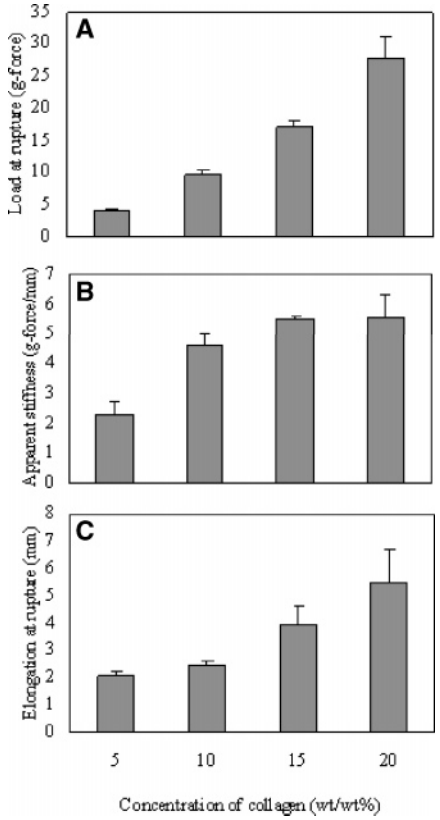

Figure 2. Effect of the collagen solution concentration on tensile properties of hydrogels, [EDC/−NH_2] = 0.5. (A) Load at rupture. (B) Apparent stiffness. (C) Elongation at rupture.

neurites grew to traverse the implant and be functional as confirmed by restoration of touch sensitivity of the eye. In vivo slit lamp and microscopy images recorded from 2 to 6 months postimplantation in 24 animals (16 rabbits and 8 mini-pigs) showed absence of inflamation without the use of any postoperative drugs (apart from an initial pain medication). The implants were optically clear by slit lamp. There were no differences between the implanted and nonoperated eyes in each animal by slit lamp biomicroscopy, and there was no change in refractive power, overall corneal thickness, or topography.[12]

Biodegradation resistance is important for long-term performance. Optically clear materials with a very high resistance to biodegradation, such as pHEMA and polyperfluoroethers, have been promoted as beneficial for corneal implants, but these implants did not promote normal epithelial overgrowth and stratification.[24−26] More cell-friendly materials such as collagen do allow re-epithelialsation and, for our materials, nerve regeneration (Figure 1A,B), but are expected to biodegrade in the long term, even if cross-linked. In vitro biodegradation rates (constant up to ~50% loss in dry weight) by collagenase are listed in Table 4 for several 9 w/w % collagen implants as a function of cross-linking stoichiometry ([EDC/−NH_2] value). (Biodegradation rates for matrices prepared under the cross-linking chemistry conditions but in the absence of EDC/NHS could not be measured because of the very weak nature of these highly opaque materials.) Although high stoichiometries lead to slower biodegradation as expected, such samples were found to be too brittle for surgical implantation. At [EDC/−NH_2] = 0.5, the biodegradation rate is about 10 times faster than the native cornea under the same test conditions. However, what this rate difference will mean in practice for a corneal implant is currently unknown. Extrapolation from the in vitro test conditions to in vivo conditions is currently impossible, and

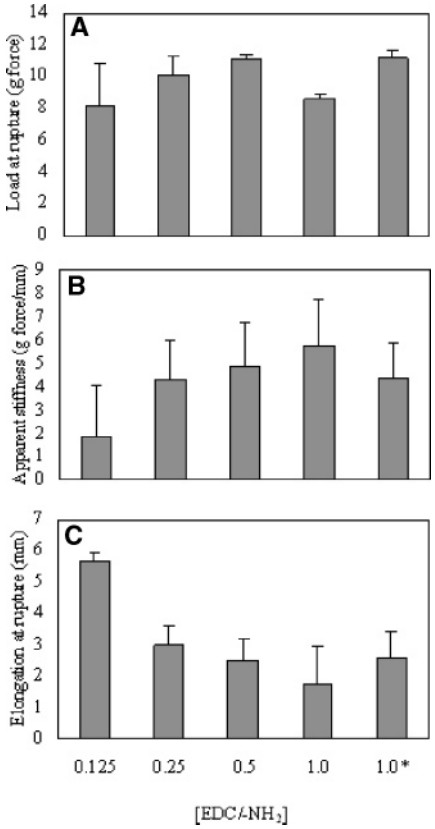

Figure 3. Effect [EDC/$-NH_2$] levels on tensile properties of hydrogels. Collagen concentration in gels = 9 w/w %. (A) Load at rupture. (B) Apparent stiffness. (C) Elongation at rupture. * rhc.

the true test of long-term performance can only come from an in vivo study. Our recent 6 month in vivo study[12] clearly indicated retention of overall corneal thickness, refractive power, and surface topography, very similar to the unoperated (contralateral) eye of each animal, with negligible haze development. Whether this performance results from absence of significant biodegradation over this time period or from progressive implant biodegradation with concomitant regeneration of collagen by the recruited stromal cells remains to be established and is under investigation.

