Collagen gel systems for sustained delivery and tissue engineering

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Abstract

Collagen gels are flowable, suggesting the possibility of an easily injectable, biocompatible drug delivery matrix. Sustained release of therapeutic molecules from collagen matrices, however, is beset with difficulties. Fibrillar collagen gels have an effective pore size of several tens of nanometers, too large to control release by hindered diffusion. To control release, it is necessary to rely on binding of the active agent to collagen, either by covalent or non-covalent bonds, or on sequestering in a secondary matrix. Such steps rapidly increase the complexity of the system. Non-fibrillar collagen has a lower effective pore size (4–6 nm), but it dissolves rapidly in vivo (~ 24 h). For tissue engineering applications, collagen gels are more attractive, since they can act as a “cage” to retain cells or as gene delivery complexes, which are larger than drugs and therapeutic proteins. The gels have limitations in terms of strength, but reinforcement with solid components and alignment during gelation and culture can improve performance.

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Keywords: Collagen gels; Fibrillar; Non-fibrillar; Hindered diffusion; Cell delivery; Tissue engineering

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1. Introduction

Collagen gels at first sight seem to be promising matrices for drug delivery and tissue engineering. They are visco-elastic; i.e. they are semi-solid when at rest, but can be induced to flow under stress (e.g., extrusion from a syringe) [1–4]. In addition, they exhibit good cell and tissue compatibility [1,5–7], and thus should not interfere with normal function at the site and systemically.

With respect to drug delivery, one can readily envision applications in which collagen plus active agent are injected into the tissue site of interest, the agent then being released in a controlled manner. The most readily available forms of such injectable collagen gels are (1) injectable suspensions of collagen fibers [1,3] and (2) non-fibrillar, viscous solutions [4] in aqueous media. Small molecule drugs and therapeutic proteins can be mixed with the fibrillar suspensions or viscous solutions. Some of the release properties of these mixtures will be described below. The major issue, as will be seen, is that the collagen network is too open to retain most active agents of interest. Secondary mechanisms of retention must be employed, with added complexity and decreased ease of use.

Collagen gels have also been employed as scaffolds in tissue engineering, and as delivery matrices for cells and genes in gene therapy. Here, happily, the collagen network easily retains cells, by physical entrapment.

In order to fully exploit the potential of collagen materials for drug delivery and tissue engineering, it is helpful to understand the structure of collagen and how to modify it, physically and chemically, to obtain desired results. The limitations and advantages of collagen gels will be presented, along with some suggestions for improving performance. Reviews covering some aspects of the subject exist [8–10]; and there are many papers which describe the use of solid-form collagen: sponges, pellets, membranes, and plugs; often cross-linked chemically, by heat, or by irradiation. This review will be confined to non-cross-linked collagen gels which can be rendered flowable under appropriate imposed stress.

2. Collagen materials: preparation, properties, and availability

2.1. Fibrillar collagen (FC)

Type I collagen protein has been studied and characterized for more than 50 years. Schmitt et al. [11,12] developed methods for isolating and purifying collagen in high yield from bovine skin, using the enzyme pepsin. This method is still preferred for obtaining a highly purified solubilized collagen, which under proper conditions can yield medical grade soluble and fibrillar collagen, suitable for human implantation. In 1958, Gross and Kirk [13] showed that acidic solutions of collagen (at 0.2 to 2 mg/ml) could be adjusted to physiological ionic strength and pH in the cold (less than 10 °C), and then warmed to 30–34 °C for an hour, forming a translucent collagen gel, which is relatively firm, but friable under applied stress. (These are referred to as “heat gels”, HG; see below for a discussion of flowability.) McPherson et al. [14] showed that acidic solutions of collagen (at 0.2 to 2 mg/ml) could be adjusted to physiological ionic strength and pH in the cold (less than 10 °C), and then warmed to 30–34 °C for an hour, forming a translucent collagen gel, which is relatively firm, but friable under applied stress. (These are referred to as “heat gels”, HG; see below for a discussion of flowability.) McPherson et al. [14] showed that acidic solutions of collagen (at 0.2 to 2 mg/ml) could be adjusted to physiological ionic strength and pH in the cold (less than 10 °C), and then warmed to 30–34 °C for an hour, forming a translucent collagen gel, which is relatively firm, but friable under applied stress. (These are referred to as “heat gels”, HG; see below for a discussion of flowability.) McPherson et al. [14] showed that acidic solutions of collagen (at 0.2 to 2 mg/ml) could be adjusted to physiological ionic strength and pH in the cold (less than 10 °C), and then warmed to 30–34 °C for an hour, forming a translucent collagen gel, which is relatively firm, but friable under applied stress.
collagen/ml. Such fibrillar suspensions could then be mixed with additional sodium phosphate and sodium chloride to a final concentration of collagen of 30–40 mg/ml and a salt environment of 20 mM phosphate and 130–150 mM sodium chloride, pH 7 (referred to as FCS). Blending and screening of the FCS was then performed to obtain a uniform, viscous paste which could be extruded through small gauge needles, in some cases easily flowing through needles 1 cm in length and approximately 150 μm (30 gauge) inside diameter [1,15].

In general, polymer gels consist of an interconnected network of polymer chains, reaching infinite molecular weight, plus accompanying solvent; the connections create the elastic character of the gel [16]. Gels in which the connections are not covalent (i.e., gels which are not cross-linked) can also have viscous properties, since, under stress, the molecular chains can slide past each other [17]. FCS possess gel properties because they consist of entanglements of collagen fibers, which are quite variable in length and thickness, suspended in aqueous buffer [3,18,19]. The fibers attract each other through hydrophobic and electrostatic bonds [3,20,21]. Under flow conditions, such as stirring in a vessel with a paddle agitator, extrusion from a tube or syringe, or pumping through tubing, the fiber entanglements yield to the imposed stress and exhibit apparent shear-thinning behaviour [1,22]; once the suspension is at rest, the fibers again entangle and exhibit gel-like behavior [23]. The behavior is temperature dependent, the gel being easily fluidized at 10 to 25 °C, but becoming relatively rigid after incubation at 30–37 °C [3]. The latter behavior is advantageous for delivery into warm-blooded animals, since the gel can be easily injected at room temperature, but after an hour in tissue it becomes more rigid, resisting displacement from the tissue site. In the case of injectable collagen fibers, the porosity of the gels is quite large, the “mesh size” being estimated to be several tens of nanometers [18,19]. In the case of HG collagen, the gel is so firm that imposed stress will “fracture” the gel and irreversibly dis-assemble the network connections [3,23].

