Structural determinants of biologically active materials

1. Engineering control of structure of collagen-based biomaterials

2. Identification of structural features that determine the biological activity of scaffolds.

Analogs of extracellular matrix

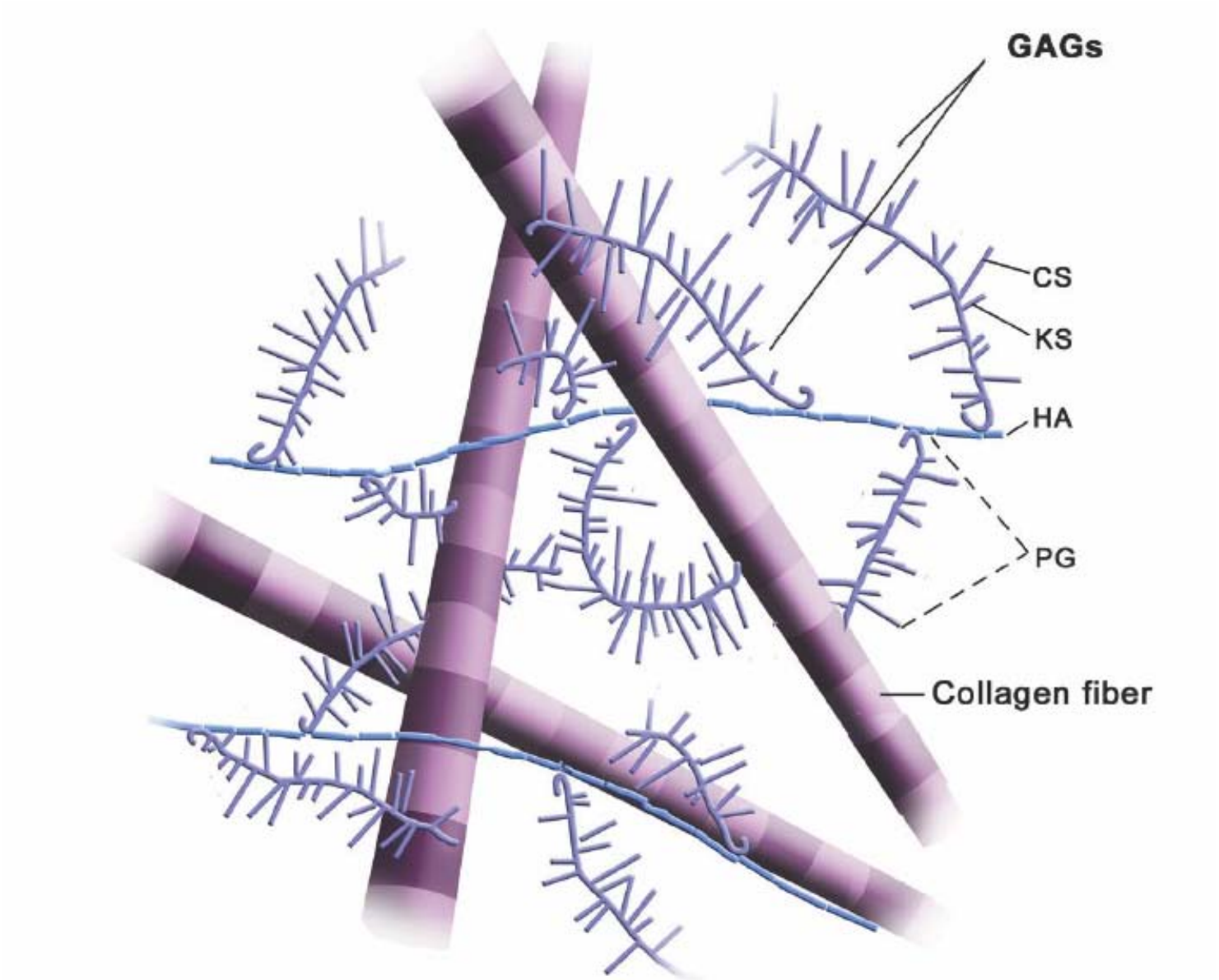


Figure by MIT OpenCourseWare. After Ricci.

1. Engineering control of structure of collagen-based biomaterials.

(outline)

A. Crosslinking of collagen fibers leads to deceleration of degradation rate.

B. Grafting of GAG molecules on collagen fibers: deceleration of degradation rate.

C. Melting of collagen tertiary structure: acceleration of biodegradation rate.

D. Melting of collagen quaternary structure: thromboresistance.

E. Porosity of scaffold can be used to control the density of ligands engaged in cell adhesion.

Regenerative activity is highest when scaffold degrades at optimal rate

<u>Reduce</u> degradation rate by:

A. **Crosslinking of collagen**
B. **Grafting collagen with GAGs**
C. **Preservation of native collagen structure (collagen vs gelatin)**

A. Crosslinking of collagen

Degradation rate of implanted polymer

The mass of undegraded polymer, M, often decreases according to the differential equation:

$$-dM/dt = kM$$

The solution is:

$$M(t) = M_o exp(-kt)$$

where *Mo* is the mass at $t = 0$ (undegraded polymer) and *k* is the rate constant for the degradation reaction.

At the half-life $\tau_{1/2}$:

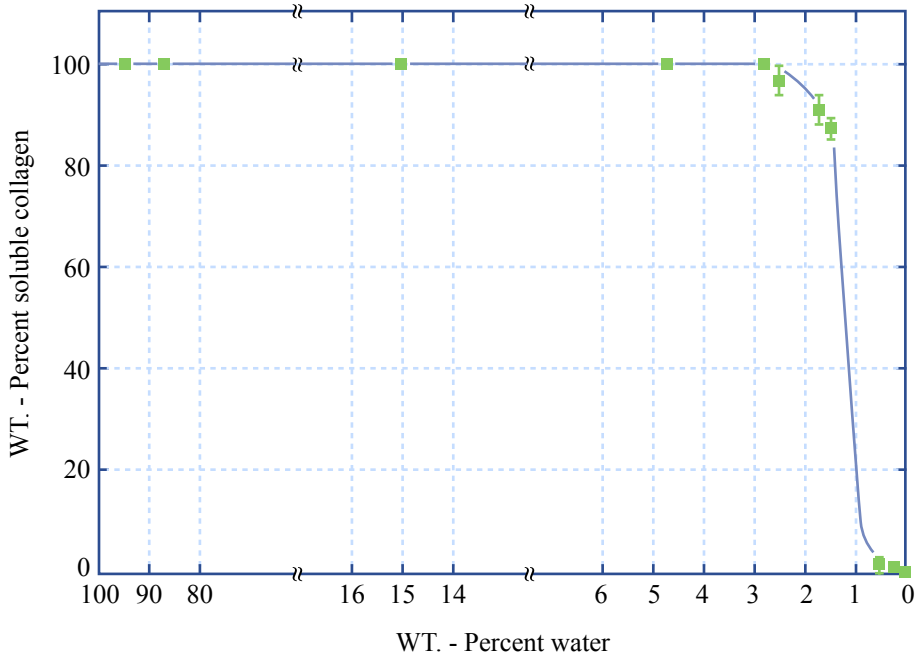
$$M = M_o/2$$

$$\tau_{1/2} = 0.693/k$$

which allows calculation of *k.*

Example of use. The regenerative activity of a scaffold was observed to be maximal when its degradation rate was approximately equal to the rate at which new tissue was being synthesized in the wound.

Collagen becomes insoluble by covalent crosslinking simply by drastic dehydration. Carboxylic groups and hydroxyl groups condense forming an ester (amide) and water, which departs, leading to crosslinking.

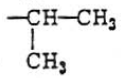
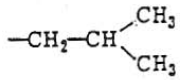
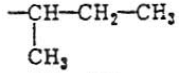
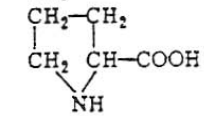
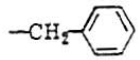
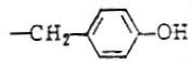
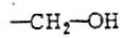
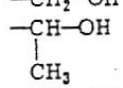
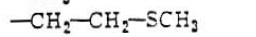
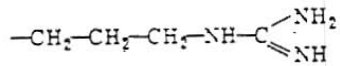
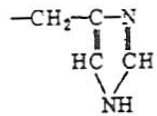
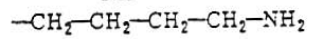
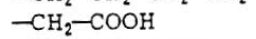
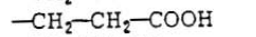
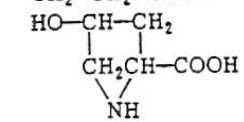
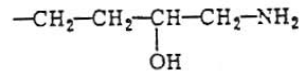


Progressive insolubilization of collagen film brought about by dehydration (left to right). Solubility determined after immersion in 0.5 M acetic acid at 23°C over 48 hr.

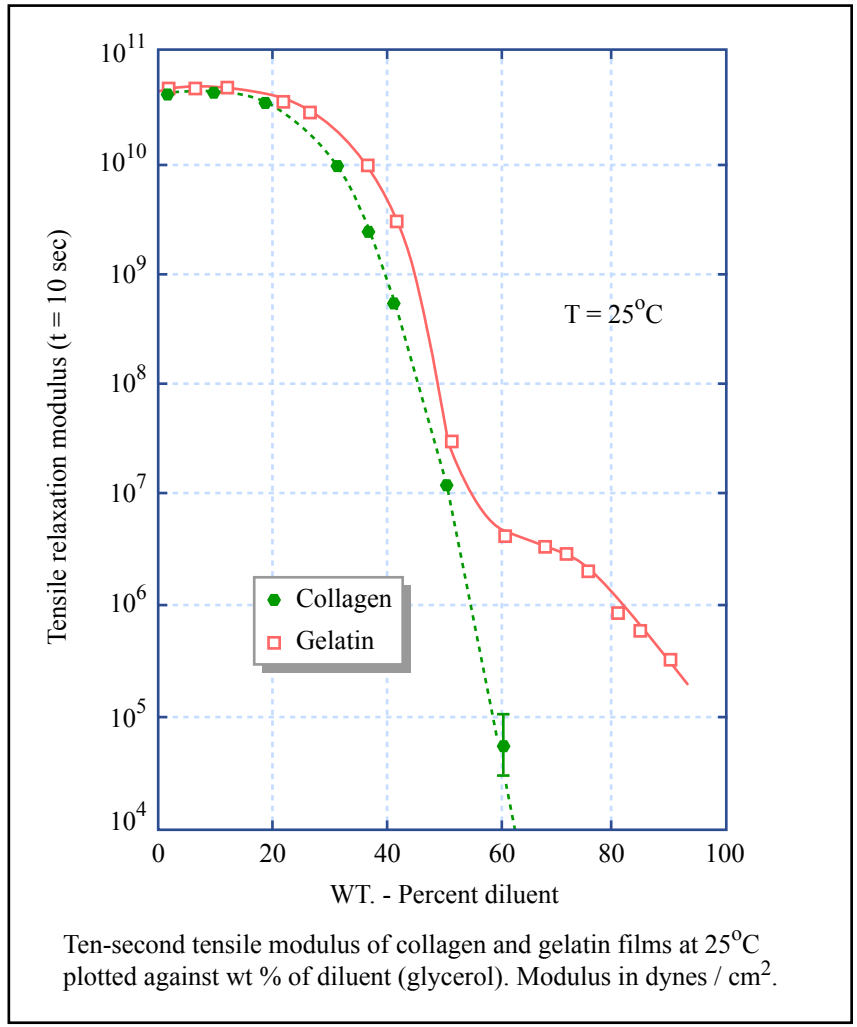
Primary structure (amino acid sequence) of type I collagen shows amino acids (e.g., lysine, aspartic acid) that can condense during severe dehydration to produce crosslinks.