Mechanical Properties. The influence of cross-linking on the mechanical properties of fully hydrated hydrogels was determined with EDC/NHS molar ratio of 2:1 and a pH of 5.0 to 5.5 for all reactions. For [EDC/$-NH_2$] fixed at 0.5, the load at break of collagen hydrogels increased from ~4 g-force to ~28 g-force when the initial collagen solution concentration increased from 5 to 20 w/w % (Figure 2A) and the apparent stiffness increased from 2.3 (g-force/mm) to 5.5 (g-force/mm) for this series (Figure 2B), but when the collagen concentration was increased from 15 to 20 w/w %, the apparent stiffness change was very small. The elongation at break of the hydrogel increased from 2.0 mm to 5.5 mm when collagen concentration increased from 5 to 20 w/w % (Figure 2C). At a fixed collagen concentration in gels (9 w/w %), increasing [EDC/$-NH_2$] resulted in only a slight change in hydrogel strength up to [EDC/$-NH_2$] = 0.5, followed by a decrease (Figure 3A). The apparent stiffness increased progressively with increasing [EDC/$-NH_2$] up to [EDC/$-NH_2$] = 1.0 (Figure 3B). The elongation at break of the hydrogel decreased steadily from 5.7 to 1.7 mm as [EDC/$-NH_2$] increased from 0.125 to 1.0 (Figure 3C). These results suggested that the gels made at [EDC/$-NH_2$] values greater than 1.0 will be brittle (greater stiffness, but lower elongation and load at break).

The increase in tensile strength and apparent stiffness and decrease in elongation at break caused by cross-linking collagen can obviously be explained by the introduction of the amide intermolecular bridges in the matrix. These bridges will hinder the collagen helices, microfibrils, and fibers from orienting during the draw process. Porcine collagen and rhc showed quite similar tensile behavior at comparable collagen concentrations and EDC stoichiometries (Figure 3). Although just strong enough to be handled with forceps, the collagen thermogel (without EDC/NHS) was too weak to allow measurement of tensile properties.

Amine Group Reaction. The TNBS method is specific for the primary amine group of lysine and hydroxylysine.[27] Our measured value for the ϵ-NH_2 content of the porcine atelocollagen (34 ± 3 equiv/1000 amino acid repeats of collagen) is consistent with the theoretical and experimental values for mammalian collagen.[1,7,13] Measurement of ϵ-NH_2 content in collagen before and after EDC/NHS cross-linking showed that the ϵ-NH_2 had been reduced, consistent with cross-link formation in the hydrogel, although only a relatively small percentage of the ϵ-NH_2 groups were consumed. The percentage of reacted ϵ-NH_2 groups in gels with 9 w/w % collagen increased from 6% to 24% when [EDC/$-NH_2$] increased from 0.25 to 1.0 (Figure 4). However, when [EDC/$-NH_2$] was further increased to 2.0, only a 21% consumption of the amine groups was detected. This decrease might result from nonuniform mixing of the very viscous collagen solution with EDC as a result of the very fast gelation (<30 s from mixing) which occurred at ~0 °C at this stoichiometry. Alternatively, the viscosity increase upon gelation at the highest [EDC/$-NH_2$] stoichiometries could have rapidly reached the point where the EDC reaction with collagen $-NH_2$ groups was suppressed. Sung et al. have also reported ~20% consumption of $-NH_2$ groups, but in fibrous collagen tissues after EDC cross-linking.[6] Their amine quantification was based on the ninhydrin colorimetric method, which indicated only 23/1000 amino acid repeats in porcine fibrous collagen before cross-linking. This contrasts with our value of 34/1000 repeats from the TNBS method that is in good agreement with a published value from the TNBS method.[1]

Water Content of Gels. When formed in the mold for all formulations studied, the collagen gels were found to entrap all of the available water (no syneresis detected). Upon immersion in PBS, some of these freshly demolded gels equilibrated to a slightly higher degree of hydration, although this effect decreased as [EDC/$-NH_2$] increased (Figure 5). As [EDC/$-NH_2$] approached 1.0, postgelation water uptake dropped to zero, consistent with the cross-link density having increased to the point where postgelation hydration was completely constrained.