2.2. Non-fibrillar collagen (NFC)

FCS can be dialyzed versus 0.01 M HCl (at 5–10 °C) to yield a viscous, transparent molecular collagen at about 20 mg/ml [4,18,19,23]. During dialysis, the bag swells, diluting the collagen from the original 30–40 mg/ml. Such highly viscous, non-fibrillar solutions can be neutralized by adding sodium hydroxide, with stirring on ice, and monitoring the pH with indicator paper. If warmed, these transparent gels will slowly form fibers. Stabilized non-fibrillar collagen can be prepared by succinylation (referred to as SC) [24,25] or methylation (referred to as MC) [24] of the collagen. Succinylated collagen has many of its lysine side chains converted to succinate esters. The molecule possesses a net negative charge at neutral pH. Methylated collagen is the reverse, in which aspartate and glutamate side chains have been modified to the methyl ester, thus removing carboxyl side chains; charged amino side chains remain, and the molecule has a net positive charge (see Fig. 1). These viscous, transparent gels do not convert to the fibrillar state at neutral pH because of charge repulsion. They are gels because the rod-like collagen molecules are entangled. The molecules are much thinner than fibers, and the resulting porosity, or “mesh” of the gel is finer, about 4 to 30 nm, depending on concentration and additives [19,26]. Since the gels are true solutions of collagen in aqueous media, they also are injectable through fine-gauge needles. The injection force is higher, compared to fibrillar collagen at the same concentration (unpublished measurements, Collagen).

Fig. 1. Collagen species of interest. (A) Collagen fibrils in neutral aqueous buffer. (B) Collagen molecules in solution; cold, neutral pH. (C) Methylated collagen with charged amino groups displayed (neutral pH). (D) Succinylated collagen with charged carboxyl groups displayed (neutral pH). (E) Collagen molecule tethered through a flexible polymer chain (such as PEG) to an active agent (black circle).
2.3. Collagens not treated with pepsin

Medical grade collagens can also be prepared from cowhide which has been extensively treated with calcium hydroxide, and other metal salts, including sodium sulfate at high pH \cite{27,28}. Treated hide is further washed with ethanol and water (very extensively) to remove fats and proteins digested by the strong base. The process is complete when the pH of the hide and water reaches 7–8 and remains there. Collagen fibers are never dissociated during this purification method. To generate a gel-like collagen, one must then comminute the purified hide collagen to filaments or particles, preferably filaments. Fragment sizes should be no more than 1–2 mm in the longest dimension. Then the fibers can be swollen in acid (dilute HCl or acetic acid) for several days, followed by vigorous shear homogenization. The solids content may be adjusted to 1% (w/w) or less to achieve manageable viscosity. Such preparations can then be returned to neutrality, and they will be extrudable through orifices or tubes, but achieving easy extrusion through standard syringe needles may be difficult. It is noted that collagen treated with strong alkali will undergo conversion of asparagine and glutamine amino acid side chains to form aspartic and glutamic acid, lowering the isoelectric point, relative to acid-extracted collagen.

Collagen can also be isolated from tissues of young animals (usually calfskin \cite{11,13} or rat tails \cite{29}) by simple extraction with acid, since cross-links have not formed. It is the cross-links which render collagen insoluble in more mature tissues.

2.4. Composites of collagen with other polymers

Collagen properties can be conveniently modified using a cross-linking agent, such as glutaraldehyde \cite{22}, or water-soluble carbo-diimides. A wide range of small molecular weight cross-linkers is sold by Pierce, Rockford, IL, USA. In general, cross-linking creates a covalently bonded gel which does not deform like a fluid during injection; rather, the gel fractures and does not recover, but remains a mass of particles. To maintain fluidity, one presumably would like to admix collagen, drug, and cross-linker at the time of injection. In this way, one could not only achieve injectability but also have an implant with modified pore size, usually much smaller pore size in situ, from which diffusion control of release would occur. Some examples of this approach will be presented below. A particularly attractive modifying/cross-linking agent is di-functional or multi-functional succinimydyl ester polyethylene glycol (PEG, 3.4 to 10 kDa) \cite{30}. Such reagents are available from Nectar Polymers, Huntsville, AL, USA. The succinimydyl ester PEG appears to be biocompatible, even when the coupling reaction of the active PEG to protein amino occurs in situ \cite{31}. The same activated PEG chemistry can be used to couple active agents to collagen, resulting in a “tethered” agent \cite{32}. Release in this case requires hydrolysis of a linking bond. The linking structures are such that hydrolyzable amide and carboxy-ester linking bonds can be present (see Fig. 1 of \cite{30}). Fig. 1 (this chapter) summarizes in schematic form the different collagen preparations discussed.

3. Release characteristics of collagen gels

3.1. Release of macromolecular drugs

3.1.1. Release from FCS

Rosenblatt et al. \cite{19} used radiolabeled proteins of varying molecular weight, admixed with FCS, and followed the kinetics of release in vitro. The proteins were iodinated to achieve about $10^6$ dpm per release experiment; 60 µg of each protein were mixed with 1 ml of FCS (35 mg collagen/ml). The release vessel had the geometry of a one-sided slab (1.25 cm for 1/2 slab), and the results were modeled using one-half of a two-sided slab with an infinite sink. The mesh size of the gel was computed from the collagen fibril size distribution and geometric arguments based on the collagen fiber as a rigid cylinder. For these specific formulations, the computed mesh size of FCS was 58 nm. Diffusion coefficients in the gel were computed based on a model of reptation of the diffusing protein confined in the collagen matrix, which generated a smaller diffusion coefficient, so-called ‘hindered diffusion’. In some examples, the protein was sufficiently smaller than the mesh porosity so that the free diffusion coefficient was used (see Table 1). Fig. 2a shows three release profiles, one for the protein chymotrypsinogen (KT, 23 kDa,
estimated “protein length [diameter]: 7 nm) in FCS, one for KT in succinylated collagen, SC; and a third for fibrinogen (a blunt-shaped rod, FBN; 330 kDa, estimated “protein length”: 70 nm) in FCS. Regions of the curve where diffusion was presumed to be the dominant release mechanism were marked with a straight-line segment. This approach was taken to avoid early regions of the curve where convective washout, release of non-bound iodine, and other complications may have governed the release, as discussed in the paper. Over the linear curve segments, marked in Fig. 2a, a release curve was computed, using Eq. (6) of Ref. [19]; with permission from Elsevier Science.