$-NH_2 + -COOH \longrightarrow -NHCO- + H_2O$

The Amino Acid Composition of Collagen (Human Tendon)

Trivial name	R in repeating sequence —NHCHRCO—; or imino acid formula	Number of amino acid residues per 1000 total residues [6]
Alanine		110.7
Glycine		324
Valine		25.4
Leucine		26.0
Isoleucine		11.1
Proline		126.4
Phenylalanine		14.2
Tyrosine		3.6
Serine		36.9
Threonine		18.5
Methionine		5.7
Arginine		49.0
Histidine		5.4
Lysine		21.6
Aspartic acid		48.4
Glutamic acid		72.3
Hydroxyproline		92.1
Hydroxylysine		8.9

Measure crosslink density using finding that when collagen melts, it produces gelatin, a rubber under certain conditions Mechanical (viscoelastic) behavior of collagen and gelatin is distinctly different. Gelatin shows a rubberlike state. Collagen does not. [Both proteins were progressively diluted with glycerol to elicit the entire spectrum of their viscoelastic behavior.]



Ten-second tensile modulus of collagen and gelatin films at 25°C plotted against wt % of diluent (glycerol). Modulus in dynes / cm^2.

Crosslink density of a network

The crosslink density of a macromolecular network is reciprocally related to the average molecular weight, M_c g/mol, of a network chain (chain length between two neighboring crosslinks).

From the rubber elasticity equation:

$$M_c = 3\rho RT/E$$

Measurement of E (under conditions where rubber elasticity holds) gives the properties of the network.

Gas constant, R = 8 .3 X 10^7 dyn·cm/deg/mol. Temperature, T, in degrees Kelvin. P, density, g/cm^3

Measure the crosslink density of collagen-based scaffolds

1. Gelatinize to confer rubberlike behavior to collagen material. E.g., heat in water at 80°C for 3 min to melt tertiary structure.

2. Use model of ideal rubberlike behavior to describe stress-strain relation for gelatin.

3. Measure tensile modulus of gelatin, $E = 3G = 3\rho RT/M_c$.

$$\sigma = \frac{\rho RT}{M_c} v_2^{1/3}(\alpha - 1/\alpha^2)$$

This formula gives the shear stress acting on network that is stretched to an extension ration, α.

σ = shear stress. α = extension ratio, L/L_o. ρ = density of rubber.

How to predict in vivo degradation rate from in vitro data? The degradation rate of collagen-based scaffolds measured in vivo (vertical axis) and in vitro (horizontal axis) are related empirically. Prediction of in vivo degradation rate from in vitro data is possible from these data.

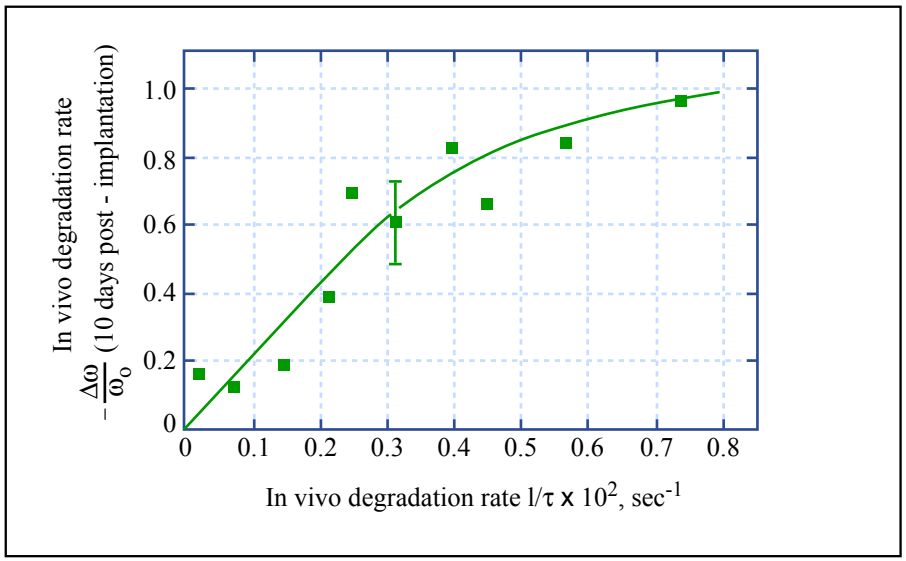


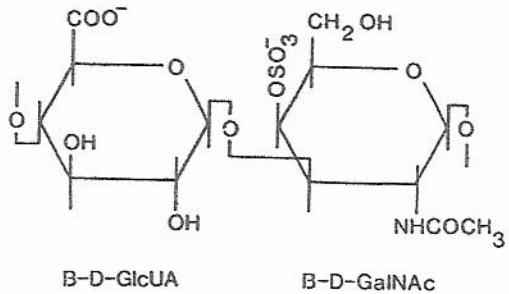
Figure by MIT OpenCourseWare.

B. Grafting collagen with GAGs

FIGURE 8.1 continued.

CHONDROITIN 4-SULFATE

(n 20-60)

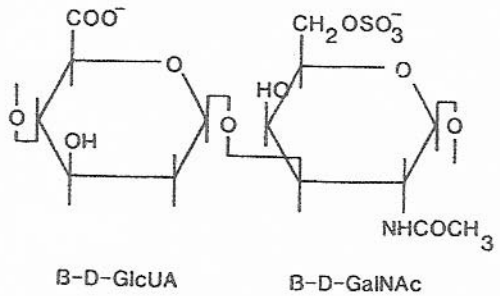


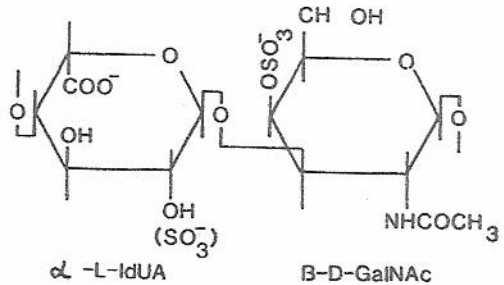
Glycosamino-glycans (GAGs)

disaccharide repeat unit

DERMATAN SULFATE

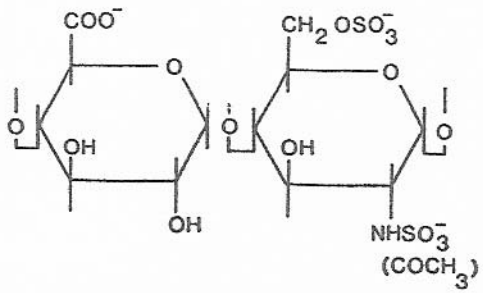
(n 30-80)

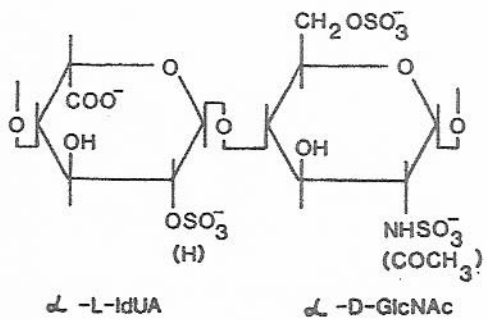




HEPARAN SULFATE

(Heparin)





<u>Ionic complexation of collagen and GAG molecules at acidic pH levels</u>. Positively charged amino groups in collagen form ionic complexes at low pH with negatively charged sulfate groups in GAG. Complex dissociates reversibly at neutral pH because charges weaken. Complex also dissociates at low pH provided that ionic strength is high enough.