Non-cross-linked collagen can absorb water up to the point where it completely dissolves. The degree of overall hydration of a gel should inversely correlate with cross-link density, because a covalent cross-linked network between the microfibrils and the helices will restrict swelling. For a fixed [EDC/$-NH_2$], the overall degree of hydration decreased progressively as the collagen concentration increased (Figure 6). The overall hydration also decreased when [EDC/$-NH_2$] increased from 0.125 to 0.25 at fixed collagen concentration in the gels (9 w/w %) (Figure 7). However, no significant changes were observed when [EDC/$-NH_2$] increased beyond 0.25. This implies that above 0.25 no further tightening of the collagen network by cross-linking occurred to prevent further swelling by water. The [EDC/$-NH_2$] dependence contrasts with the collagen $-NH_2$ depletion (Figure 3), where at above [EDC/$-NH_2$] = 0.25, the

Table 1. DSC and Hydraulic Permeation Data of Different Concentrations of Collagen before and after Crosslinking[a]

before crosslinking (solutions)			after cross-linking (hydrogels)[b]				
collagen concentration (w/w %)	denature temperature T_d (°C)	ΔH_d collagen (J/g of dry collagen)	collagen concentration in gel (w/w %)[c]	denature temperature T_d (°C)	ΔH_d (J/g of dry gel)	hydraulic permeation coefficient (cm^2)	average pore diameter (nm)
5	37.4 ± 0.5	66.7 ± 2	5.0	48.9 ± 0.5	31.7 ± 5	$(3.5 \pm 1.1) \times 10^{-14}$	11 ± 2
10	36.6 ± 0.5	67.7 ± 2	9.0	48.8 ± 1	32.0 ± 5	$(1.7 \pm 0.8) \times 10^{-14}$	8 ± 2
15	37.6 ± 0.5	71.3 ± 2	12.5	46.6 ± 1	42.4 ± 3	$(0.8 \pm 0.4) \times 10^{-14}$	6 ± 2
20	38.7 ± 0.5	71.9 ± 2	16.0	46.7 ± 0.5	44.3 ± 3	$(0.4 \pm 0.3) \times 10^{-14}$	4 ± 2
13.6[d]	35.8 ± 0.5	62.9 ± 2					

[a] Theoretical ΔH_d = 60−70 J/g for 100% triple helix.[28−30] [b] [EDC/-NH_2] = 0.5. [c] From degree of hydration measurements. [d] rhc.

Table 2. DSC and Hydraulic Permeation Data for Collagen Solutions and for Hydrogels at Different EDC/−NH_2 Levels

[EDC/−NH_2] level	denature temperature T_d (°C)	ΔH_d collagen (J/g of dry collagen)	hydraulic permeation coefficient (K_s) (cm^2)	average pore diameter (nm)
0[a]	36.6 ± 0.5	67.7 ± 2	NA	NA
0[b]	35.8 ± 0.5	62.9 ± 2	NA	NA
0.125[c]	44.3 ± 0.5	46.4 ± 3	$(1.9 \pm 0.7) \times 10^{-14}$	9 ± 2
0.25[c]	43.8 ± 0.5	33.9 ± 3		
0.5[c]	48.8 ± 1	32.0 ± 5	$(1.7 \pm 0.8) \times 10^{-14}$	8 ± 2
1.0[c]	58.9 ± 1	10.6 ± 5		
1.0[d]	55.0 ± 1	15.2 ± 5	$(1.3 \pm 0.6) \times 10^{-14}$	8 ± 2

[a] Collagen porcine solution (10 w/w %). [b] Collagen rhc solution (13.6 w/w %). [c] Porcine collagen concentration in gels 9 w/w %. EDC/NHS = 2:1. [d] Rhc collagen concentration in gels 9 w/w %. EDC/NHS = 2:1.

Table 3. Collagen Concentration Effect on Optical Transmission and Backscatter of Gels[a]

collagen solution concentration (w/w %)	collagen concentration in gel[b] (w/w %)	transmission (white light, %)	backscatter (white light, %)
2.8[c]	2.8[c]	30[c]	20[c]
5	5	90.5 ± 1	0.6 ± 0.1
10	9	90.1 ± 1	1.1 ± 0.2
15	12.5	86.7 ± 1.5	2.9 ± 0.3
20	16	71.7 ± 2	2.3 ± 0.3
human cornea[d]		76.0	6.2

[a] [EDC/-NH_2] = 0.5 except for thermogel. Thickness of implants = 500 μm. [b] From degree of hydration measurements. [c] Thermogel, no EDC cross-linker.[9] [d] For comparison purposes. Thickness of the human cornea = 550 μm.

collagen −NH_2 concentration continued to decrease. These contradicting trends might imply that the observed −NH_2 loss came increasingly from intramolecular reactions as [EDC/−NH_2] increased above 0.25.

