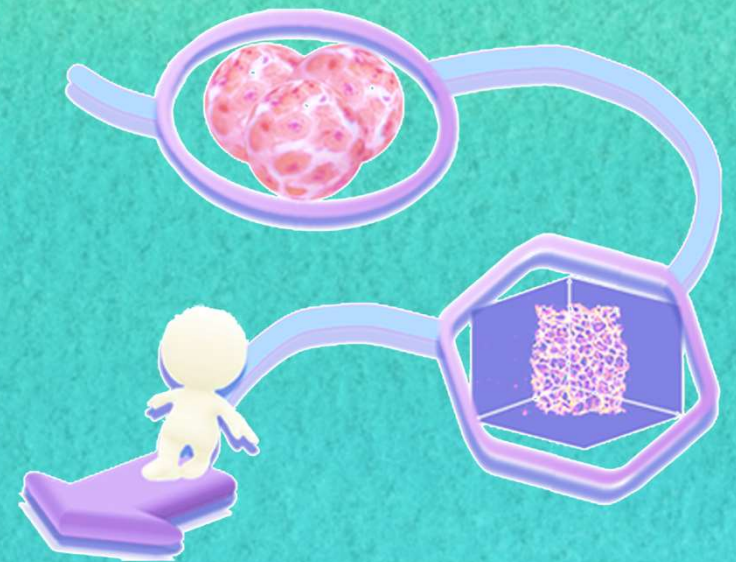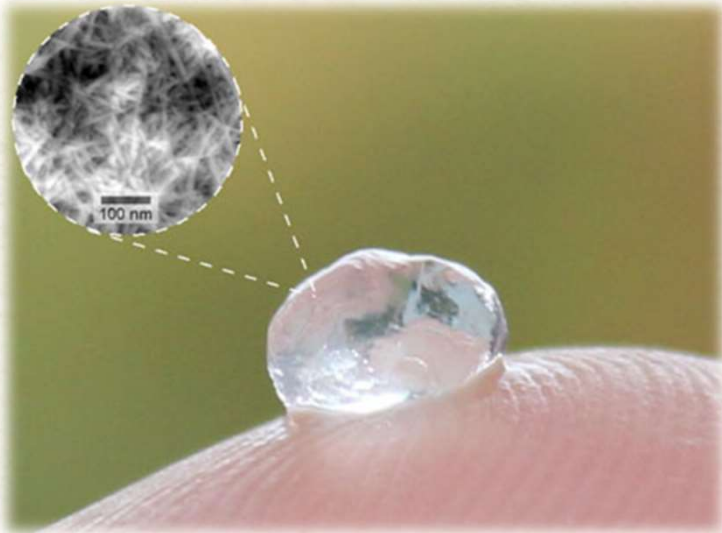# Hydrogel Stiffening

Corso LM 'Materiali intelligenti e biomimetici' – Prof. Ahluwalia
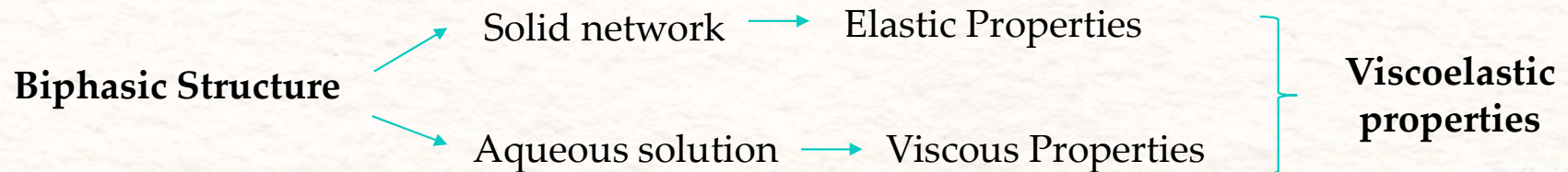
15/03/2017

Ludovica Cacopardo – PhD Student

Hydrogels, Biological material & soft tissues..

Hydrogels **are hydrophilic polymer networks** which may absorb from 10–20% up to thousands of times their dry weight in water.

As the term 'network' implies, **crosslinks** have to be present to avoid dissolution of the hydrophilic polymer chains/segments into the aqueous phase.

Biphasic Structure

Solid network → Elastic Properties

Aqueous solution → Viscous Properties

Viscoelastic properties

Hydrogels & Crosslinking types

Hydrogels can also be described in a **rheological way:**

Aqueous solutions of hydrophilic polymers at low or moderate concentrations, where **no substantial entanglement of chains occurs,** normally show <u>**Newtonian behavior**</u>.