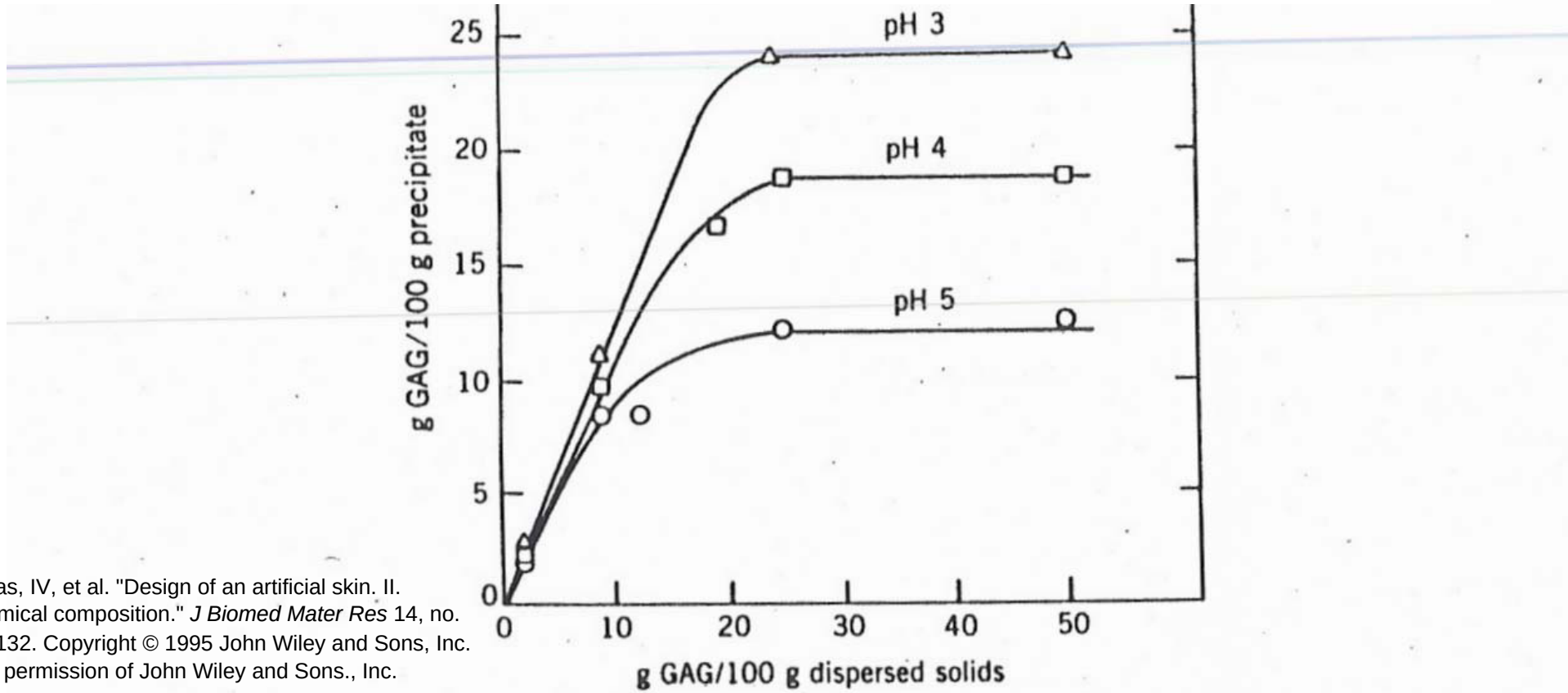


Fig. 1. The composition of collagen–GAG coprecipitates, plotted on the ordinate, depends on the amount of GAG added to the bovine hide collagen dispersion (abscissa).

The GAG is chondroitin 6-sulfate. Adsorption isotherms were obtained at 4 degree C at the pH levels indicated. No chondoitin 6-sulfate coprecipitated with collagen at pH7.

Charge interaction among polyelectrolytes. Ionic strength and Debye length of an electrolytic solution

The ionic strength of an electrolytic solution is:

$$I = (1/2)\Sigma m_i z_i^2$$

where m = molarity of ionic species, and z is its electric charge.

I is closely related to the Debye length via the factor $\Sigma n_i z_i^2$.

The distance over which the electrostatic field of an ion extends with appreciable strength can be calculated using the equation for the Debye length, 1/b:

$$b^2 = (4\pi e^2/\varepsilon kT)\ \Sigma n_i z_i^2$$

1/b has dimensions of length ~ thickness of ionic atmosphere. It is a measure of thickness of the ionic atmosphere. E.g., in a 1M aqueous NaCl solution, 1/b = 0.3 nm. Increase in ionic strength leads to shrinking of Debye length.

e, electronic charge

ε, dielectric constant of medium

k, Boltzmann's constant

T, temperature (absolute)

n_i, number of ions of type I per unit volume

z_i, valence of ion type i

Conclusion: In solutions of very high ionic strength, the electrostatic interaction between ions becomes very weak. This results in dissociation of collagen-GAG ionic complex and makes it necessary to induce covalent crosslinking of GAG to collagen to maintain the association at neutral pH.

Crosslinking binds GAG covalently to collagen and produces a graft copolymer. Solvents with high ionic strength fail to separate the grafted polymers from each other. The copolymer is stable at neutral pH. Crosslinking GAG to collagen decreases degradation rate.

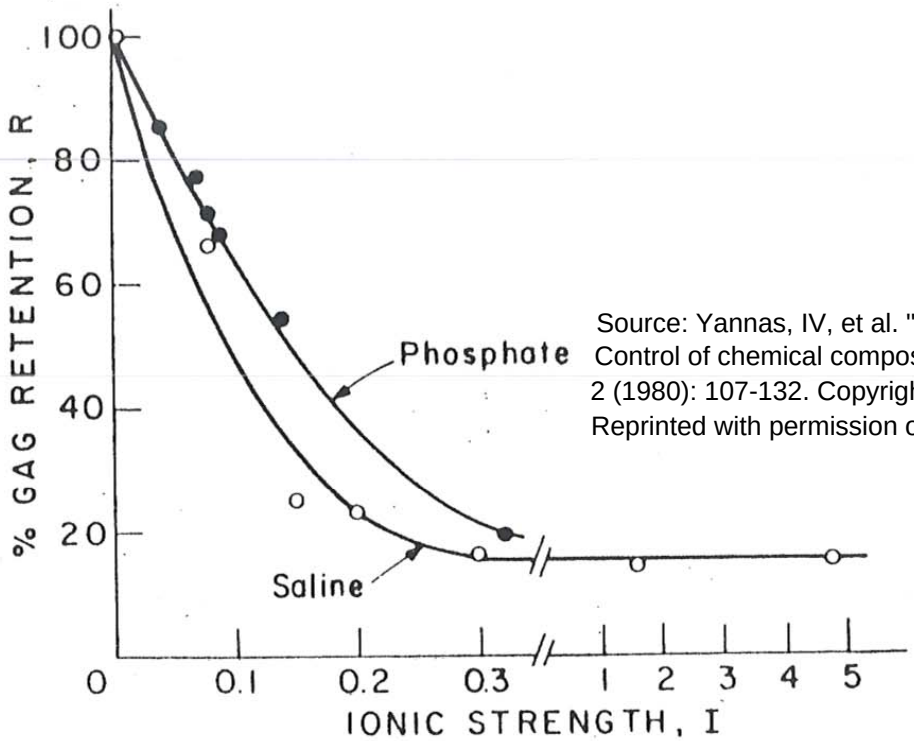
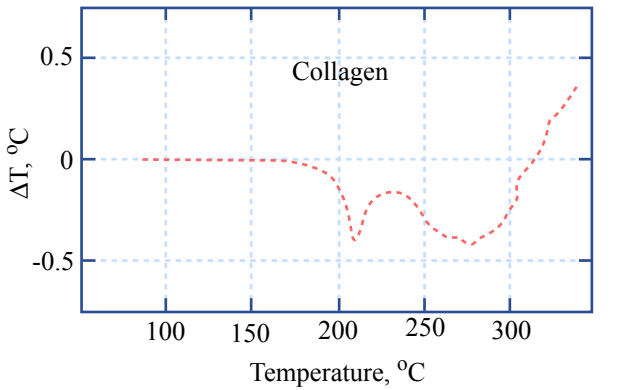


Figure 4. Effect of ionic strength on GAG retention in bovine hide collagen–GAG membranes. Original chondroitin 6-sulfate content, 10 wt. %. Eluted at 4°C in saline and in mixed phosphate buffer (pH 7.4) over 24 hr. Crosslinked with 0.25 wt. % glutaraldehyde in 0.05 *M* acetic acid, pH 3.2, at 22°C over 24 hr.

C. Preservation of native collagen structure (collagen vs gelatin)

Melting of tertiary structure of collagen (triple helix) is a helix-coil transition to the randomly coiled gelatin. It occurs near 210°C (see endothermic peak in differential analytical data below). Above that temperature collagen is pyrolyzed (reacts destructively with oxygen).



Differential thermal analysis of anhydrous rat-tail tendon.
Specimen heated at 5°C/min under a nitrogen atmosphere.

Melting of collagen fibers

- **Dilute solution of collagen single molecules. Helix → Coil transition. Single collagen molecules melt at ca. 37°C in dilute solution.**
- **Hydrated collagen fiber → Hydrated Gelatin fiber. "Shrinkage", "gelatinization". Many helices melt into coils in hydrated state (over 30% water) . Transition at > 60°C.**
- **Anhydrous collagen fibers → anhydrous gelatin fibers. Solid state transition at 205°C. Melting point of anhydrous collagen fibers.**
- **Thermodynamics of melting:**

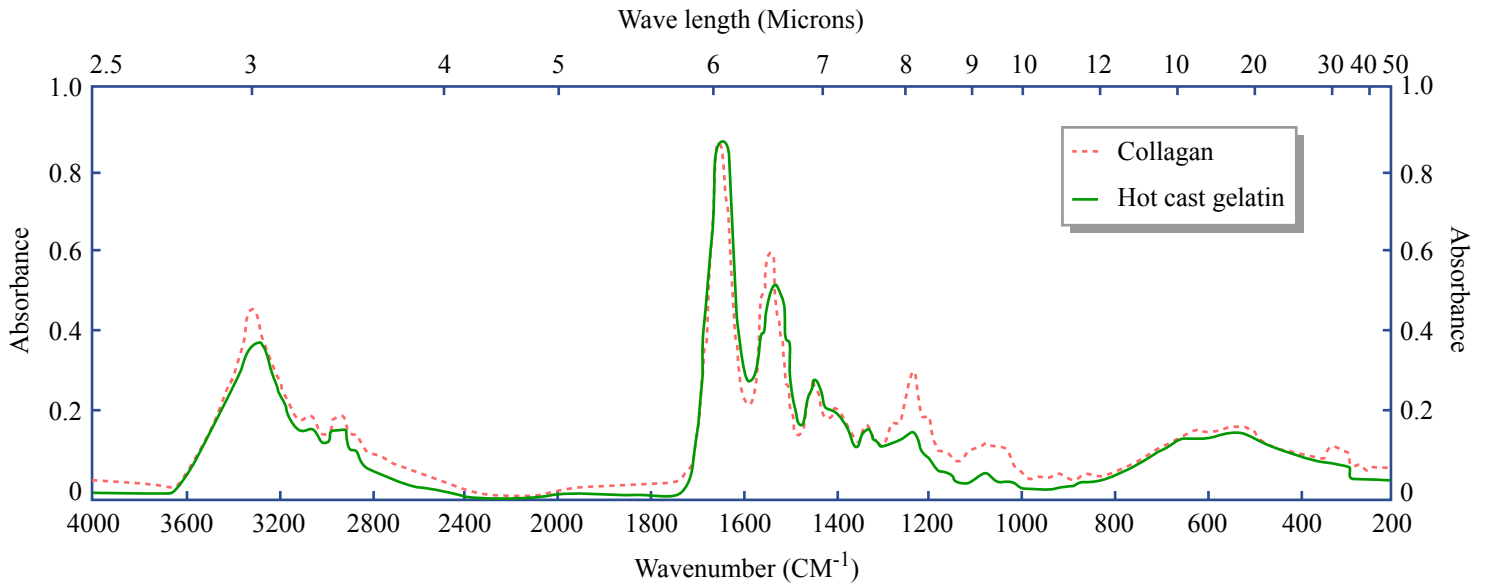
$$\Delta G = \Delta H - T\Delta S$$

G is Gibbs' free energy, the enthalpy is H = E + PV, T is absolute temperature and S is the entropy.