Complete drying of hydrogel implants for long-term sample storage and then rehydration just before use may be advantageous for production, shipping, and storage of TE scaffolds and implants. The extent to which rehydration to the predried level can be achieved is shown in Figure 5 as a function of [EDC/−NH_2]. For a fixed collagen concentration (9 w/w %) at the higher [EDC/−NH_2], close to quantitative rehydration was possible; at lower ratios, ~90% rehydration was found. At all stoichiometries, with careful manipulation it was possible to dry and rehydrate cornea-shaped implants completely without their distortion. This means that dried TE matrices or corneal implants could potentially be stored and shipped, facilitating distribution and reducing shelf life problems as compared to donor tissue.

Porcine collagen and rhc showed very similar hydration performance at comparable collagen concentrations and [EDC/−NH_2] values (Figures 5 and 7).

Thermal Properties. Heating of type I collagen solutions or hydrogels leads to denaturing, that is, a largely irreversible transition of the native triple helical structure into a single-chain random coil (gelatin) conformation, readily measured by DSC (Figure 8). For solutions, ΔH_d of porcine collagen and rhc were both close to the theoretical value for 100% triple helix conformation (Table 1). The denaturing transition occurs at a temperature (T_d) dependent on the nature and degree of cross-linking. Introduction of covalent cross-links is known to increase the stability of the triple helix and so increase the denaturing temperature.[7]

To allow the practical analysis of many samples by DSC, a heating rate of 2 °C/min was chosen for all samples. This allowed meaningful intersample comparison, but is appreciably faster than the rate of <0.005 °C /min required to record the absolute endotherm for the very slow denaturing process.[31] Use of a 2 °C /min heating rate will cause the observed T_d values to be ~2 °C above the very slow heating rate values.[31] ΔH_d is largely independent of heating rate and only slightly pH dependent,[28,29] but it decreases rapidly with increasing cross-linking, whereas T_d increases markedly.[7] DSC measurements on collagen *solutions* over the 5−20 w/w % collagen range indicated only slight changes in both T_d (~1 °C) and in ΔH_d (~5 J/g, calculated on the basis of the dry collagen weight in each sample) (Table 1). At a fixed [EDC/−NH_2] value of 0.5, *hydrogels* showed a slight decrease in T_d (~2 °C) and a modest increase in ΔH_d (~13 J/g) when the concentration of collagen in gels increased from 5 to 16 w/w %, but in all cases, ΔH_d was much less than found for the non-cross-linked collagen solutions (Table 1). This increase in ΔH_d with increasing collagen concentration implied that the EDC cross-linking became less efficient, possibly because of increased intramolecular reaction or inefficient mixing caused by fast gelation, as suggested by our hydration data. In contrast, the T_d increased significantly from 36 to 59 °C when [EDC/−NH_2] increased from 0 to 1.0 at a fixed collagen concentration (9 w/w %, Figure

Table 4. [EDC/$-NH_2$] Effect on Biodegradation, Optical Transmission, and Optical Backscatter of Gels[a]

[EDC/-NH_2] level	transmission (white light, %)	backscatter (white light, %)	average rate of biodegradation by collagenase[b,c] (%/h)	biodegradation time (h) to 50% loss in dry weight[c]
0.125	87.1 ± 0.5	0.5 ± 0.1	22 ± 4	2.3 ± 0.4
0.25	87.2 ± 0.5	1.5 ± 0.1	ND	ND
0.5	90.1 ± 1	1.1 ± 0.2	5.5 ± 0.3	9.1 ± 0.6
1.0	79.5 ± 1	2.3 ± 0.2	2.5 ± 0.0.4	21 ± 5
1.0[d]	90.0[b] ± 1	ND	ND	ND
human cornea[e]	76.0	6.2	0.58 ± 0.07	85 ± 11

[a] Collagen concentration in gel 9 w/w %. Thickness of implants = 500 ± 20 μm. [b] Incubated at 37 °C with 5.0 units of collagenase Type 1. Biodegradation rate expressed as the percentage loss in dry weight of collagen/initial dry weight in each hour. [c] For all samples, loss of dry weight was measured at four points up to ~50% dry weight loss; weight loss increased linearly over each indicated time period. [d] rhc. [e] For comparison purposes, center thickness of the human cornea = 550 ± 5 μm.