On the other hand, once **crosslinks** between the different polymer chains are introduced, the so obtained networks show <u>**viscoelastic and sometimes pure elastic behaviour.**</u>
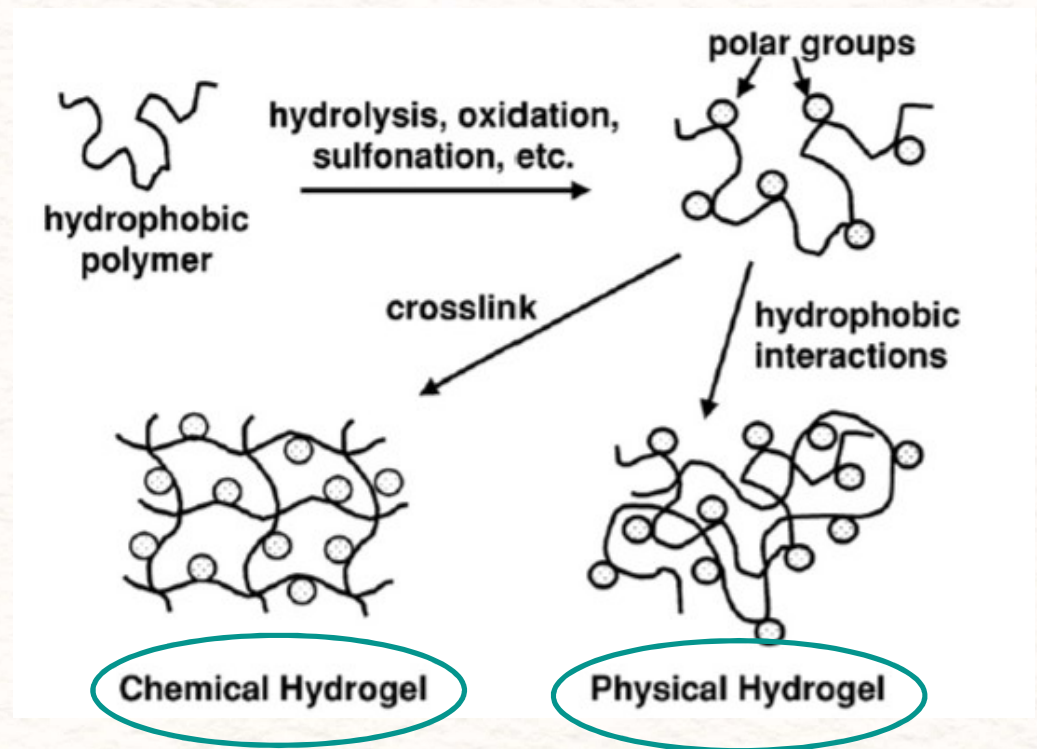
Physical Hydrogels

Hydrogels are defined **'reversible' or 'physical' gels** when the <u>networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces</u>.

Physical hydrogels are not homogeneous, since clusters of molecular entanglements or hydrophobically- or ionically-associated domains can create inhomogeneities.

When a polyelectrolyte is combined with a multivalent ion of the opposite charge, it may form a physical hydrogel known as an 'ionotropic' hydrogel. Further, when polyelectrolytes of opposite charges are mixed, **they may gel or precipitate depending on their concentrations, the ionic strength, and pH of the solution.**

<u>**All of these interactions are reversible**</u>, and can be disrupted by changes in physical conditions such as ionic strength, pH, temperature, application of stress, or addition of specific solutes that compete.

Chemical Hydrogels

Chemical Hydrogels are based on **covalently-crosslinked networks**. Chemical hydrogels may also be generated by crosslinking of water soluble polymers or by conversion of hydrophobic polymers to hydrophilic polymers plus crosslinking to form a network.

In the crosslinked state, **crosslinked hydrogels reach an equilibrium swelling level** in aqueous solutions which depends mainly on the crosslink density.

Equilibrium Water Content:
$$EWC = \frac{W_w}{W_t} \cdot 100\%$$

Like physical hydrogels, chemical hydrogels are not homogeneous. They usually contain regions of low water swelling and high crosslink density, called 'clusters', that are dispersed within regions of high swelling, and low crosslink density.
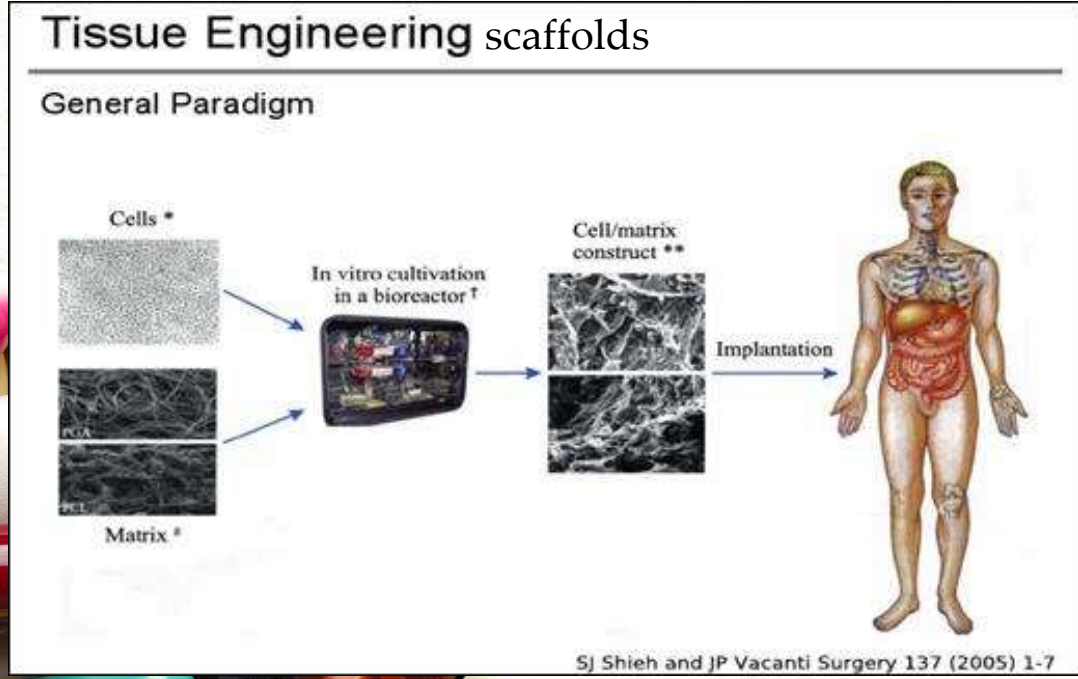
Applications