Equilibrium when $\Delta G = 0$. $T_m = \Delta G / \Delta S$. Implications of large vs small entropy of melting.

Analyze gelatin content of collagen materials.

Tertiary structure of Type I collagen (triple helix) viewed by infrared spectroscopy. Solid line: Collagen spectra. Broken line: Gelatin spectra.



The IR absorption spectrum of collagen and hot-cast gelatin.

Calculation of collagen/gelatin ratio in transparent films based on IR spectroscopy

When a substance absorbs radiation the relative amount absorbed can be used to determine the concentration of the component that absorbs. Beer's law gives:

$$A = kc$$

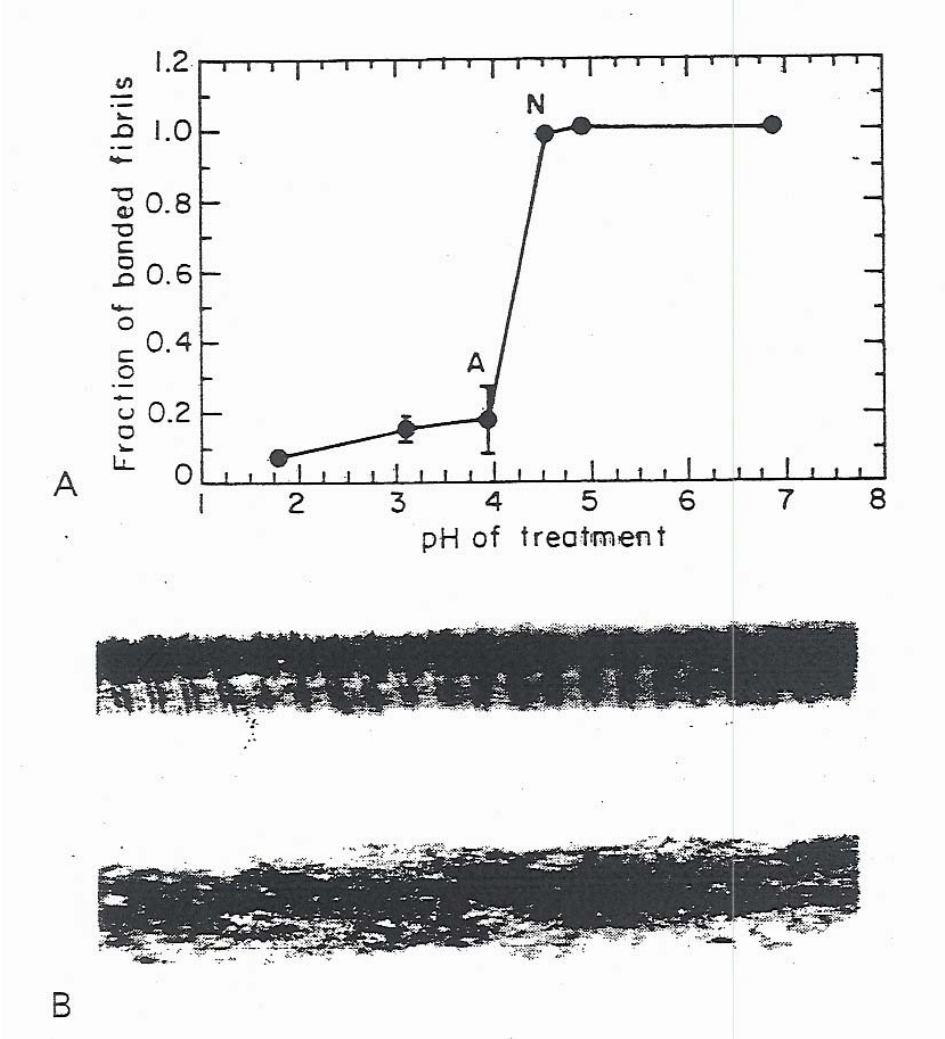
where A is the absorbance at a particular wavelenth, *k* is a constant and *c* is concentration of the absorbing component.

Example of assay for gelatin content. Measurement of *A* at the infrared frequency of 1230 cm^{-1} ("amide III band") for thin films of collagen and gelatin shows that collagen absorbs more strongly and eventually allows calculation of the relative mass of collagen/gelatin in an unknown film using the ratio $A_{collagen}/A_{gelatin}$.

D. Melting of collagen quaternary structure: thromboresistance.

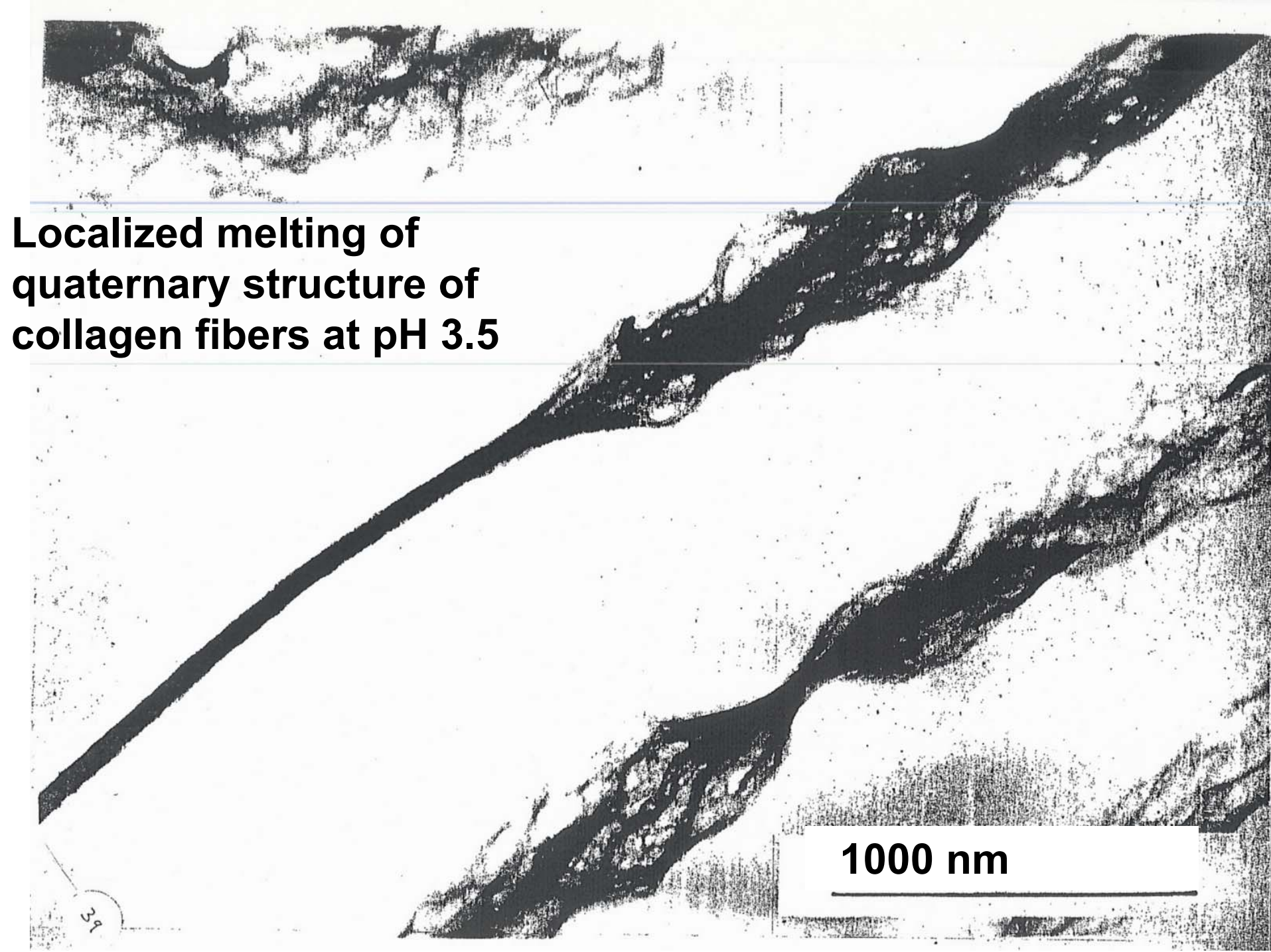
Melting of quaternary collagen structure downregulates platelet clotting and controls thrombosis. It also controls the inflammatory response.

Platelets are cells involved in blood clotting. They form clots in contact with collagen quaternary structure (banding). Platelet clots release inflammatory factors (cytokines, growth factors that kick off the inflammatory response. Melting of banded structure prevents clotting and downregulates the inflammatory response.