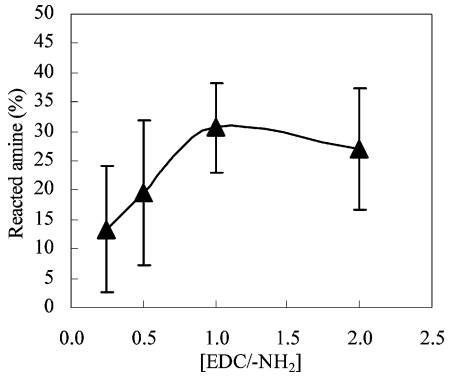

Figure 4. Effect of [EDC/$-NH_2$] level on extent of collagen's amine group reaction. Collagen concentration in gel = 9 w/w %. Before cross-linking, collagen amine concentration = 35 ± 3 equiv/1000 amino acid residues.

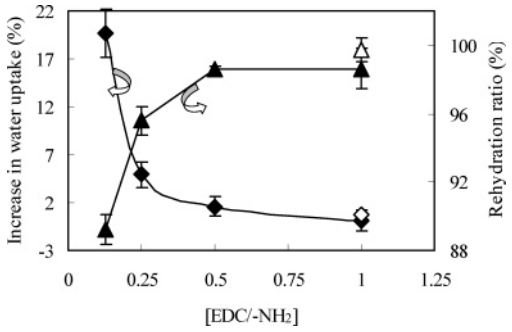

Figure 5. Effect of [EDC/$-NH_2$] levels on hydrogel rehydration and post-gelation hydration. Collagen concentration in gel = 9 w/w %. Diamonds: on rehydration from dryness. Triangles: increased water uptake after gelation. Closed symbols: porcine collagen. open symbols: rhc.

8 and Table 2). Furthermore, in these gels ΔH_d decreased dramatically from 67 to 10.6 J/g of dry collagen. This is consistent with the collagen triple helices in the gel being more difficult to denature when [EDC/$-NH_2$] was increased, presumably because the cross-link points blocked the unzipping of the collagen triple helix.[7]

Yang et al. have reported a T_d of ~38 °C for very dilute (0.02 w/w %) solutions of their rhc at pH 7.0 by circular dichroism at 0.1 °C/min heating rate.[8] This T_d is somewhat higher than our value (35.8 °C) for our 13.6 w/w % solution concentrate at pH 1.5. This T_d difference might result from a combination of differences in concentration, pH, and heating rate. Collagen solutions (non-cross-linked) in general had narrower denaturing endotherms than hydrated, cross-linked gels consistent with solutions being more homogeneous than gels

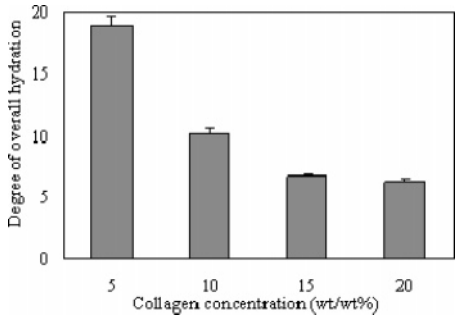

Figure 6. Effect of collagen concentration on degree of overall hydration of hydrogels. [EDC/$-NH_2$] = 0.5.

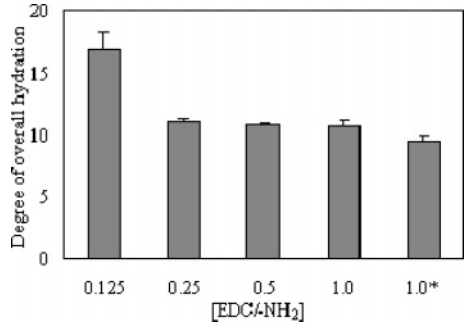

Figure 7. Effect of [EDC/$-NH_2$] levels on degree of overall hydration of hydrogels. Collagen concentration in gel = 9 w/w %. * rhc.

where the endotherm broadened and moved to higher temperature as the degree of cross-linking increased (Figure 8). Cross-linking of lower concentrations of collagen (5 w/w % and below, data not shown) gave hydrogels with symmetrical endotherm peaks, while cross-linking of higher concentrations progressively increased asymmetry toward the high-temperature side. Porcine collagen and rhc solutions and gels showed very similar DSC behavior at comparable concentrations (Figure 8, Tables 1 and 2).