soft contact lenses

pills or capsules for oral ingestion

Tissue Engineering scaffolds

General Paradigm

Cells *

In vitro cultivation in a bioreactor †

Cell/matrix construct **

Implantation

Matrix *

SJ Shieh and JP Vacanti Surgery 137 (2005) 1-7

Adequate scaffold design and material selection for each specific application depend on several variables, including physical properties (e.g. **mechanics, degradation, gel formation**), mass transport properties (e.g. **diffusion)**, and biological properties (e.g. **cell adhesion** and signaling).

Examples

Hydrophilic Polymers used to synthesize hydrogels				
Natural Polymers	**Anionic polymers:** HA, <u>alginic acid,</u> pectin	**Cationic polymers**: chitosan, poly-lysine	**Amphipathic polymers**: <u>collagen</u> (and <u>gelatin</u>), fibrin	**Neutral polymers**: dextran, <u>agarose</u>
Synthetic Polymers	**PEG** (polyethylene glycol), **PVA** (Polyvinyl alcohol), **PCL** (Polycaprolactone), **PolyHEMA** (Poly-hydroxyethyl methacrylate), **PU** (polyurethane) , **PA** (Polyacrylate), PVP (Polyvinylpyrrolidone)			

Example 1 - Collagen

Collagen is an attractive material for biomedical applications as it is the most abundant protein in mammalian tissues and is the **main component of natural ECM**.
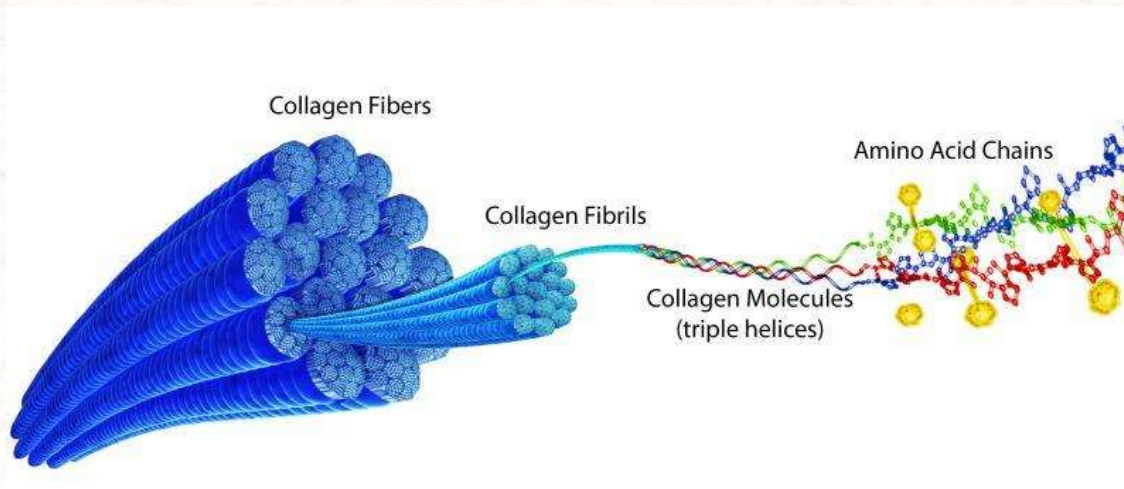
There are at least 19 different types of collagen, but the basic structure of all collagen is composed of three polypeptide chains, which wrap around one another to form a **three-stranded rope structure** [30]. The strands are held together by both hydrogen and covalent bonds. Collagen strands can self aggregate to form **stable fibers**.

Mechanical properties of collagen hydrogel can be enhanced by introducing various *chemical crosslinkers* (i.e. glutaraldehyde, formaldehyde, carbodiimide), by *crosslinking with physical treatments* (i.e. UV irradiation, freeze-drying, heating), and by *blending it with other polymers* (i.e. HA, PLA, PGA, PLGA, chitosan, PEO).

Collagen is naturally degraded by metalloproteases, specifically collagenase, and serine proteases [29], allowing for its degradation to be locally controlled by cells present in the engineered tissue.

Example 2 - Gelatin

Gelatin derives from **collagen denaturation**, resulting in a biodegradable, biocompatible and nonimmunogenic product, suitable for medical applications.

At a temperature of about 40°C, gelatin aqueous solutions form physical **thermo-reversible gels on cooling**. During gelling, the chains undergo a conformational disorder-order transition and tend to recover the collagen triple-helix structure.

With respect to collagen, which is also known to have wide biomedical applications, gelatin *does not express antigenicity in physiological conditions*, and it is much *cheaper and easier to obtain* in concentrate solutions.