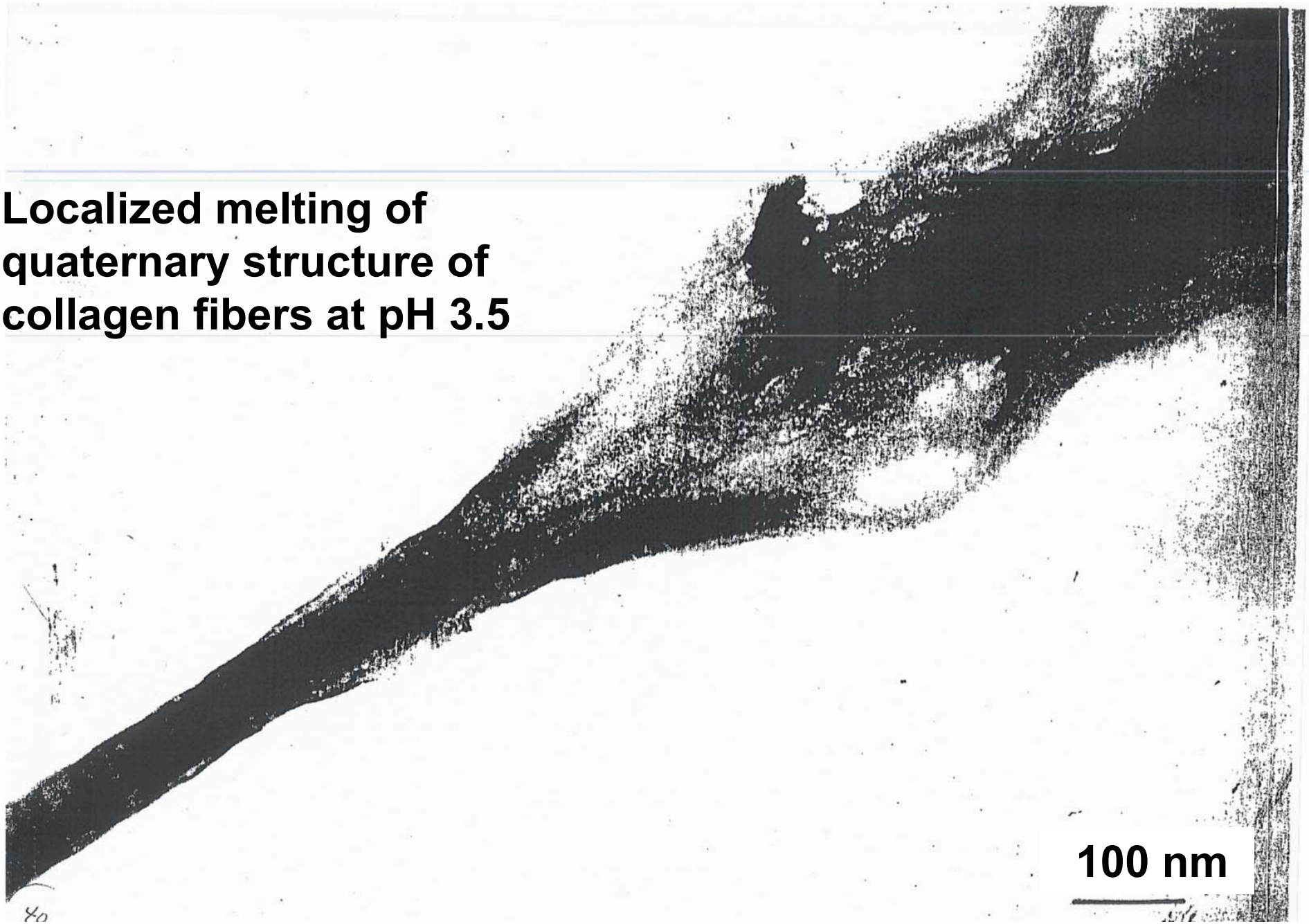


Melting of quaternary structure of collagen fibers occurs below pH 4.5. Melting confers thromboresistance to the scaffold. Platelets Do not aggregate unless the quaternary structure Is intact. Blocking of platelet aggregation leads to downregulation of the inflammatory response at the site of grafting or implantation.

Forbes, M. J. “Cross-flow filtration, transmission electron micrographic analysis and blood compatibility testing of collagen composite materials for use as vascular prosthesis.” S.M. Thesis, MIT, 1980.


Localized melting of
quaternary structure of
collagen fibers at pH 3.5
1000 nm

Localized melting of quaternary structure of collagen fibers at pH 3.5



E. Pore structure of scaffold can be used to control the density of binding sites for cells (ligands)

- Porous materials possess much higher specific surface (mm^2/mm^3) than nonporous materials.
- Porosity characterized by pore volume fraction and average pore diameter. Each affects the specific surface.
- Decrease in pore diameter leads to increased specific surface.
- Pore size decreases with increase in cooling rate.

Density of ligands increases with decrease in average pore size of scaffold

The surface density of binding sites (ligands) is

$$\Phi_b = N_b/A = \rho_b/\sigma$$

where Φ_b is the number of ligands N_b per unit surface A of template. It is also equal to the volume density of ligands ρ_b (number of ligands per unit volume of porous template) per unit specific surface of template, σ (in units of mm^2/mm^3).

If a cell is bound to χ ligands, there will be N_b/χ bound cells per unit surface. The volume density of cells is $\rho_c = \rho_b/\sigma$ and the surface density of cells is:

$$\Phi_c = \Phi_b/\chi = N_b/\chi A = \rho_b/\chi\sigma = \rho_c/\sigma$$

Density of ligands increases with decrease in average pore size of scaffold (cont.)

Example. In a scaffold with average pore diameter 10 μm that was grafted inside a skin wound a volume density, ρ_c, of myofibrolasts of order $10^7/cm^3$ porous scaffold was observed. For this scaffold the specific surface σ is calculated (using a simple geometric model) at about 8 X 10^4 mm^2/mm^3; therefore, 1 cm^3 of scaffold is characterized by a cell surface density of $\Phi_c = \rho_c/\sigma = 10^7/(8 \text{ X } 10^4) = 125$ cells/mm^2. For another scaffold, with identical chemical composition but with pore size as large as 300 μm, Φ_c is the same as above; however, the specific surface σ is calculated at only 3 X 10^3 mm^2/mm^3 and the volume density is only $\rho_c = \Phi_c\sigma = 125 \text{ X } 3 \text{ X } 10^3 = 3.75 \text{ X } 10^5$ per cm^3 scaffold.

Conclusion. The scaffold with the smaller pore diameter (10 μm) has a volume density of myofibrolasts that is about 27 times higher than that with the scaffold that has the larger pore diameter (300 μm).