Table 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Matrix type</th>
<th>Diffusivity used in release rate modeling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free solution diffusivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(cm²/s)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>fibrillar</td>
<td>2.02 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>fibrillar</td>
<td>9.5 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>succinylated</td>
<td>9.5 × 10⁻¹⁰</td>
</tr>
</tbody>
</table>

Hindered diffusivities calculated from Eq. (14) of Ref. [19]. With permission from Elsevier Science.

Diffusion is, however, not the only mechanism controlling release from FCS. Fig. 2a shows that, for KT in FCS, as much as 25% of the total load was released at a much slower time scale. The most likely explanation for this observation is that KT was binding to collagen. Since collagen, like all proteins, is a poly-ampholyte, containing both charged and hydrophobic amino acid side chains, weak binding reactions with proteins and drugs is not surprising. When the active agent binds to the matrix, the diffusion coefficient, D, is expected to be of the order: \( D/(1 + K) \), where K is the association constant [33]. Association constants of order \( K = 10–100 \) are sufficient to affect drastically the rate of release.

Singh et al. [34] modeled drug release rates from monolithic matrices where simultaneous diffusion and drug binding interactions were present. Binding mediated absorption and desorption processes were represented by Langmuir kinetics dependent on an association constant K. Drug release predictions for the coupled mechanism model were computed numerically by solving spatially for concentration profiles using finite elements and stepping temporally by finite differences. In agreement with earlier work cited above, model predictions showed that binding interactions do not begin to substantially retard drug release until K becomes greater than 1. Significant modulation of release profiles occurred when K was greater than 10. The other parameter significantly effecting drug release was the ratio of drug loading to the binding capacity of the matrix. Underloaded matrices released much slower than those loaded to capacity or overloaded. The effect was especially significant when loadings were 10% or less of capacity. The binding capacity was also a strong function of the binding strength where stronger binding yielded a greater potential maximum mass of drug adsorbed per unit mass of matrix. For fibrillar collagen gels, the practical implications, given the limited ability to modulate release profiles through hindered diffusion alone, are that strong binding interactions between collagen and drug are required to attain prolonged release durations. Since primary binding mechanisms for collagen systems are electrostatic or hydrophobic in nature, the strength of these interactions must give \( K > 1 \) to be
of practical value. The magnitude of $K$ for a specific drug–collagen system can be defined from binding isotherm measurements.

Fig. 2a (from Ref. [19]) also provides a caveat in the release behavior of fibrinogen from FCS. Here, fibrinogen, because of its large size, should have...
been fully trapped in the collagen network, but in fact more than half of it exited very rapidly, apparently due to convective washout. This observation points up the need to fully equilibrate the active agent with the collagen, and not just have surface association. Overloading the available binding sites may also have played a role in this case.

3.1.2. Release from non-fibrillar collagen

In SC (Fig. 2d), the mesh size was estimated to be 5.6 nm (at 38 mg collagen/ml), and KT (chymotrypsinogen) appeared to exhibit hindered diffusion in this matrix (see also Table 1). One might therefore expect SC to be of use in release of protein drugs. One could also envision preparing PEGylated forms of small molecule drugs to exceed the ~6 nm mesh dimension, thus extending the range of diffusion-dominated release in SC [30,32]. However, succinylated collagen gels do not appear to have a long lifetime in vivo, usually disappearing within 24 h, depending on the degree of succinyllation (unpublished results, Collagen, and Ref. [24]). Highly succinylated collagen gels (>80% derivatized) can dissolve even in vitro, if there is agitation or mechanical disturbance of the gel. Therefore, a simple admixture of SC and an active agent exceeding 4–6 nm in Stokes radius would probably not achieve a sustained release profile in vivo.

In an attempt to remove this drawback to SC, one could propose stabilizing the SC matrix with a biocompatible cross-linker, such as di-succinimidyl ester PEG, admixed at the time of application. For such a formulation, one could envision a small drug, modified by PEGylation to achieve 50–70 kDa molecular weight, mixed with SC in one syringe. In another syringe, one would have the dry di-succinimidyl ester PEG (not stable in water). One would mix the two syringes and inject. In addition, one would need to prepare the SC with only partial derivatization [25], so as to preserve some free amino groups for cross-linking. The use of methylated collagen, MC, would present similar advantages and difficulties, except that MC contains an abundance of free amino groups, and is readily cross-linked. Thus, the proposed solution to the limitations of non-fibrillar collagen gels is complex, and complexity usually precludes commercialization, unless the medical need is very high.

Nevertheless, Rosenblatt and Berg [35] applied the above principles, adding a flexible polymer to the mixture to further control the effective pore size of the matrix. They proposed the possibility of constraining the effective pore size to less than 3 nm by appropriate concentration of SC and flexible chain polymer. In their examples, they used a 400-kDa fluorescein-labeled Ficoll (a polysaccharide) as a model drug. Release profiles out to 200 h were presented, using matrices of SC, di-functional PEG, and hyaluronic acid (HA) as the flexible polymer. The long survival of the SC matrix suggested that cross-linking had played a role in stabilizing the matrix to dissolution.

Experimental measurements of mesh size in SC collagen matrices were made by Shenoy et al. [26] using an electron spin resonance (ESR) technique. This employed the spin label 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO) coupled to polyacrylic acid (PAA) of varying molecular weights via a carbo-diimide condensation reaction. Hydrodynamic radii of the PAA spin probes were measured by dynamic light scattering and ranged from 4 to 30 nm. These probes were placed in succinylated collagen gels ranging in concentration from 5 to 43 mg/ml. For cases where the probe diameter was smaller than the mesh, rotational correlation times (as measured from the first derivative ESR spectra) were unchanged from the probe alone. Rotational correlation times decreased when the mesh size of the gel approached the probe size, presumably due to electrostatic repulsion between the anionic SC and anionic PAA which induced the flexible chain PAA to contract. The measured mesh sizes for SC measured with this technique were 4 nm for a 43-mg/ml concentration, 6 nm for a 28-mg/ml concentration, 11 nm for a 22-mg/ml concentration, and 30 nm for a 5-mg/ml concentration. These measurements were in agreement with earlier theoretical estimates [19].