On the other hand, gelatin exhibits **poor mechanical properties**. *In order to create stable gelatin hydrogels at 37°C, chemical crosslinking agents such as glutaraldehyde are typically used.*

Example 3 - Agarose

Agarose is a typical naturally-occurring **polysaccharide** that is known to form **thermoreversible gels** when a homogeneous solution is cooled from 99°C to a temperature below 35°C. The melting and gelling temperatures may be dependent on the concentration of the gel.

The major drawbacks of agarose are that it shows significantly *low cell adhesiveness* and cell proliferation, as it does not contain any moieties associated with cellular adhesion and adsorption of cell adhesive proteins.

Modification of polymers with peptides containing the cell recognition motif RGD (R, arginine; G, glycine; D, aspartic acid) has recently attracted much attention for enhancing the cell adhesiveness of substrates in tissue engineering

Example 4 - Alginate

Alginate is a linear **polysaccharide extracted from brown algae** has been used in a variety of medical applications including cell encapsulation and drug stabilization and delivery, because it gels under gentle conditions, has low toxicity, and is readily available.

Gels are formed when **divalent cations** such as Ca2+, Ba2+, or Sr2+ cooperatively interact with monomers to **form ionic bridges between different polymer chains**.

Ionically crosslinked alginate hydrogels do not specifically degrade but undergo **slow, uncontrolled dissolution**. Mass is lost through ion exchange of calcium followed by dissociation of individual chains, which results in loss of mechanical stiffness over time.
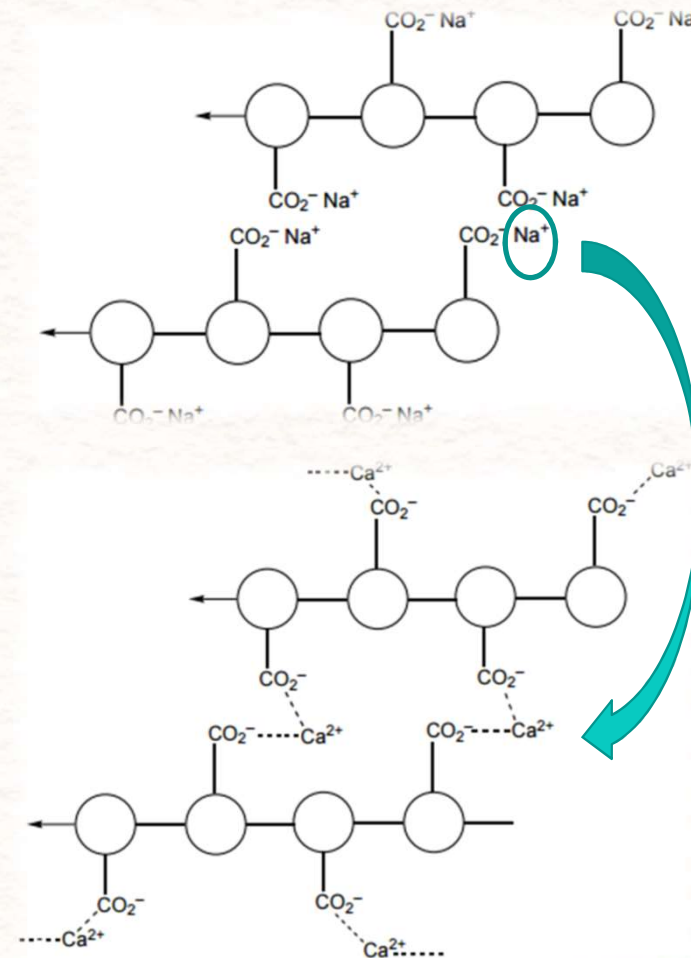

Calcium ions replace the sodium ions in the polymer. Each calcium ion can attach to two of the polymer strands.

Example 5 – Liver ECM

Decellularization maintains **microstructures of native extracellular matrices and its biochemical compositions**, providing tissue-specific microenvironments for efficient tissue regeneration.