Optical Properties. The optical transmission and backscatter of the collagen hydrogels and human corneas were determined for white light and at several incident wavelengths (latter data not shown) by the same methods.[9] Transmission and backscatter for white light are compared in Tables 3 and 4 for collagen gels of increasing concentration and for increasing [EDC/$-NH_2$], respectively. As the collagen concentration increased at a constant [EDC/$-NH_2$] value of 0.5, the transmission decreased. The highest transmission was obtained from hydrogels made from a 10% collagen solution at an [EDC/$-NH_2$] of 0.5 as [EDC/$-NH_2$] changed from 0.125 to 1.0. The transmissions of most of the gels were higher than for the human cornea, and the backscatter percentages of all the gels were lower than for

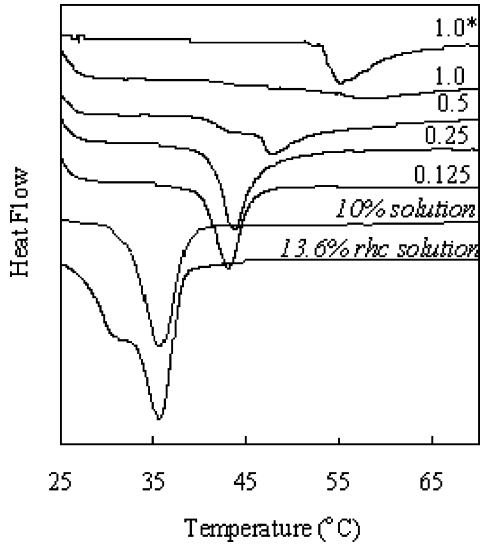

Figure 8. Effects of EDC/$-NH_2$ levels on DSC of cross-linked hydrogels. Collagen concentration in gel = 9 w/w %. Labels on curves indicate sample composition. All samples are ~15 mg of solution or gel.

Table 5. Refractive Indices of the Collagen Hydrogels[a]

for [EDC/-NH_2] fixed at 0.5		for collagen concentration in gel fixed at 9 w/w %	
concentration of collagen (%, w/w)	refractive index of hydrogel	[EDC/$-NH_2$] g equiv ratio	refractive index of hydrogel
5	1.3412 ± 0.001	0.125	1.3489 ± 0.002
9	1.3505 ± 0.001	0.25	1.3500 ± 0.001
12.5	1.3570 ± 0.001	0.5	1.3505 ± 0.001
16	1.3649 ± 0.001	1.0	1.3512 ± 0.001
		1.0[b]	1.3511[b] ± 0.001

[a] Thickness of implants = 500 μm. For comparison, refractive indices: human stroma = 1.373−1.380; PBS = 1.3370. [b] rhc.

the human cornea. Gels of rhc had high white light transmission (Table 4). Collagen gels prepared without EDC/−NHS (thermogels resulting from incubation at 37 °C) were very opaque (low transmission, high backscattering) (Table 3). Presumably, this results from refractive index fluctuations caused by aggregation of collagen into fibrils with thicknesses comparable to wavelengths of visible light.[32]

The refractive indices of the hydrogels increased progressively as the collagen concentration increased, but remained largely unchanged for a fixed collagen concentration in the gels (9 w/w %) as [EDC/$-NH_2$] changed (Table 5). The refractive index of an implant depended both on the high refractive index of dry collagen (1.547) compared to PBS (1.335) and on the increased collagen contribution to the solids volume fraction of the gel.[19] EDC cross-linking chemistry adds little structural change, being zero length, accounting for the virtual absence of refractive index change with increased [EDC/$-NH_2$] stoichiometry. Our cross-linked hydrogels at 16 w/w % collagen in the gels had a refractive index (1.365, Table 5) a little lower than that of the natural stroma (1.376, ~15% collagen).[19] The lower value in EDC cross-linked collagen hydrogels possibly results from the absence of GAG chains found in the natural stroma and from the absence of the highly ordered natural stromal structure. The optical properties of the hydrogel implants were unchanged by drying followed by rehydration. At 9 w/w % collagen in gels, porcine collagen and rhc both had very similar refractive indices (Table 5).