Mesh sizes were measured in mixed SC and HA gels using the same ESR technique described above [36]. Mesh sizes of mixed gels were reduced from those of SC alone (at similar concentrations). For example, the mesh size of 40 mg/ml SC was approximately 4 nm; however, the mesh size of a 30-mg/ml SC and 10-mg/ml HA gel was less than 4 nm. Similarly, a 20-mg/ml SC and 20-mg/ml HA gel
had a mesh size less than 4 nm while a 10-mg/ml SC and 30-mg/ml HA gel had a mesh size between 4 and 6 nm. The authors concluded that a single-phase molecular dispersion formed between the rigid anionic SC and more flexible anionic HA which accounted for the reduction in mesh sizes. In the same paper, the diffusivities of bovine serum albumin (BSA, 67 kDa) and dextran sulfate (DS, 69 kDa) in these matrices were calculated from measured release kinetics from the gels. The diffusivity of BSA was reduced from its free solution value of \(5.9 \times 10^{-7}\) to \(0.93 \times 10^{-7}\) cm\(^2\)/s in the mixed matrices. In matrices containing SC alone or HA alone, SC appeared to have a more retarding effect on BSA diffusivity (although neither gel retarded BSA as much as the mixed gel). The enhanced hindrance was attributed to the greater rigidity of the SC molecules. The diffusivity of DS was reduced from its free solution value of \(4.5 \times 10^{-7}\) to \(1.08 \times 10^{-7}\) cm\(^2\)/s in the mixed gel. For more flexible DS, there was little difference in diffusional hindrance between SC-only and HA-only gels. In this and related release studies using SC in vitro, special care was exercised to avoid disruption of the gel matrix; SC was centrifuged into the bottom of the release tube, and eluting buffer was carefully layered on and removed. The gels remained clear, and the concentration of SC at the end of the study did not show evidence of dilution.

Electrostatic effects also play a role in non-fibrillar collagens. Singh et al. [37] measured the strength of electrostatic binding interactions between cations and anionic collagen gels as well as their influence on release rates. In one study, the effects of charge valence and charge density on binding interactions between polylysine and collagen gels were investigated. SC gels with approximately 50% and 85% modification of available lysines on collagen were prepared and a phosphonylated collagen (diacid) gel with approximately 25% of lysines modified was also prepared. Polylysine (MW 15 kDa) was spin labeled and the binding isotherms measured via an ESR technique. There was little effect of the degree of succinylation on \(K\) values attainable \((K = 0.5\) for 50% modified and 0.6 for 85% modified). Valence exerted a more significant effect where the \(K\) value for polylysine binding to phosphonylated collagen gel was 1.1. Release measurements showed that the electrostatic binding interactions in the phosphonylated collagen gel could slow the rate of release by approximately a factor of 2 relative to native collagen gels. The SC gels exerted less of a retarding effect.

### 3.2. Release of small molecules

Based on the above reasoning about collagen structure, drug molecules (mol. wt. 0.5 to 2 kDa, molecular dimensions of 0.5 to 1.5 nm) should not experience hindered diffusion in transport through FCS (mesh size estimate: 58 nm) or SC (measured mesh size 4–6 nm). Diffusion-dominated release of dissolved small molecules from collagen gels may be predicted to resemble that in Fig. 3, which is for pilocarpine nitrate in a synthetic polymeric network (Hydron contact lens) [38]. The drug was 80% depleted in only about 8 min. The curve fitted to the experimental points was derived from the early- and

![Fig. 3. Rate of desorption of pilocarpine nitrate from a 70-mg Hydron contact lens previously equilibrated with an aqueous solution of 4% pilocarpine nitrate. From Ref. [38], with permission from Plenum Press.](image)
late-time approximations for diffusional release from a slab, Eqs. (24) and (25) of Ref. [38]. In Fig. 3, the “slab” was a swollen contact lens of unknown dimensions. In an analogous hypothetical experiment, one could inject FCS as a bolus (5-cc volume) into a tissue site, with a roughly spherical shape and radius of 1 cm. Then release could be estimated from the late-time approximation for a sphere, Eq. (36) of [38]:

\[ \frac{M_t}{M_i} = 1 - \frac{6}{\pi^2} \exp(-\pi^2Dt/r^2) \]

where \( M_t \) is the mass at time \( t \), and \( M_i \), the mass at infinite time. \( D \), the diffusivity is taken as \( 1 \times 10^{-5} \) cm²/s (typical for small molecular weight non-electrolytes in water), and \( r \) is the radius of the sphere. This expression yields a time of about 210 min for 80% depletion. Such short release times may or may not be of clinical utility.

Longer release times, however, may occur, due to binding of the drug to collagen, as discussed above for macro-molecular active agents. From this perspective, Cussler [33] gives an illuminating example. He considers the analogous problem of dyeing of wool. Here, the diffusion of dye into wool can be very slow, with apparent diffusion coefficients as low as \( 10^{-12} \) cm²/s, probably due to a binding reaction. Wool, like collagen, is a protein with an assortment of ionic and hydrophobic binding sites; the dye is analogous to a drug. Thus, one can easily imagine that many drugs will have weak binding reactions with collagen and be hindered in diffusion, relative to free diffusion.

Such binding, however, may not be simple. Proteins have so many potential binding sites that it is likely that there will be a distribution of site number and affinity. Tanford [39] describes the binding of iodide ion to serum albumin; the following number of sites per molecule (\( n \)) and molar affinities (\( K \)) were found: \( n_1 = 1, K_1 = 9250, n_2 = 8, K_2 = 385, n_3 = 18, K_3 = 12.7 \). These considerations, taken together, suggest each drug must be taken as a separate case. Fortunately, preliminary release experiments, using simple admixtures of drug and collagen, are relatively easy to do. A likely scenario is analogous to that shown for the large molecule KT in FCS (Fig. 2a): approximately 1/3 to 1/2 of the drug exits quickly (in a few hours) due to convective transport and free diffusion; another 1/3 is released over 2–5 days due to binding, and the remainder may require weeks to emerge, being subject to strong binding. For some clinical applications where an initial burst of drug is desirable, followed by a maintenance level, such release kinetics may be of value. Nevertheless, since binding constants from \( K = 1 \) to perhaps \( K = 10^8 \) (on the scale of antibody–antigen affinities) are conceivable, a very wide range of behavior is possible. Achievement of zero-order release will be rare, and the approach is not general, but strictly case-by-case.

Another important point to be noted is that many drugs of interest will be only sparingly soluble in water. FCS, on the other hand, are only biocompatible in water; there are no organic solvents which are suitable for suspension of collagen and which are safe for injection. If one wants to load large quantities of drug into FCS, one immediately is faced with the situation of crystals of drug which one must disperse into the collagen suspension. In general, the kinetics of release are slower, sometimes considerably slower, if the crystallized drug must first dissolve and then disperse [38].