Digestion, its necessary to solubilize decellularized ECM (i.e. breaks down proteins into smaller peptides).
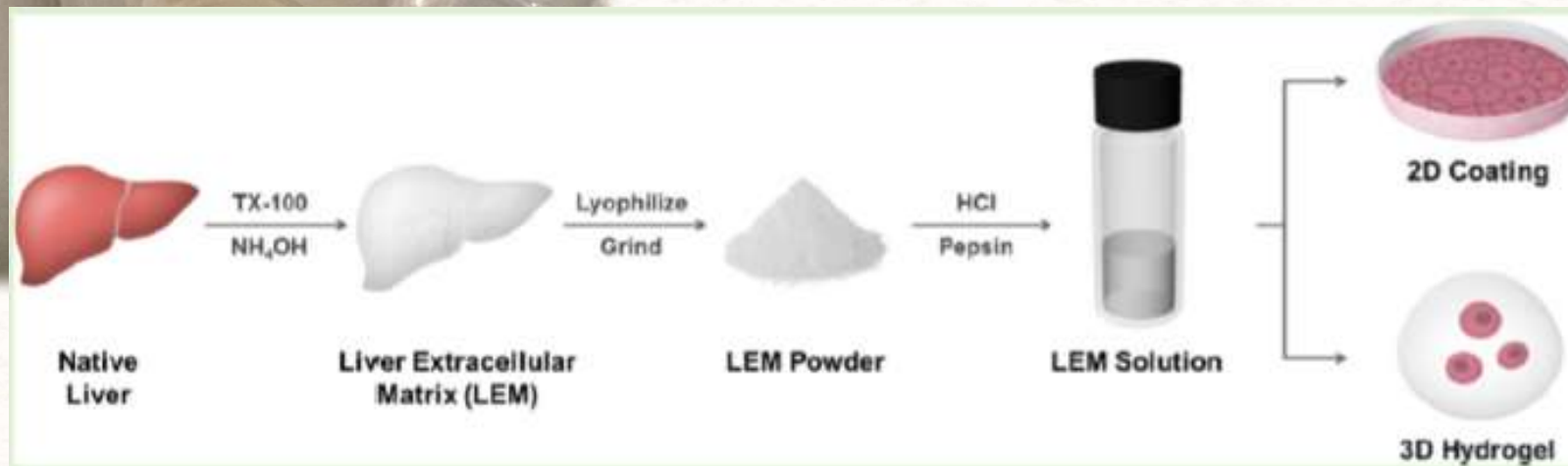
The digested ECM solution is brought from 4°C to 37°C to form hydrogels.

Methods for synthesizing physical and chemical hydrogels

Physical gels

- <u>Warm a polymer</u> solution to form a gel
- <u>Cool a polymer</u> solution to form a gel (e.g., agarose or gelatin in H2O)
- 'Crosslink' a polymer in aqueous solution, using freeze–thaw cycles to form polymer microcrystals
- <u>Lower pH</u> to form an H-bonded gel between two different polymers in the same aqueous solution
- Mix solutions of a polyanion and a polycation to form a complex coacervate gel (e.g., sodium alginate plus polylysine)
- Gel a polyelectrolyte solution with a multivalent ion of opposite charge

Chemical gels

Crosslink polymers in the solid state or in solution with:

- <u>Radiation</u>
- <u>Chemical crosslinkers</u> (e.g., treat collagen with glutaraldehyde)
- <u>Copolymerize a monomer+crosslinker</u> in solution/multifunctional macromer
- Chemically convert a hydrophobic polymer to a hydrogel
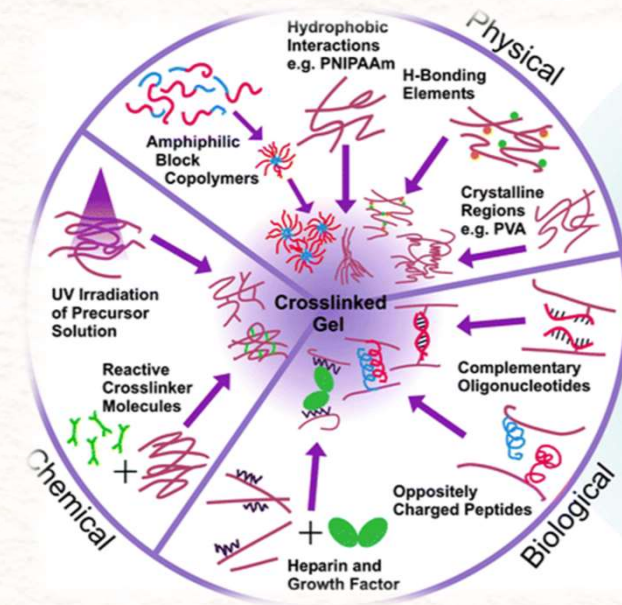
Why Stiffening?

- Stabilize hydrogels

- Enhance mechanical properties

- Modulate Mechanical Properties

Pathophysiological models of foetal growth, ageing, fibrosis

Crosslinker types

- Chemical/UV → Toxics effects
- Biological
- Enzimatic

In general, non toxic

Stiffening depends on concentration/dose

Stiffening depends on concentration and time

More stable hydrogels can be created by using either UV-light or chemical crosslinkers (e.g. glutaraldehyde). Despite the improved mechanical strength and proteolytic stability of synthetically crosslinked hydrogels, the crosslinkers often elicit either cytotoxic side-effects or immunological responses from the host. Photocrosslinked hydrogels may also encounter a limitation in applications of deep tissue implants, where light is unable to penetrate the host tissue.

Chemical stiffening: GTA

Crosslinking of amine containing polymers (i.e. collagen, gelatin, ecm) with GTA (glutaraldehyde) involves the *reaction of free amino groups of lysine* or hydroxy-lysine amino acid residues of the polypeptide chains *with the aldehyde groups of GTA*