Glucose and Albumin Permeability. Matrix permeability is critical for healthy encapsulated cells and for the anterior epithelial layer of the cornea, because glucose and other nutrients such as albumin and other small proteins must diffuse to the cells. In the case of the avascular cornea, nutrients diffuse largely from the aqueous humor and to a lesser extent from the limbal vasculature.[33] The glucose diffusive permeability coefficient was $(2.7 \pm 0.8) \times 10^{-6}$ cm^2/s at 35 °C for cross-linked collagen hydrogels made with 9 w/w % collagen and an [EDC/$-NH_2$] of 0.5. This is comparable with the estimated human cornea permeability coefficient ($\sim 2.5 \times 10^{-6}$ cm^2/s).[34] The albumin diffusion coefficient was found to be $(1.6 \pm 0.6) \times 10^{-7}$ cm^2/s for a 9 w/w % collagen hydrogel with [EDC/$-NH_2$] of 0.5. This is much lower than for glucose, as is to be expected for a protein of 67 kDa molecular weight, but close to the value (2.2×10^{-7} cm^2/s) reported for human serum albumin diffusion through rabbit corneas.[35] The relative ratio of glucose-to-albumin diffusion coefficients for collagen gels (~20:1) is consistent with values reported for other membranes, but can be pore size dependent.[33]

Hydraulic Permeability Coefficient and Pore Diameter. Permeability coefficients (K_s) are listed in Tables 1 and 2 and were largely independent of the hydrostatic pressure (10−100 cm liquid column). K_s decreased steadily with increasing collagen concentration at a fixed [EDC/$-NH_2$] value of 0.5 but was little affected by increasing the [EDC/$-NH_2$] value from 0.125 to 0.5. Average pore diameters were calculated from K_s (Tables 1 and 2) and the measured specific water content at equilibrium hydration (Figures 6 and 7). Pore diameter values in Tables 1 and 2 are useful for intercomparison, but should not be viewed as absolute because of the inherent assumption, especially of cylindrical, parallel channels through the hydrogels, in the derivation of the average pore diameters.[16,36] However, all methods for estimation of average pore diameter have been suggested to be problematic.[16]

Comparison with Other Collagen-Based, Transparent Matrices. We have previously reported the use of collagen I cross-linked with a *N*-isopropyl acrylamide (NiPAAm) copolymer (designated TERP), designed to spontaneously react with the pendant amine groups of collagen.[4,9] Quite robust hydrogels were produced at modest collagen concentrations (~4 w/w %), with good clarity (no opacity at up to >55 °C) and encouraging short-term in vivo implantation results when used for lamellar keratoplasty in pigs.[4,9] Very rapid regrowth of functional nerves was particularly novel. The amine-reactive site in this copolymer was an acryloxysuccinimide ester repeat, which essentially is the carbodiimide/NHS reaction intermediate. The synthetic copolymer increased the hydrophilicity of the matrix, functioning somewhat like the natural GAG materials. Although implants from these formulations could withstand careful suturing, mechanical properties were quite poor. Increasing the collagen concentration in the starting solutions to improve toughness was not practicable, because opacity problems ensued. These were traced to phase separation caused by the insolubility of the synthetic copolymer at concentrations required for adequate cross-linking at collagen solution concentrations above 5%. However, our matrix of collagen cross-linked by direct EDC/NHS chemistry in water yielded materials that were equally cell-friendly and optically very clear, even at 9−16 w/w % collagen concentration in gels (Tables 3 and 4). Consequently, collagen I alone is adequately hydrophilic and cell-attractive to allow epithelial cell overgrowth, keratocyte ingrowth, and neurite extension, provided that the matrix has a high enough permeability to nutrients.

EDC cross-linked atelocollagen hydrogels reported in this study were highly transparent, probably because gelation occurred in less than 60 s from mixing, and prevented the collagen auto-association process (fibrillogenesis) that is known to result in aggregates of collagen fibrils[37] which scatter light.[32] Stress at rupture was a maximum for EDC cross-linked collagen at [EDC/$-NH_2$] = ~0.5, whereas for the polymeric cross-linker (TERP), stress at rupture was a maximum when the TERP acryloxysuccinimide group to collagen $-NH_2$ group ratio was ~1.5:1.[9] This might result from the diffusive differences between a small molecule reagent and a polymeric reagent. Load at rupture and aggregate stiffness were both much higher for collagen/EDC as compared to collagen TERP (load at rupture 28 and ~5 g-force, aggregate stiffness 5.5 and 1.5 g-force/mm at collagen concentrations in gels of 16 and 2.4 w/w % for collagen/EDC and collagen/TERP, respectively). However, these differences can be attributed both to greater cross-linking efficiency and to higher collagen concentrations for the collagen/EDC materials. The glucose diffusive permeability constant at 35 °C was lower in the WSC cross-linked collagen gels (2×10^{-6} cm^2/s) as compared to the TERP cross-linked gels (4×10^{-6} cm^2/s)[9] as expected from the much higher collagen concentrations in the former gels. This is consistent with the average pore diameters being much smaller for the WSC cross-linked gels (4−11 nm, Tables 1 and 2) as compared to the TERP gels (60−70 nm), measured by hydraulic permeation in both cases. Glucose and albumin diffusion were however adequate for epithelial cell health in vivo in EDC cross-linked gels and in the range of that for the animal corneas. The rate of neurite extension through WSC cross-linked collagen gels (~18 μm /day for 9% collagen gel with [EDC/$-NH_2$] = 0.5) was only slightly slower than that observed for neurite extension through a collagen−TERP gel (~25 μm /day).[4]