Even in cases where the drug is soluble, the volume required, to achieve desired dosing when mixed with FCS, could be inconvenient, amounting to 10–30 cc or more. Only injections of 1–5 cc of FCS into most tissue sites are acceptable, although larger volumes can be slowly infused. Smaller volumes are required for small structures, such as those found in ophthalmics or nerve repair. Experimental data were reported by Sutton et al. [40], in which vinblastine dissolved in aqueous media was mixed with FCS. Radiolabeled vinblastine (apparently only a few mg dissolved in saline) was mixed with FCS at 65 mg collagen/ml to achieve a final collagen concentration of 33 mg/ml. Aliquots of 0.1 ml each were placed in micro-centrifuge tubes and buffer was exchanged at intervals for counting (Fig. 4). These reviewers attempted to fit the data to a diffusion model with slab geometry. The dimensions were estimated based on the volume given and the shape of the tube. The model curve was derived from the early and late time approximation equations of [38], with no assumptions about burst or lag. The resultant diffusion coefficient, \( D = 2 \times 10^{-6} \) cm²/s, is roughly consistent with free diffusion of a small molecule (vinblastine mol. wt.: 900 Da), at least in the early part of the release curve. The later
part of the curve seemed to follow a more retarded mechanism, possibly due to drug–collagen interaction. Vinblastine possesses amine and other polar groups, as well as aromatic character, all of which could interact weakly with corresponding groups on collagen. In vivo, in mouse tumors, the drug–collagen system exhibited greater retention of the drug at the site, compared to injection of free drug, resulting in a 3-fold enhancement of tumor exposure to drug.

In a related study [41], 5-fluorouracil (5-FU, dissolved in aqueous media) mixed with FC plus epinephrine (5-FU at 30 mg/ml, epinephrine at 0.1 mg/ml, and collagen at 20 mg/ml; again with 0.1 ml aliquots in the bottom of a micro-centrifuge tube, but no explicitly specified dimensions) exhibited slower in vitro release kinetics (Fig. 5). The same model fitting by these reviewers suggested a diffusion coefficient of about $5 \times 10^{-7}$ cm$^2$/s, which seems too slow for free diffusion of a small molecule. The drug 5-fluorouracil is quite polar and possibly is bound to collagen. In addition, it is possible that the epinephrine affected the collagen fibril structure or participated in weak binding interactions between 5-FU and the matrix. Again, retention of drug in mouse tumors was enhanced, compared to soluble drug alone. Some of the in vivo retention was presumably due to vasoconstriction by the epinephrine. Thus, in these two examples, the general behavior was consistent with plausible mechanisms discussed above. The examples cited above for vinblastine and 5-fluorouracil were part of a larger effort, associated with the firm Matrix Pharmaceuticals, to develop clinically effective localized delivery systems for anti-tumor drugs. Early work in this area had been done by Daniels et al. [42], using collagen injection through catheters to limit blood supply to tumors, and anti-tumor drugs were also included in the therapy. Subsequently, Luck et al. tested anti-tumor drugs plus FCS in large animal models [43], and in humans [44], with reduction in tumor growth, compared to controls.

Singh et al. [45] conducted a study of the effect of electrostatic charge interactions on release rates of gentamicin from collagen matrices. The charge distribution on collagen was altered by using SC and phosphonylated collagen to yield net negative charges which exhibited binding interactions with positively charged gentamicin. Adsorption isotherms were measured to characterize binding interactions. Release of gentamicin from native and modified collagen was measured, and release rates were compared to a mathematical model based on an instantaneous desorption coupled with diffusion mechanism. Ninety percent of the gentamicin loaded was released

![Fig. 4. Release kinetics of tritiated vinblastine from collagen matrix carrier in vitro at 37 °C. The data were expressed as the percent recovered of the initial total amount. Triplicate samples were taken and the mean was plotted for each time point (diamonds). Experimental points re-plotted from Ref. [40], with permission from Mary Ann Liebert Publications. The solid lines show the early- and late-time approximation equations for one-half of a two-sided slab; Eqs. (24) and (25) of Ref. [38].](image1)

![Fig. 5. Release kinetics of tritiated 5-fluorouracil from a collagen gel as determined in vitro. Each data point represented the mean value ± S.D. for triplicate samples (squares). Data replotted from Ref. [41], with permission from Springer Verlag. Solid line is the composite for the early- and late-time approximation model, as in Fig. 4.](image2)
from native collagen matrices in 2.5 days (one-sided slab geometry in vitro). SC matrices released 70% in 2.5 days and phosphorylated collagen matrices released 50% in 2.5 days. Excellent agreement between model predictions and experimental results were obtained.

3.3. Release of therapeutic proteins, including complexed, conjugated and tethered proteins

Bentz et al. [30] studied local delivery of transforming growth factor beta-2 (TGF-beta2) from FCS. The growth factor was admixed and also tethered covalently to FCS, using difunctional succinimidyl ester PEG (3.4 kDa). Approximately 20 μg of TGF-beta2 was present per 0.35 cc volume of FCS. In vitro release experiments were performed by placing 0.35 cc of each mixture into wire cages, and exchanging with 10 ml aliquots of buffer at 24-h intervals (Fig. 6). Free TGF-beta2, being very poorly soluble in water, did not exhibit appreciable release during 5 days. TGF-beta2 coupled to PEG (but not tethered to collagen) was 80% depleted in 36 h. These TGF-beta2–PEG conjugates had apparent molecular weights of 30 to 46 kDa in gel electrophoresis. Each PEG molecule added has a molecular weight of 3.4 kDa and an approximate diameter of 2.8 nm, using Eq. (14) of Flory [46]. The increase in apparent molecular weight suggests that 1–4 PEG chains are attached to the TGF-beta2 (protein alone, 26 kDa [30]). From the known X-ray structure of TGF-beta2 [47], one can estimate an effective diameter of about 5 to 7 nm for the conjugate and a diffusion coefficient similar to that of the protein beta-lactoglobulin, $7.8 \times 10^{-7} \text{ cm}^2/\text{s}$ (using Tables 21-1, 21-2, pp. 358–359, Ref. [39]). A priori, one would not expect these conjugates to be hindered in diffusion out of FCS; free diffusion and convective washout would be the anticipated mechanisms.

These reviewers performed a rough estimate of free diffusion release kinetics for a spherical geometry, using early and late-time approximations Eqs. (35), (36) of Ref. [38]. The roughly fitted model curve shown in Fig. 6 utilized a diffusion coefficient of $1.7 \times 10^{-7} \text{ cm}^2/\text{s}$, which is too low to be consistent with free diffusion of PEGylated TGF-beta2. TGF-beta2 is a very hydrophobic protein [30], and PEGylation is necessary to improve its solubility in aqueous media. Nevertheless, some hydrophobic or polar parts of the molecule could still be exposed and bind weakly to collagen, retarding the release.