Since glutaraldehyde is a **toxic** compound that even at low concentration shows cell-growth inhibition, hydrogels need to be careful washed before use.
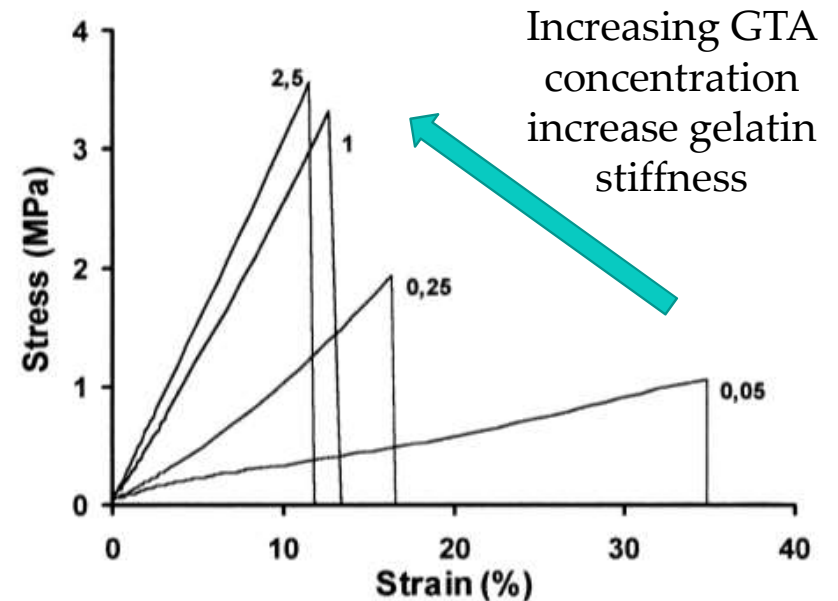

Increasing GTA concentration increase gelatin stiffness
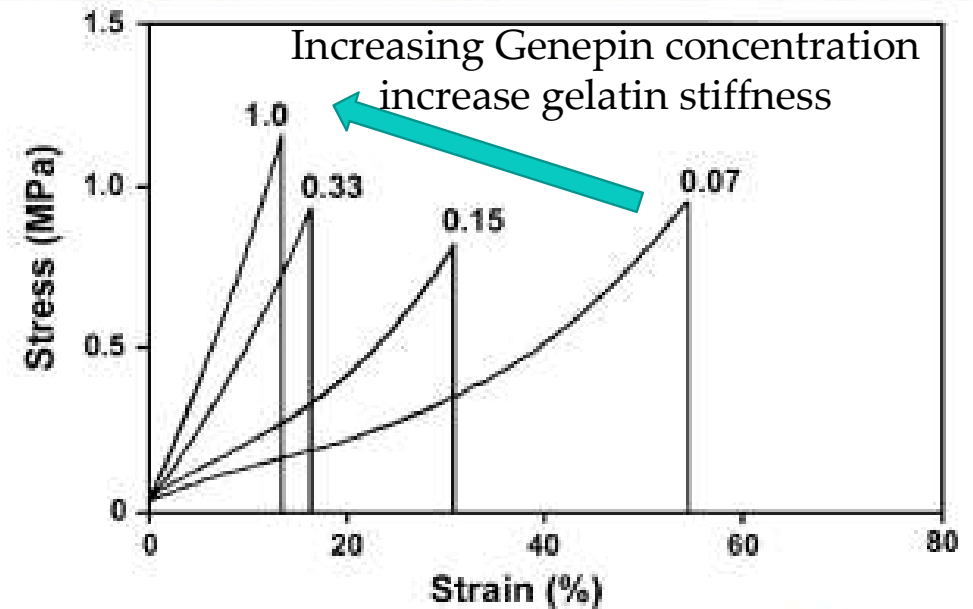
Fig. 1. Typical stress–strain curves recorded from gelatin films crosslinked with GTA. The numbers near the curves indicate the concentration of GTA, expressed as wt%.

Biological crosslinkers: Genepin

Toxicity of chemical reagents such as GTA is the reason of the increasing demand for a crosslinking agent able to form stable and biocompatible crosslinked products.

Genipin is a **naturally occurring crosslinking agent**, which seems to display promising characteristics.

Genipin can be obtained from an iridoid glucoside, geniposide, abundantly present in **gardenia fruits**. Genipin has been widely used in herbal medicine, and the *dark blue pigments obtained by its spontaneous reaction with amino acids* or proteins have been used in the fabrication of food dye.

Increasing Genepin concentration increase gelatin stiffness

Fig. 1. Typical stress–strain curves recorded from gelatin films cross-linked with genipin. The numbers near the curves indicate the concentration of genipin, expressed as wt%.

Enzymatic stiffening: mTG

A **naturally occurring protein crosslinking enzyme**, transglutaminase, was used to form a thermally stable hydrogel from gelatin. This enzyme is ubiquitous in nature, being found in many species of the plant and animal kingdoms (e.g. peas, oysters, shrimp, tuna, chickens, cows, and humans).

Transglutaminase functions by **catalysing the formation of covalent N e-(g-glutamyl) lysine amide bonds** between individual gelatin strands to form a permanent network of polypeptides.