Chen et al. have reported in vitro and in vivo results for collagen I−chitosan−sodium hyaluronate ionic complexes as cornea implants.[10] Some of these complexes had excellent light transmission and biocompatibility when implanted in corneal stromal pockets in rabbits. Endothelial and epithelial cell attachment was found in vitro, although full stratification of the epithelium was not reported. Although chitosan was reported to protect collagen I from collagenase digestion, extensive degradation of the ionomer matrices was found after 3 months implantation. This might result from the reversibility of formation of the ionomer complex, rather than direct enzymatic attack.

The type I rhc used in our matrices was made from a "multigene process" which generates both of the collagen chains required for the triple helix [two α1(I) chains and one α2(I) chain] as well as coexpression of human prolyl 4-hydroxylase enzyme (P4H).[8] This enzyme causes the hydroxylation of proline groups to the level in natural collagen, critical for thermal stability of the triple helix.[30] This makes rhc a desirable material for TE scaffolds and for corneal implants if its gel properties (especially optical and toughness) can be controlled. The rhc is reported to be 90−95% triple helix "monomer", whereas native collagens can contain over 50% of dimer and trimer aggregates.[8] Although pepsin-treated during production, rhc contains a portion of the terminal telocollagen sequence on each triple helix as compared to the porcine atelocollagen, which is free of telocollagen sequences.[5] Both of these differences from porcine collagen (monomer purity and telocollagen composition) could influence production of tough, clear matrices. In fact, tensile (Figure 3) and optical (transparency, Table 3; refractive index, Table 5) properties were very similar to porcine atelocollagen matrices at the same final collagen concentration.

Our DSC results confirmed that the thermal stability of the rhc solution and gel were comparable to those of the porcine material (Figure 8, Table 1). Because of the observed similarity in chemical, physical, and in vitro properties of porcine collagen and rhc, acceptable in vivo performance of rhc is expected and is currently under study. Yang et al. have already reported production of EDC cross-linked rhc materials, but in the form of membranes and sponges for bone, cartilage, and skin TE scaffolds.[8] Also, Olsen et al. have discussed the use of opaque membranes and sponges from self-assembled (thermogelled) rhc for vascular grafts, for coatings, and for drug delivery.[5]

We are currently unable to differentiate in vivo between negligible biodegradation of our implants or regeneration of the matrix by recruited cells. However, our in vivo data[12] clearly indicated persistence of a functional cornea for the 6 months duration of our in vivo animal studies. Completely biodegradation-resistant corneal implants (pHEMA, polyperfluoroethers, etc.) have been reported,[24,25] but do not support the natural regrowth of the stratified epithelium or nerves. Interestingly, collagen I-coated polyfluoroether implants have been used to resolve this problem.[38] In vivo use of these coated synthetic polymers leads to the question of the resistance to biodegradation of the thin (5−10 nm) surface coating.

Conclusions

Although the use of TE scaffolds based on bovine collagen are widely reported, the potential risks with the use of bovine collagen, such as the possibility of transmitting diseases (viral and prion-based, for example) and many immunological or allergic reactions, are a significant drawback for manufacturing TE biomaterials based on this material. Our successful production of matrices from porcine collagen and rhc indicate that these materials could serve as an alternative approach, minimizing medical risks in, for example, cornea implantation. We have demonstrated that the properties of the cross-linked, rhc, and porcine collagen hydrogel matrices can now be controlled at physiological concentrations (10−15%) by variation of the concentration of the collagen solution and the ratio of EDC to accessible amine groups in the collagen solution. In general, property changes correlated logically with increased degree of cross-linking as measured by amine group consumption, but each parameter had its own peculiarities. Implants of porcine collagen produced in this study showed increasing biodegradation resistance with increasing EDC/−NHS cross-linking and excellent in vivo persistence of optical, thickness, and topographical properties.[12] Both porcine collagen and rhc matrices had very good transparency and mechanical properties, strong enough to withstand the suturing procedure for implantation. Such materials could be a partial answer to shortfalls in human donor tissue and may possibly lead to regeneration of most corneal functions.

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