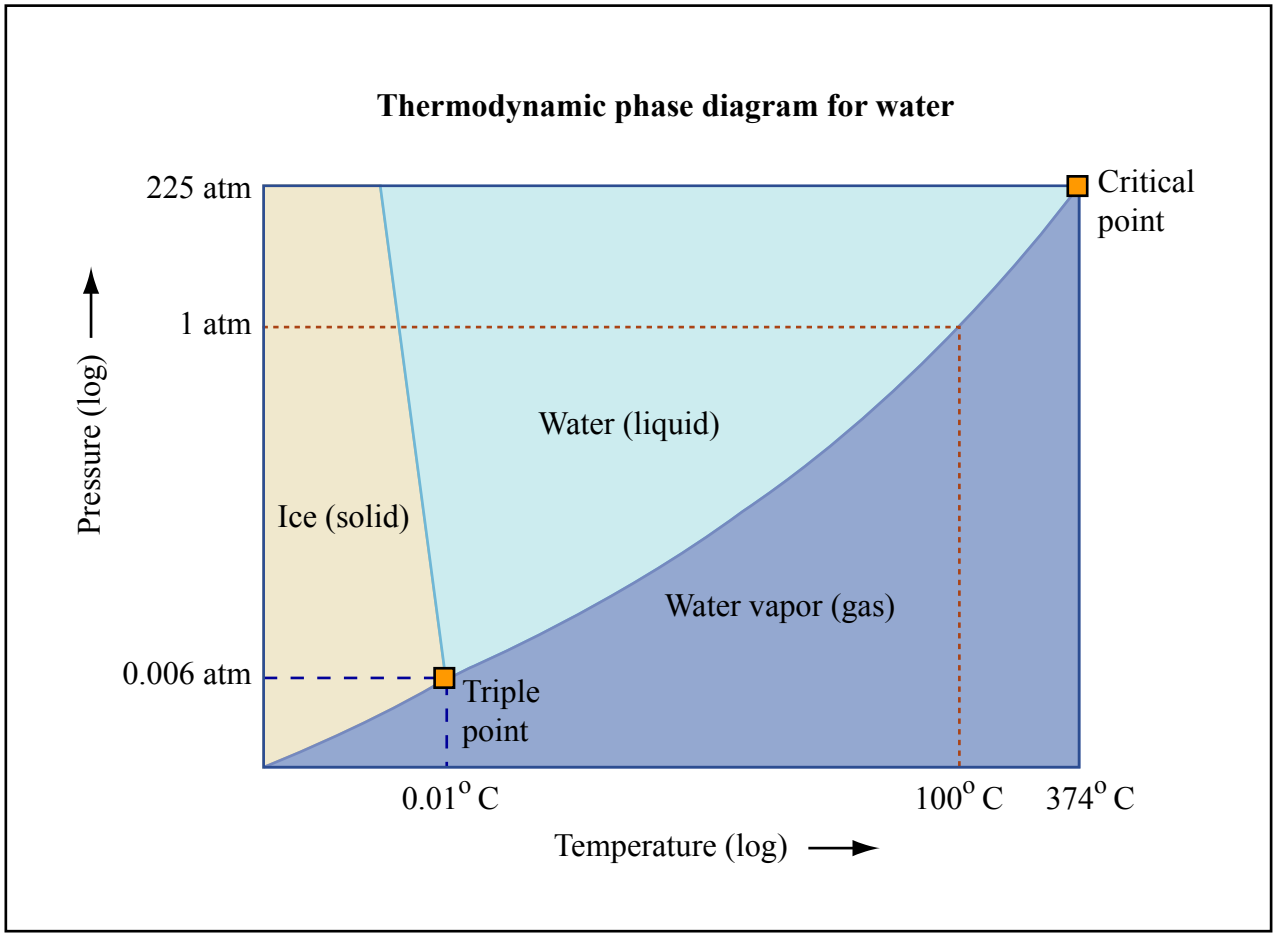
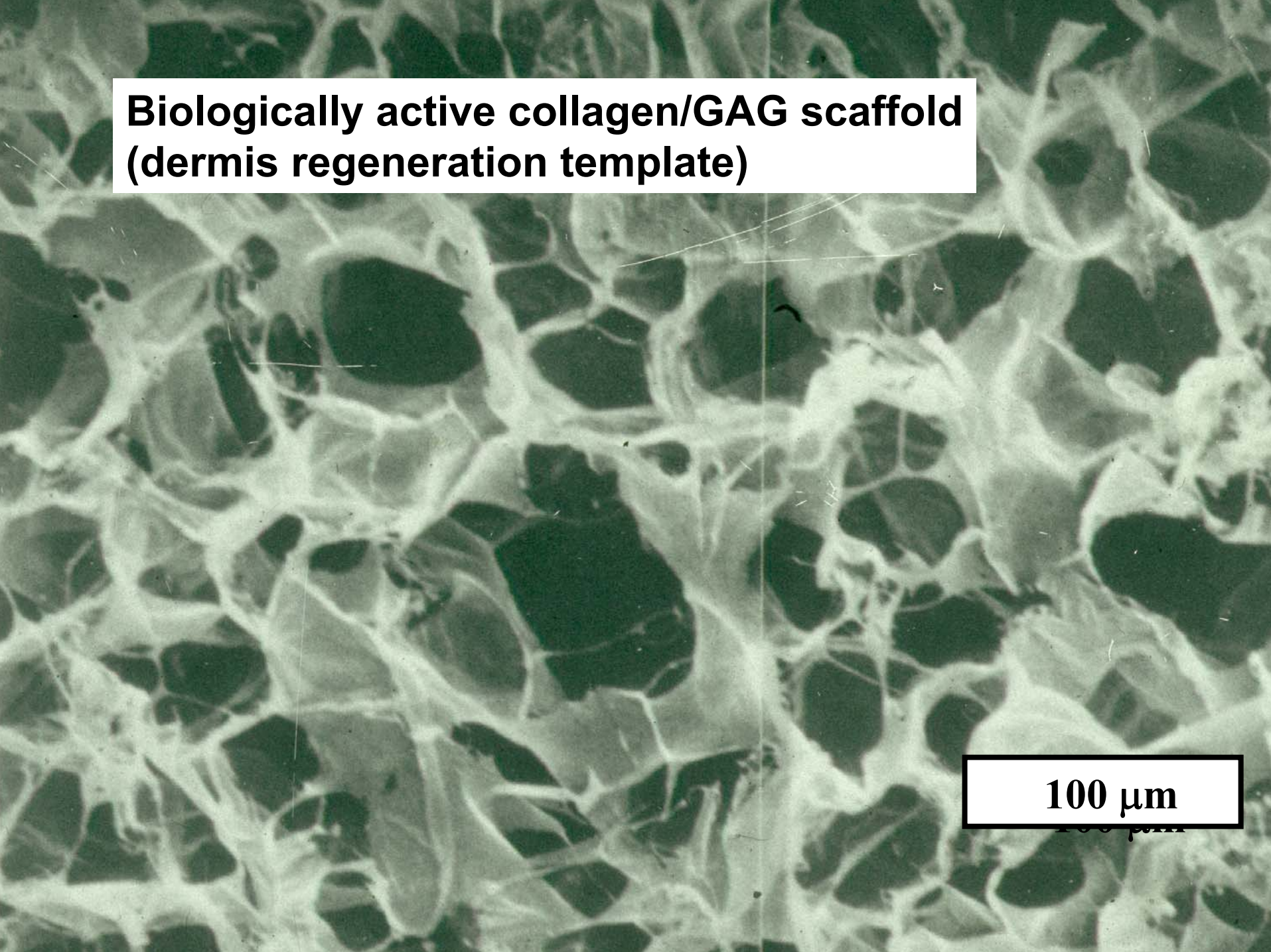
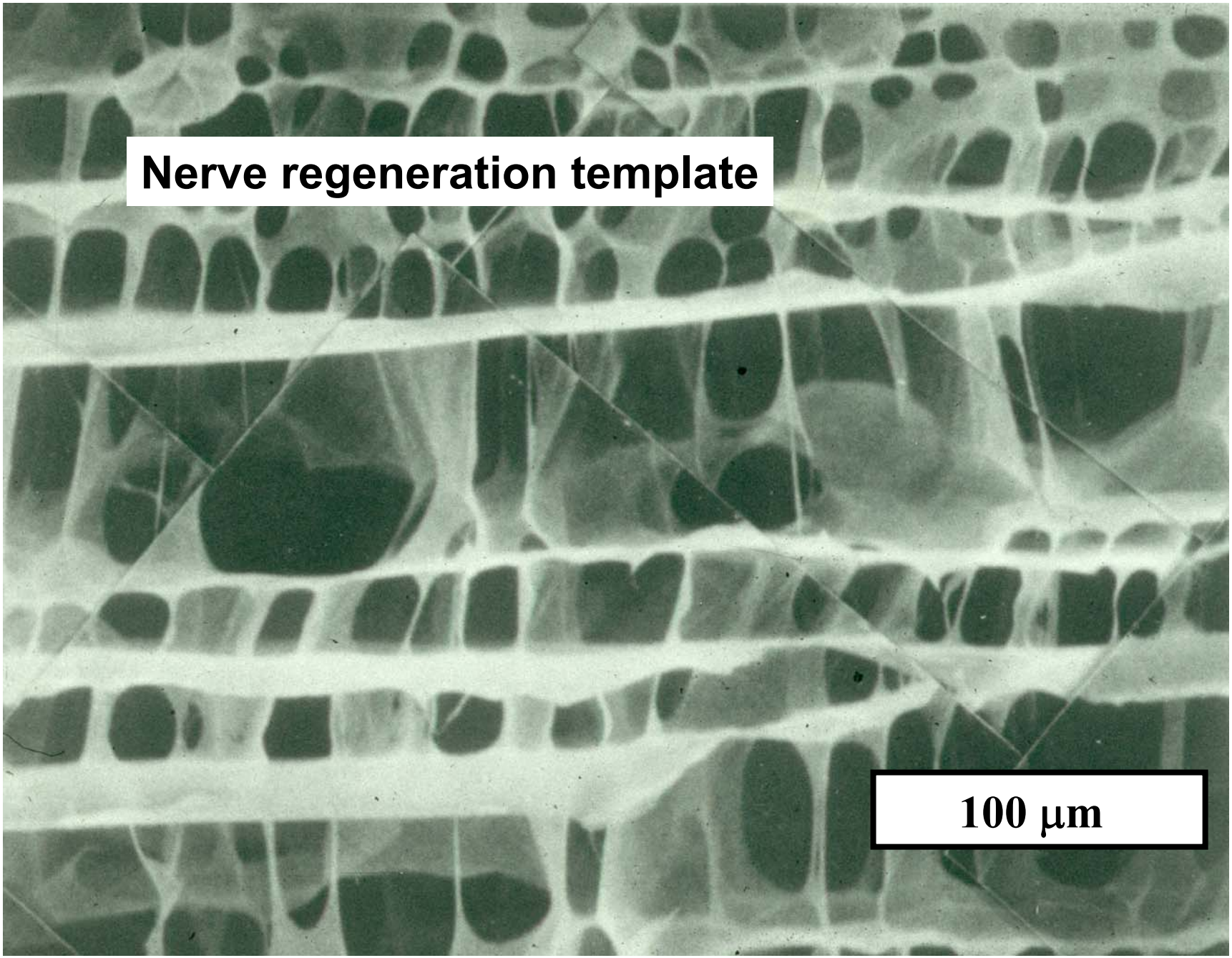


Image by MIT OpenCourseWare.

Collagen/GAG precipitate is freeze-dried (lyophilized) by dehydration at –40°C under vacuum to form pores of controlled size


Biologically active collagen/GAG scaffold
(dermis regeneration template)
100 μm


Nerve regeneration template
100 μm

Procedures used to study the pore structure of scaffolds. Unlike collagen sponges (used as hemostatic agents), regeneration templates have very high pore volume fraction. typically >95%.

Diagram removed due to copyright restrictions.

Summary of process for synthesis of active ECM analogs:

---- Ionic complexation of collagen/GAG.
---- Formation of pore structure.
---- Crosslinking.

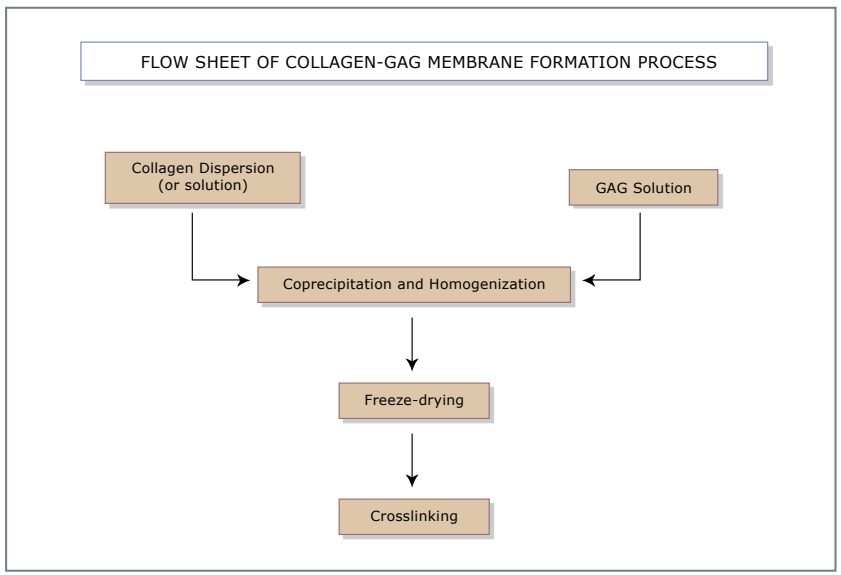


Figure by MIT OpenCourseWare.

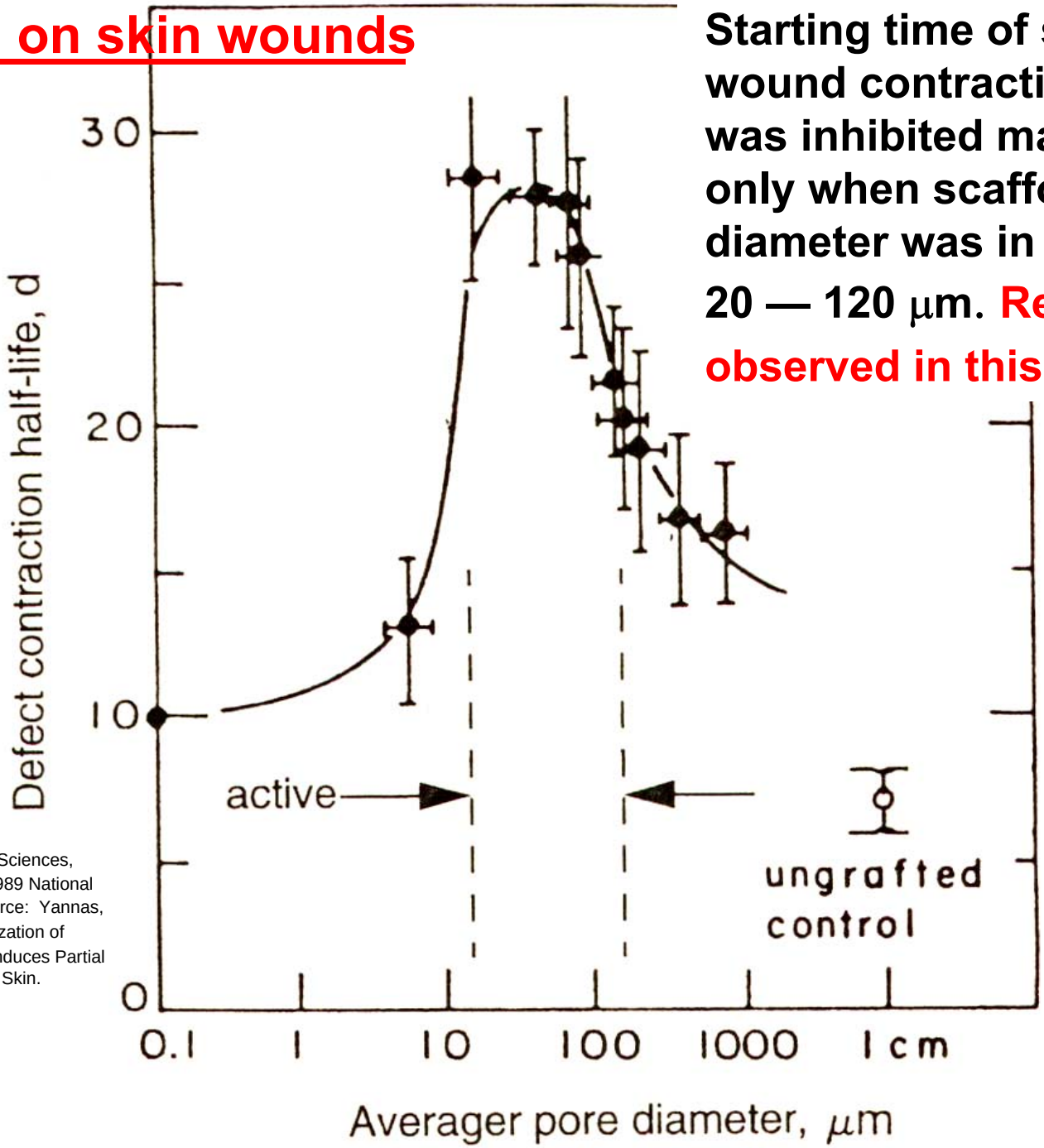
2. Identification of structural features that determine the biological activity of scaffolds. (outline)