Microbial transglutaminase (mTG) is a native protein that is innocuous and **commonly used in food manufacturing processes** approved for human consumption by the U.S. Food and Drug Administration.
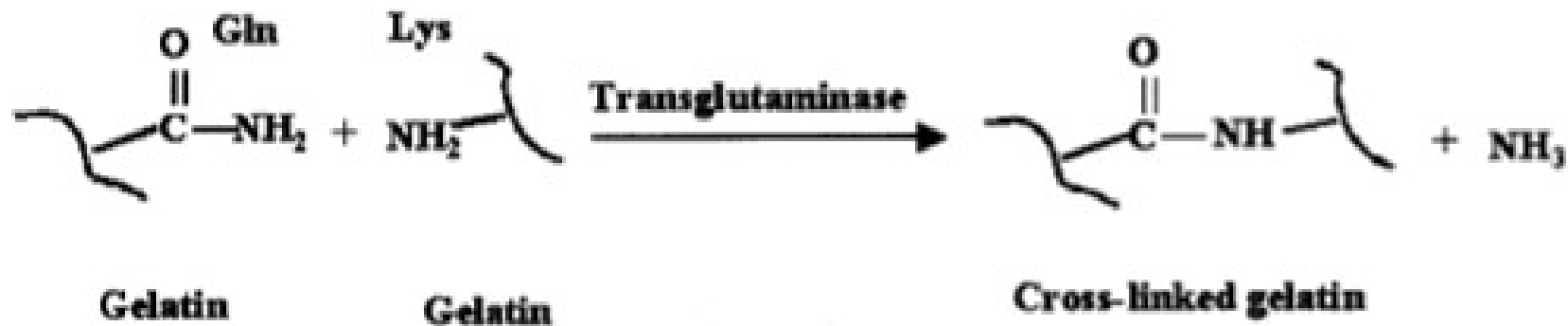
# Example: mTG-Gelatin Hydrogels

1 U/g

Inside the gel

10 U/g

0 U/g

10 U/g

100 U/g

Solution (outside the gel)
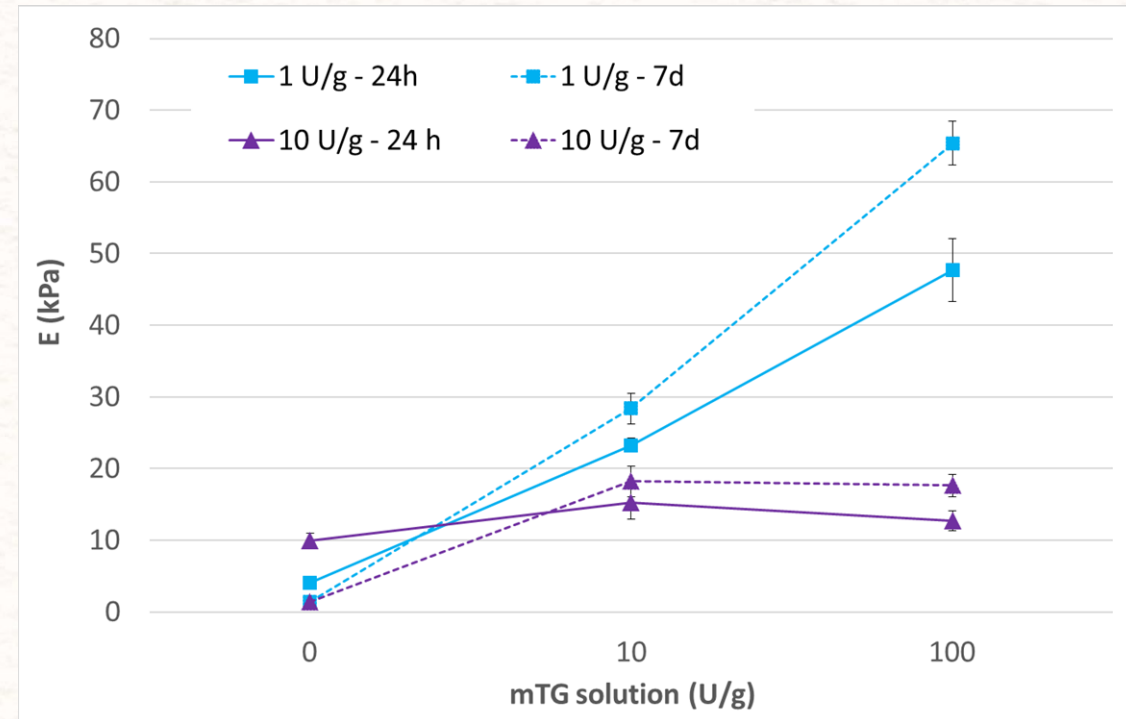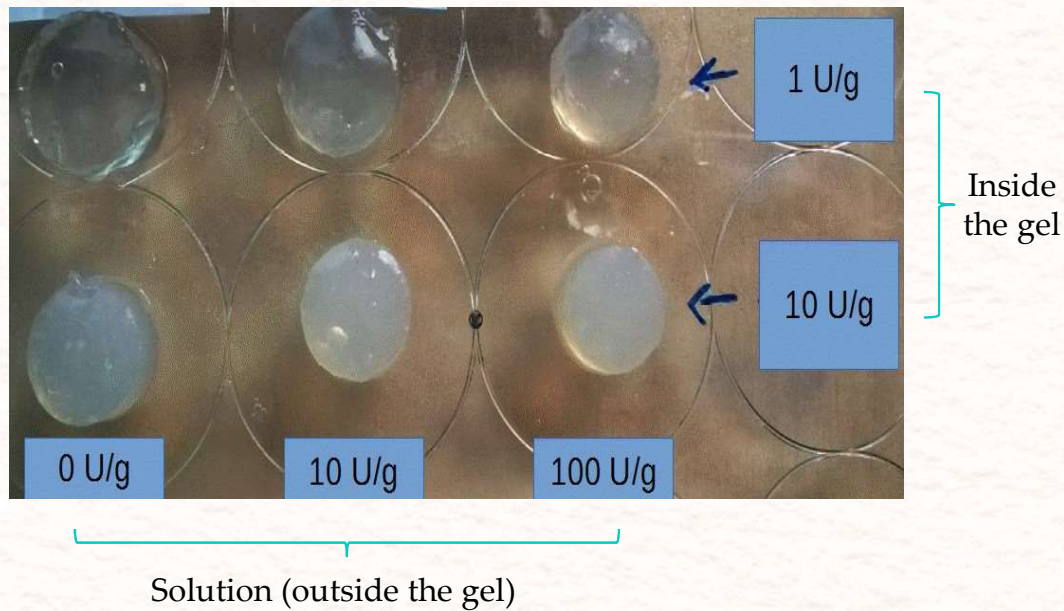
*One **U (unit)** is defined as the **amount of the enzyme that produces a certain amount of enzymatic activity** (i.e. the amount that catalyzes the conversion of 1 micro mole of substrate per minute).*
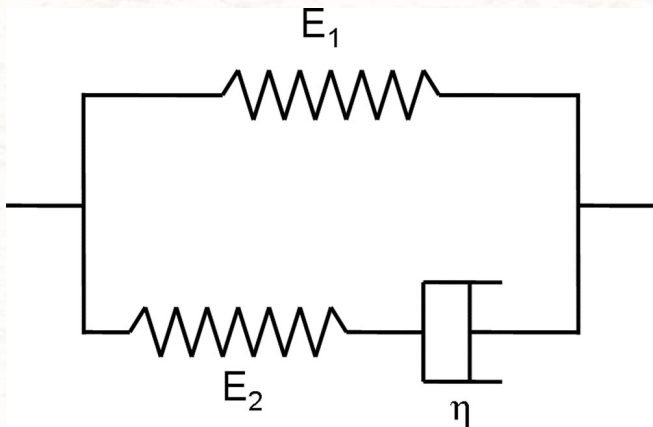
E increase both with mtg concentration on incubation time