For tethered TGF-beta2, about 20% exited in a burst within 1 day, with the remainder showing a very slow release rate, probably due to hydrolytic cleavage of the amide or ester bonds present. PEG hydrogels formed from the same coupling chemistry can hydrolyze in vitro in several hundred hours [31], suggesting that tethered TGF-beta2 in the above example will be released over 10–20 days for cleavage of ester links, but perhaps over longer intervals if cleavage of amide links is rate-limiting.

These formulations were studied in vivo for their ability to generate new collagen (due to the activity of TGF-beta2). TGF-beta2 tethered through PEG to collagen showed the highest activity. TGF-beta2 simply admixed with FCS was only slightly more active than FCS controls. TGF-beta2 coupled to PEG (3.4 kDa mol. wt.) and admixed to FCS was also poorly reactive. It appears that a slow delivery rate and persistence locally for more than 5 days is important to achieve the desired response. TGF-beta2 coupled to a larger PEG molecule (40 kDa) admixed with FCS did show some enhanced activity over controls; this may be due to slower release of this larger molecular weight species.
For some applications, such as creating newly vascularized tissue or in bone repair, it has proved advantageous to form a complex of collagen with active agents which are released in trace quantities (ng/ml), and act as cytokines to attract cells into the implant over an extended period.

Injected TGF-beta2 produces its characteristic response in vivo by recruiting surrounding tissue fibroblasts and stimulating them to deposit new collagen at the site. However, this growth factor rapidly loses biological activity in physiological buffers [48]. Schroeder-Tefft et al. [48] showed that TGF-beta2 activity could be stabilized by complexing it with heparin. The complex is apparently due to ionic bonding, since TGF-beta2 has a net positive charge at neutral pH, while heparin is negatively charged. Such complexes were then mixed with FCS to form an injectable tissue scaffold formulation. In a rat subcutaneous model, the persistence of the biological response to TGF-beta2 was measured at 7, 21, and 42 days post-injection. At days 21 and 42, TGF-beta2 complexed with heparin and mixed with FCS showed more extensive collagen deposition than did TGF-beta2 simply mixed with FCS. Both TGF-beta2 formulations had higher responses than controls at the two time points. Here, FCS was serving as a depot for the stabilized growth factor, which then directed the desired local tissue response.

No quantitative measurements of TGF-beta2 release were presented, but it is known that it must be present at 1 ng/ml or greater to elicit an effect [48]. For injection, 0.2 ml boli of FCS at 30–40 mg collagen/ml containing approximately 0.01–0.1 mg heparin and 0.01–0.1 mg TGF-beta2 were employed. From these data, one may conclude that the depot of growth factor, if released at only a few nanograms per day, would have been more than adequate to sustain the observed response for 42 days.

In a related study, Pieper et al. [49] used collagen–heparan sulfate matrices to complex basic fibroblast growth factor; in vivo this formulation promoted angiogenesis into the matrix. In this latter study, the collagen was not in gel form, but was cross-linked; nevertheless, the analogous injectable formulation using FC [48] could have been prepared without difficulty. Such formulations could offer a solution to the need for rapid vascularization in large tissue constructs (see Section 4.3).

### 3.4. Release of therapeutic proteins from liposomes in FC

Human growth hormone (hGH), molecular weight 27,000 Da was sequestered in liposomes, which were then admixed into non-fibrillar collagen at 0.3% or 0.9% (w collagen protein/v) [50]. The non-fibrillar collagen originally was an acidic solution; it had been mixed with buffers and sodium hydroxide to achieve neutral pH. When such neutral solutions are maintained near 4 °C, fibril assembly is retarded, and the system is a flowable liquid. Liposomes containing hGH were added to the neutral collagen solution, and incubated at 37 °C, whereupon a fibrous gel (heat gel; HG) formed in 5 to 15 min. Acidic collagen (1.7 ml) was mixed with 0.4 ml buffer/sodium hydroxide, and 0.1 to 0.6 ml (usually 0.3 ml) of liposomes were added. The 0.3-ml liposome solution contained approximately 100 mg of lipid and ~ 10^6 cpm of hGH (no specific activity given, but this probably amounted to 0.1 to 1 mg hGH). Thus, the concentration of hGH in final collagen gels was 0.04 to 0.4 mg/ml, depending on the specific activity. The final collagen gel was at 2.1 or 6.4 mg collagen/ml, which is typical for heat gels. Such gels are usually relatively firm, but they are friable. This is in contrast to FCS, which is usually a suspension of collagen fibrils at 30–60 mg/ml and has a paste-like consistency. For animal studies, the cold liposome/non-fibrillar collagen mixture was injected into intra-muscular or subcutaneous tissue sites; in the animal at 37 °C, gelation then occurred. The radiolabeled hGH was retained at intramuscular sites in mice longer when sequestered in liposomes plus collagen gel, compared to injection of free hormone or hormone in liposomes (Fig. 7).

As a variant, the protein fibronectin was covalently bound to the liposomes. Since collagen binds to fibronectin [51], it was anticipated that liposome anchoring would be more secure in that system. Indeed, at days 1 and 7 after implantation, about 30–50% more radiolabel was retained at the site in the fibronectin-stabilized formulation, compared to liposomes in collagen without fibronectin. Finally, the authors compared the performance of a pepsin-treated collagen versus rat-tail tendon collagen, which was not enzyme-digested. It is well known that pepsinized collagen forms weaker gels than
non-pepsinized [52], and in fact the rat-tail tendon collagen provided higher retention of label, compared to pepsinized collagen. From a practical point of view, obtaining commercial quantities of non-pepsinized medical-grade collagen may be difficult and expensive. In general, it should be possible to increase gel strength in pepsinized collagen by simply raising the total collagen concentration.

3.5. Conclusions

The above examples illustrate systems utilizing collagen for drug and protein delivery. In general, because of the open weave of FC, practical diffusional control of release is rare. Binding interactions, especially to FC, can retard release, but are specific to each drug and hard to predict; if the binding interaction is weak, little improvement is observed. Modified collagens may be useful in this regard since they may have additional binding sites for drugs. Drugs tethered to collagen with labile bonds are another useful approach, but the complexity of such systems is a disadvantage from a commercial perspective. For SC, macromolecular agents may undergo hindered diffusion, and the addition of flexible polymers may be used to tune the release; however, SC may need to be stabilized in vivo by cross-linking.