A. Data from in vivo studies of regenerative activity.

B. Table of structural determinants.

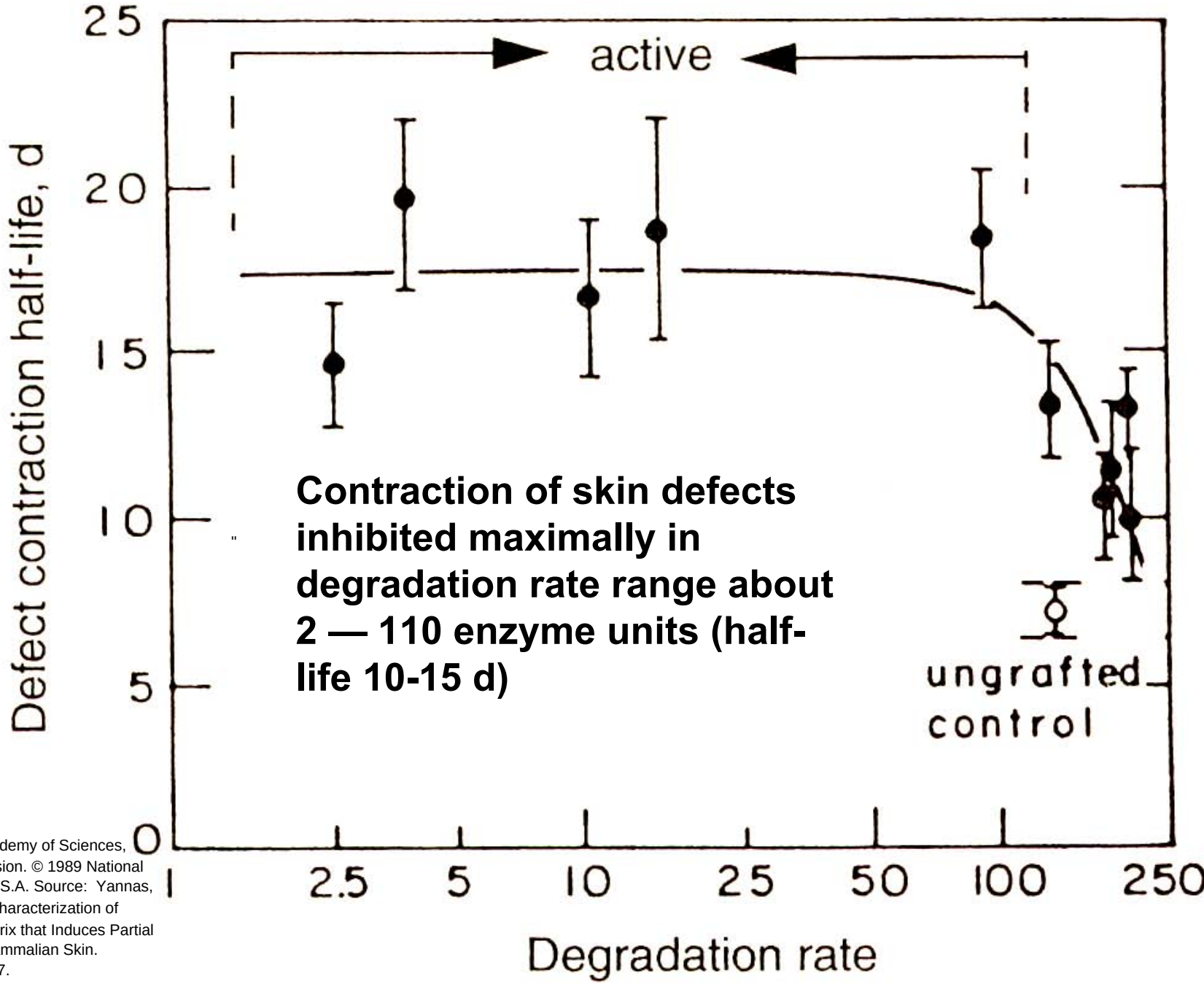
C. Conclusions.

Scaffolds on skin wounds



Starting time of skin wound contraction was inhibited maximally only when scaffold pore diameter was in range 20 — 120 μm. Regeneration observed in this range only..

Scaffolds on skin wounds



Contraction of skin defects inhibited maximally in degradation rate range about 2 — 110 enzyme units (half-life 10-15 d)

Scaffolds on peripheral nerves

Nerve chamber (tubulation) model. Gap length between nerve stumps is fixed.

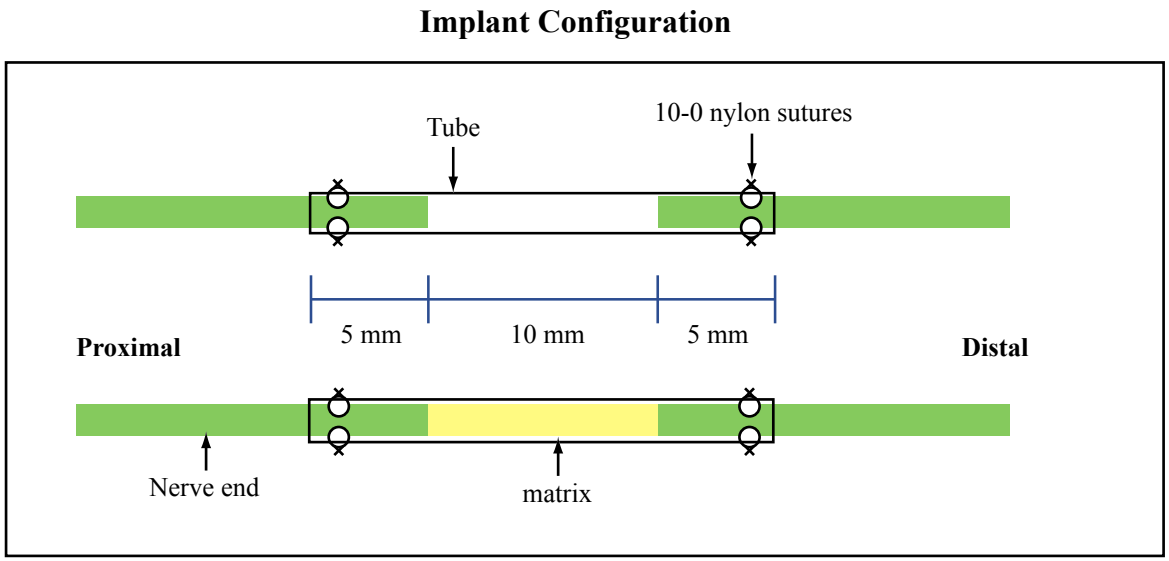
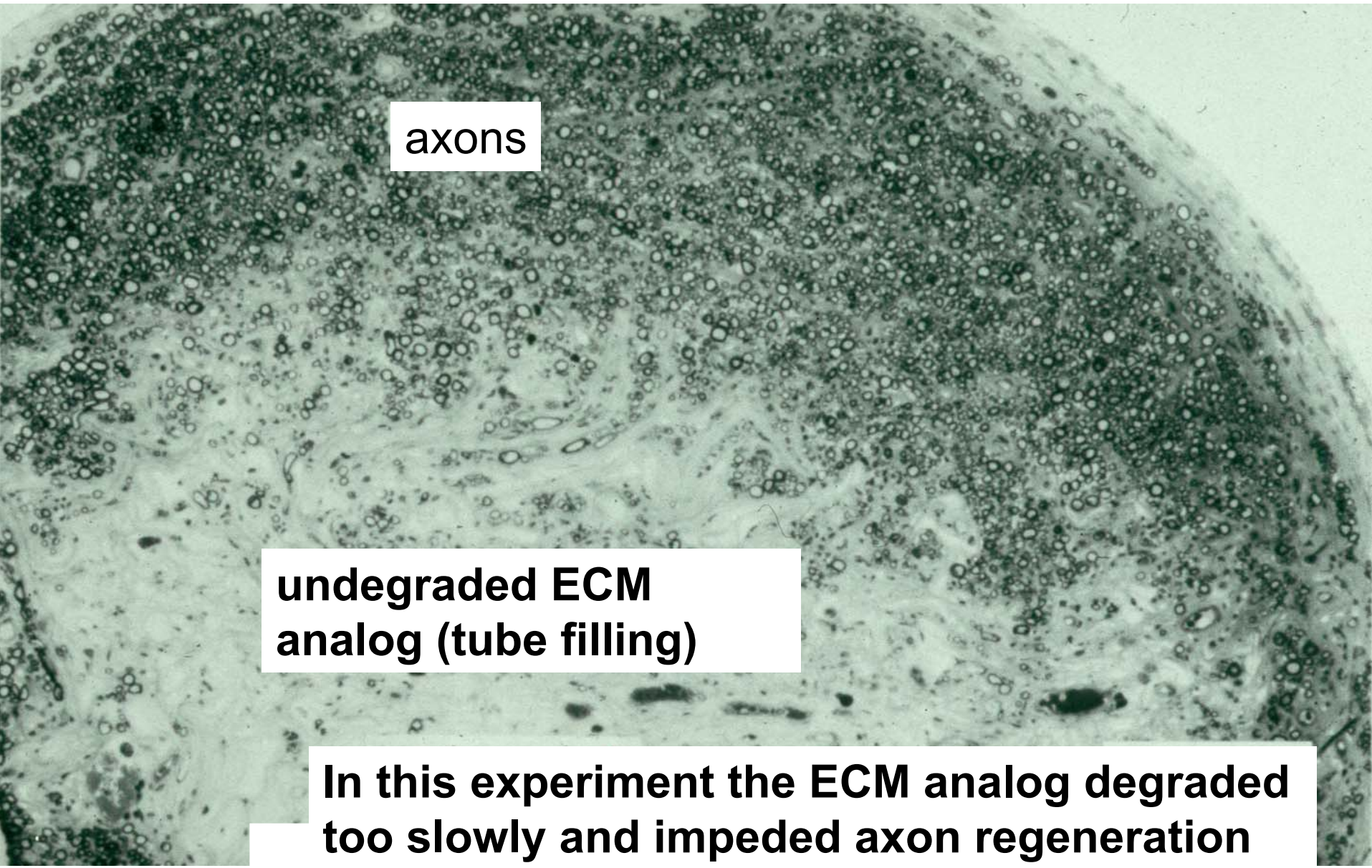


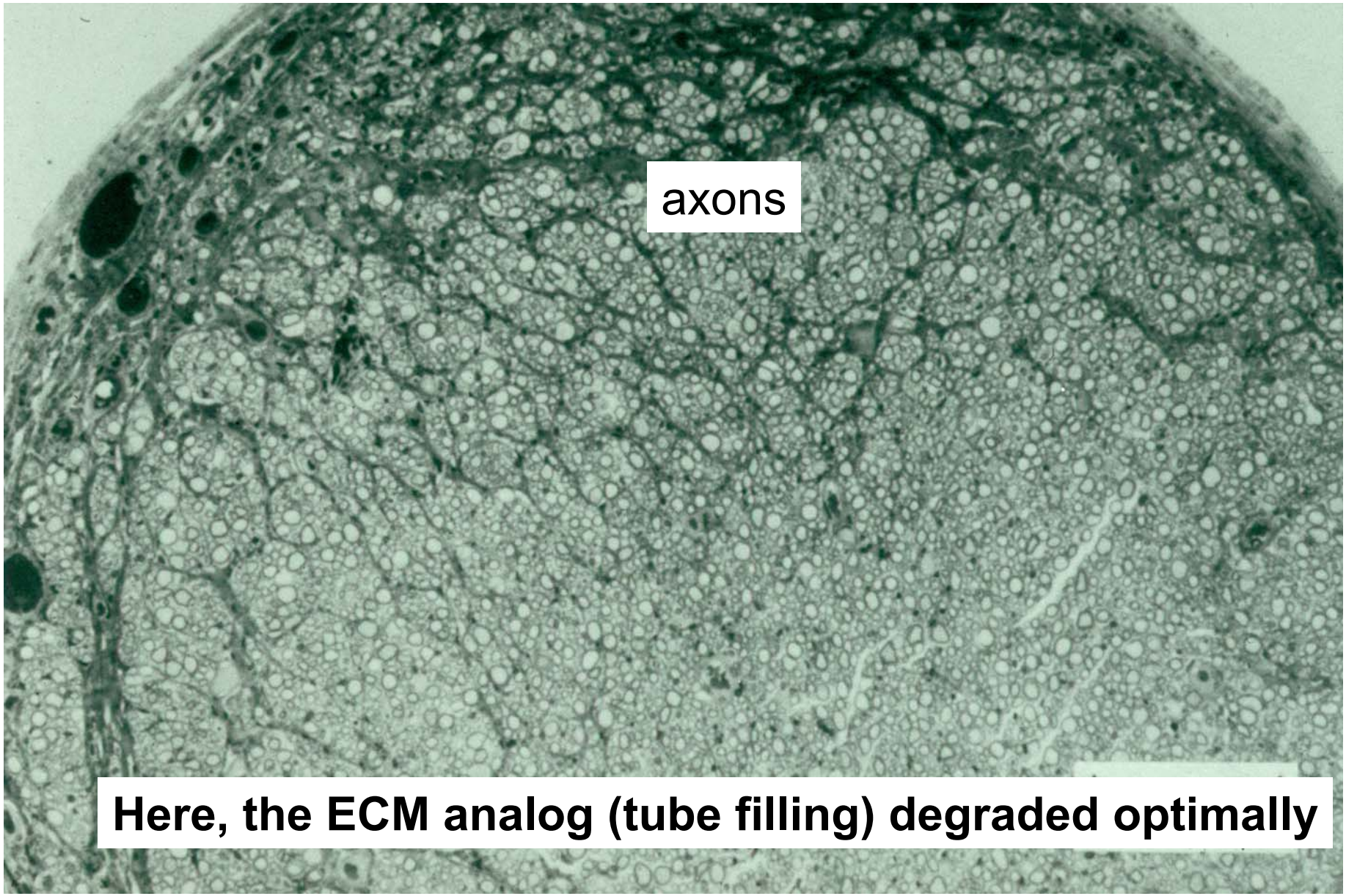
Figure by MIT OpenCourseWare.

In this experimental configuration it is possible to study the effect of structure either of the nerve chamber (tube) or the effect of a tube filling.

Scaffolds on peripheral nerves



Scaffolds on peripheral nerves

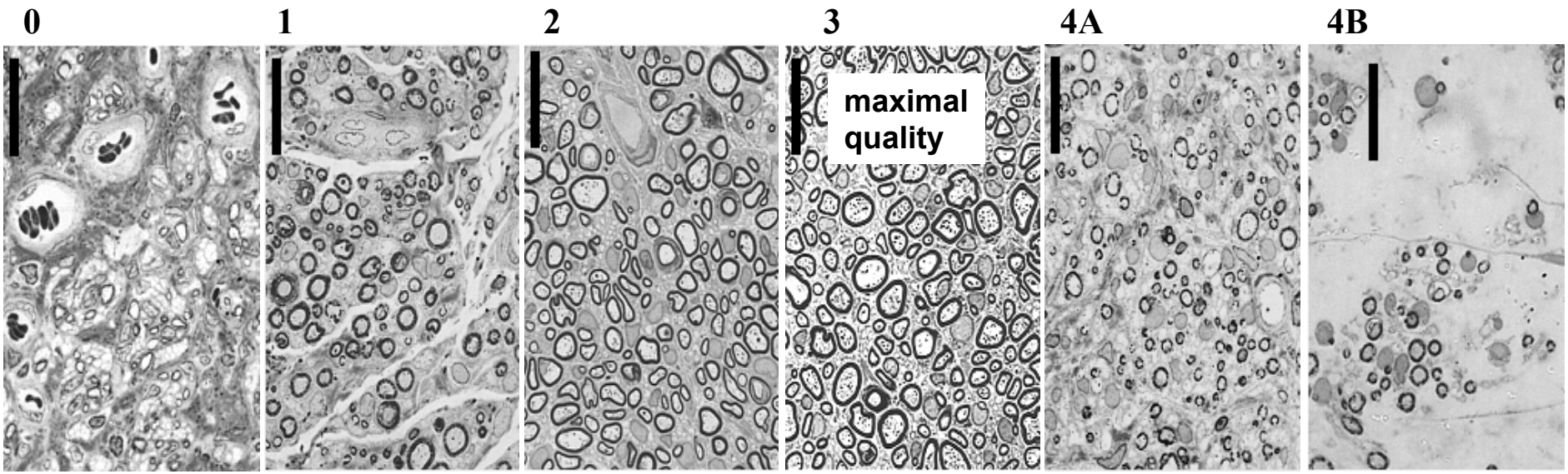


Scaffolds on peripheral nerves

Effect of collagen tube degradation rate. Maximal quality of nerve regeneration observed at intermediate tube degradation rate.

Scale bars: 25 μm

Regenerated nerves (Harley, B. MIT Thesis, 2002)



Continuously decreasing degradation rate of collagen tube

Scaffolds on peripheral nerves

Conduction velocity of regenerated nerve became normal at scaffold pore diameter about 5 μm

Graph removed due to copyright restrictions.
See Figure 10.9 in [TORA].

[TORA] = Yannas, I. V. *Tissue and Organ Regeneration in Adults*. New York, NY: Springer-Verlag, 2001. ISBN: 9780387952147.
[Preview in Google Books]

Table. Structural determinants of regenerative activity of two scaffolds based on ECM analogs

Structural parameter of scaffold that is required for regenerative activity	SKIN regeneration (DRT)[1]	NERVE regeneration (filling of silicone chamber)[2]	Structural features of scaffold involved in contraction blocking
Type I collagen/GAG[3], w/w	98/2	98/2	Ligand identity required for binding of $\alpha 2\beta 1$ integrin and other fibroblast integrins
Residual collagen fiber banding) [Yannas, 1990]	ca. 5% of native collagen	ca. 5% of native collagen	Platelet aggregation downregulated
Average molecular weight between crosslinks, M_c, kDa	5-15	40-60	Scaffold maintains undegraded structure during contraction process
Average pore diameter, μm	20-120	5-10	Max. ligand density
Pore channel orientation	random	axial	Ligand orientation specific for stroma of organ

[1] Yannas et al., 1989. [2] Yannas, 2001. [3]Glycosaminoglycan.

Conclusions on biological activity of scaffolds used as regeneration templates

1. Certain ECM analogs are biologically active scaffolds (regeneration templates) that induce regeneration of tissues and organs: skin, peripheral nerve and the conjunctiva (eye) in humans and experimental animals.
2. Regeneration templates lose their activity if the following structural features fall outside a narrow range: chemical composition, collagen quaternary structure, pore diameter, degradation rate.
3. The data suggest that templates induce regeneration in a defect by blocking selectively the contraction process that leads to closure of the defect in adults.
4. Templates block contraction by two basic mechanisms. First, by downregulating differentiation of fibroblasts to myofibroblasts. Second, by binding most of the contractile cells in the defect over a period corresponding to the duration of contraction in that defect. Binding requires the presence of appropriate ligands (chem. composition) at a minimal density (pore diameter) over a critical duration (degradation rate).

Questions

- **How to design steps in manufacturing process?**
- **How to set up quality control in a plant manufacturing collagen-GAG scaffolds?**
- **What happens to biological activity of the scaffold product if quality control fails?**

20.441J / 2.79J / 3.96J / HST.522J Biomaterials-Tissue Interactions

Fall 2009