4. Tissue engineering

4.1. Implant fabrication and cell culture

As early as 1962, Grillo and Gross [53] described the preparation of collagen gels from neutralized acidic collagen solutions, and proposed their use as medical implants. They showed that gels, sheets, tubes and various articles could be prepared, and they formed gels in animal tissue by injecting cold neutralized collagen solutions. They also studied the immunological reactivity of bovine collagen (without pepsin treatment) in other vertebrate species, demonstrating the relative safety of collagen implants. Collagen gels for culture of living cells in vitro came into wide use (see, for example, Ref. [54]). Stopak et al. [55] showed that collagen gels formed after injection in developing chick limb buds re-arranged to conform with the host tissue, and formed parts of tendon, perichondria, perineuria, and other structures. Such work set the stage for the use of collagen gels plus living cells, capable of being remodeled to generate new functional tissue. One of the earliest collagen tissue engineering applications was the development of an artificial skin for treatment of burns [56], which was a membrane, and not a collagen gel. Below are presented further examples of the use of collagen gels. The list is not exhaustive; more references could be cited, using much the same methods.

4.2. Examples of simple constructed tissues utilizing collagen gels

Wakitani et al. [57] embedded chondrocytes in collagen gels to repair articular cartilage defects. Acidic collagen at 3 mg/ml was neutralized by adding 100 ml 2 × concentrated DMEM to 100 ml of collagen solution at 4 °C. Chondrocytes were added as a pellet (1 × 10^6 cells), the mixture was gelled in an incubator at 37 °C for 10 min or more, and then implanted in cartilage defects in rabbits. The chondrocytes maintained their phenotype and new cartilage was observed as early as 4 weeks. The grafted tissue remained as cartilage over 48 weeks.

Orwin and Hubel [58] attempted to construct corneal tissue, using epithelial, endothelial, and keratocyte cells plus collagen sponges. They cited an earlier study in which collagen gels were tried for
this application [59], but the implants lost transparency, due to extreme contraction of the collagen gel by keratocytes. To overcome the contraction, Orwin and Hubel resorted to a collagen sponge, which was sufficiently rigid to resist cellular contraction forces. To achieve translucency of the sponge, they prepared a dispersion of collagen in dilute acid, pH 4, followed by freeze-drying. Such acid-swollen collagen should have less fibrillar structure (thinner fibrils) and should scatter less light than fibrils formed at neutral pH.

Awad et al. [60] incorporated autologous mesenchymal stem cells into collagen gels for tendon repair. In order to avoid problems of gel contraction in vivo, they incubated cells and gel for 18 h prior to implantation; this approach allowed contraction to occur before insertion into the tissue site. In the tendon defect, the stem cells differentiated into tenocytes. Compared to controls (collagen gels with no cells), the stem-cell/collagen gels yielded repaired tendons with improved mechanical properties. The authors expressed an interest in having collagen matrices with aligned fibers. In the typical heat gel (HG), collagen fibers are randomly oriented.

Another approach to the problem of collagen gel contraction was the study of Moriyama et al. [61], in which a collagen gel plus cells was cast into a pre-formed collagen sponge. The sponge provided rigidity. Nevertheless, within the sponge pores, it was observed that the gel elements did contract. Such matrices were prepared with fibroblasts in the collagen gel phase, but later keratinocytes were cultured on one surface of the gel, to simulate oral mucosa. The tissue constructs were implanted onto skin wounds in nude mice. The grafts epithelialized and exhibited limited wound contraction, compared to wounds with no grafts.

4.3. Enhancing functionality

The examples below utilize multiple constituents to construct tissues, including stratagems to strengthen the gel, and achieve adhesion of components.

4.3.1. Artificial myocardial tissue

Myocardial infarction damages regions of heart tissue, leading to patient morbidity and mortality. Engineering and transplantation of artificial myocardial tissue to replace ischemic and infarcted areas of the heart is an alternative to whole organ transplantation [62]. Kofidis et al. [62] described the production of collagen devices colonized with cardiomyocytes that showed continuous, rhythmic, and synchronized contractions for up to 13 weeks in vitro. Such devices are close to possessing the properties necessary for repair of the heart, which include uniform colonization of the implant with viable cells, and adequate mechanical strength for manipulation in surgery. To form the constructs, 1 ml of cell suspension in soluble collagen containing $2 \times 10^6$ cells was added to a sheet of collagen fleece and gelled for 4 h at 37 °C. Strips of the tissue construct could be cut, mounted with cyanoacrylate glue to metal holding bars, and subjected to electrical stimulation. One of the bars was connected to a force transducer to measure contractile forces. Stretching forces could also be applied to the artificial tissue. Electrocardiograms of the constructs could also be monitored, after placement of electrodes in the strips. The whole system was immersed in culture medium in which physiologic oxygen and carbon dioxide levels were maintained by an external supply. A similar experimental system was described in detail in an earlier publication [63], in which the collagen gel was anchored to test bars through Velcro, as an alternative to glueing with cyanoacrylate. The filaments of the Velcro were embedded in the cast gel. The cells contracted the rectangular gel to a bi-concave shape (Fig. 8). The gel formed a net-like, coordinate beating heart-like tissue. Such tissue constructs showed electrocardiogram patterns and responded to drug and mechanical stimuli in a fashion similar to normal heart tissue [62,63].

4.3.2. Small diameter vascular grafts

There have been several different approaches to the fabrication of vascular grafts, including use of (1) de-cellularized tissues, (2) synthetic polymer tubes seeded with cells, (3) sheets of cells plus cells-synthesized extra-cellular matrix, and finally (4) reconstituted collagen gels, formed with and compacted by cells [64]. To be successful as replacement grafts, artificial constructs need to possess adequate burst strength to withstand blood pressure and sufficient compliance match with native arteries, including elasticity without creep. Early constructs contained smooth muscle cells and fibroblasts in a collagen gel, seeded on the surface with endothelial
cells. The collagen gel possessed inadequate strength to sustain arterial pressures, and it was reinforced with a Dacron sleeve [65]. Later versions were prepared by casting the cell-seeded collagen gel around a mandrel. During subsequent culture, cell-generated contraction forces strengthened the tube-shaped artificial artery [66].

Seliktar et al. [67] built on this concept, forming a tubular gel around a silicone mandrel which could be inflated cyclically to provide dynamic mechanical conditioning during cell growth. To assure adhesion of the cell-seeded gel to the silicone tube, the silicone was activated by etching with 10 N sulfuric acid, and a layer of collagen and chitosan were bonded to it. Collagensolution was seeded only with smooth muscle cells (10^6  cells/ml) and the final collagen concentration of the cast gel was 2 mg/ml. After 2 days of culture on the mandrels, the cell constructs were placed in a reactor in which culture medium was supplied, and pressurized culture medium was pumped through the silicone tube to produce a 10% change in the outer diameter (Fig. 9). Dynamic conditioning was achieved by repeated inflation and deflation at 1 Hz frequency. At 4 and 8 days, constructs were detached from the apparatus, removed from the silicone tubes, and cut into 5-mm-wide rings. Beads were embedded in the rings to allow visualization of deformation, and the rings were mounted in a tensile testing device and subjected to cyclic loading and strain to failure. By monitoring stress and visual displacement of the embedded beads, stress–strain curves were generated. Samples with and without dynamic conditioning were compared. Ultimate tensile strength for the dynamic conditioned tissue rings was 265% higher than for non-conditioned rings. The highest values for conditioned rings were 58 kPa at 8 days. It is worth noting that 58 kPa ultimate tensile strength is about 2-fold higher than that of hydrogels formed from synthetic polymers [31]; gels with this level of strength can be manipulated, but they are still rather fragile. Other mechanical parameters (modulus, stress and strain at a yield point) were also higher in dynamically conditioned vascular constructs. Histological analysis showed marked orientation of smooth muscle cells and collagen fibers in the conditioned samples. These studies show that optimization of tissue engineered vascular grafts will require proper structural orientation of cells and matrix; i.e., mechanical factors are crucial.

Ultimate success may not involve collagen gels at all; L’Heureux et al. [68] first grew intact confluent monolayers of human vascular smooth muscle cells and fibroblasts, wrapped these around a mandrel to form a tube, cultured the tubes to maturity over 8 weeks, and seeded human endothelial cells onto the lumen, again to form a confluent monolayer. Burst strengths above 2000 mm Hg (266 kPa) were recorded, with good histology.

4.3.3. Composite neotrachea

In pediatric airway surgery, an autologous tracheal construct is needed to correct stenosis of the airway [69]. This is a composite tissue, comprising both bronchial epithelium and cartilage. Doolin et al. [69] attempted to construct an in vitro model of this tissue by growing epithelium and cartilage separately and fusing the two tissues with fibrin glue. The authors state that co-culturing two cell types, the alternative, is difficult and time-consuming because of different growth and differentiation requirements.

Rat tail tendon collagen in Eagle’s minimal essential medium at 2.0 mg/ml was gelled at room temperature, and 1 × 10^4 human bronchial epithelial cells/cm^2 area of gel were seeded on top of the gel. The cells and gel were incubated for 6 days before being bonded with TISSEEL (fibrin glue) to a construct of
chondrocytes. The chondrocyte construct was prepared with normal human articular chondrocytes mixed with a 3% sodium alginate solution, gelled with calcium ion at 5 × 10^6 cells/ml, and grown in a differentiation medium for 2 to 3 days to form cartilage. The composite tissue was incubated for up to 5 days to test bonding. The two tissue types remained firmly bonded during the period of observation. Living epithelial cells and viable cartilage were maintained throughout. In neither tissue was the cell morphology or tissue dimensions altered by the application of the TISSEEL. The authors noted that such composite tissue constructs must remain quite small until some means for introducing a blood supply is found. The next step is to find a vehicle for providing such a micro-vasculature. Some possible approaches to solve this problem were discussed above (Section 3.3).

4.4. Improving gel strength

Although collagen gels are widely used in tissue engineering [70], the nature of their application has changed little in more than a decade. Despite their advantages of convenience and biocompatibility, they have limitations. The chief drawback, as mentioned above, is the inability to control final gel properties beyond a relatively narrow range. Randomly oriented gels are too weak for surgical manipulation or to bear tensile loads in vivo [65].

One way to improve gel strength is with various composite forms, in which collagen gels are combined with solid form collagen articles, such as sponges [61], sheets [56], and pre-formed fibers [71]. Another is to devise some means to align growing collagen fibrils and cells. Ref. [63] above describes casting the cells and gel so that the ends of the gel are anchored in Velcro and attached to metal bars. Such a gel could be cast, detached from its mold and allowed to incubate for a few days with no tension applied. This would permit gel contraction by the cells, which reduces the volume (raising the collagen concentration to about 4 mg/ml and aligning the collagen fibers to some extent). Using the attached metal bars, the gel plus cells could then be mounted in a device which would apply cyclic loading to the gel. The device would be analogous to that of Eschenhagen et al. [63]; it would not only measure contractile force, but also apply force. Based on the results of Seliktar et al. [67], one would anticipate that cyclic loading in culture, including increased deformation of the construct, would promote even more alignment of cells and collagen fibers, enhancing the strength of the construct, as much as three-fold (see Sections 4.3.2 and 4.3.3). The final gel could have better overall me-

Fig. 9. An experimental bioreactor is used to impart cyclic strain onto cell-seeded constructs. The tubular constructs are cultured over thin-walled silicone sleeves that are inflated with pressurized culture medium under pneumatic control to produce a 10% change in outer diameter of the silicone. Gas exchange takes place through 0.2-μm syringe filters placed on the top of the bioreactor. From Ref. [67] with permission from the Annals of Biomedical Engineering.
mechanical properties, especially when subject to stretching, than a gel reinforced with a collagen sponge or collagen fleece.

Simply preparing the gel with higher concentrations of collagen probably will inhibit movement of cells and nutrients, while providing only modest increases in strength. Gels formed from FC (20–60 mg/ml), while biocompatible and injectable, are easily broken (Friable) [3]. In addition, they are dense and form a barrier for cell movement [1]. In vivo, FC gels condense to higher collagen concentrations and are poorly penetrated by surrounding fibroblasts or other cells [1]. The use of cross-linkers or reactive polymers added to FC, soluble collagen, or collagen gels, although they can improve mechanical strength, will reduce porosity (limit cell movement), and may have other negative influences on cells (See Section 2.4). Reactive polymers can, however, be useful in bonding constructed tissues together [30,31,72]. Rhee et al. [72] have described an adhesive prepared from di-succinimidyl PEG and methylated collagen (MC), which was useful for attaching an in situ polymerizable lenticule to bovine cornea. This collagen gel adhesive could have been substituted for TISSEEL in the example given in Section 4.3.

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References

[23] D. Wallace, W. Rhee, B. Weiss, Shear creep of injectable


[57] J. Zieske, V. Mason, M. Wasson, Basement membrane assem-