Hydrogel viscoelasticity

- Biphasic structure (solid/aqueous phase)
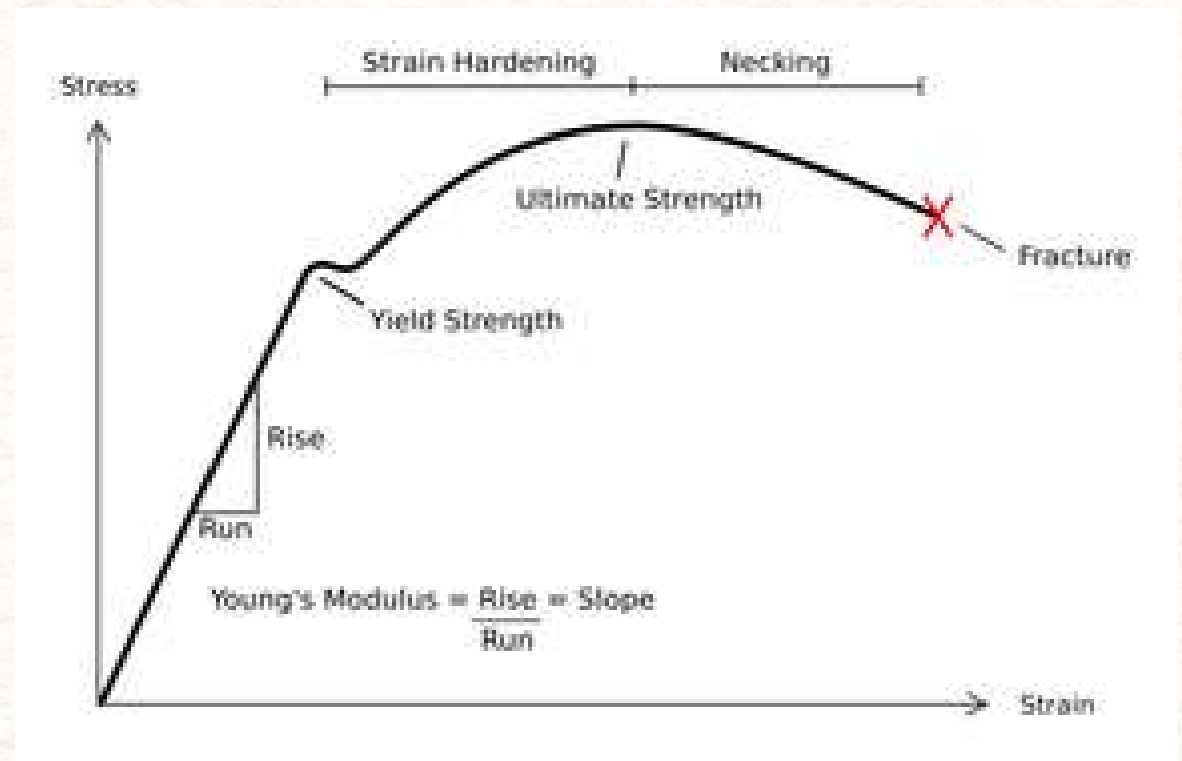- Entanglements behaviours
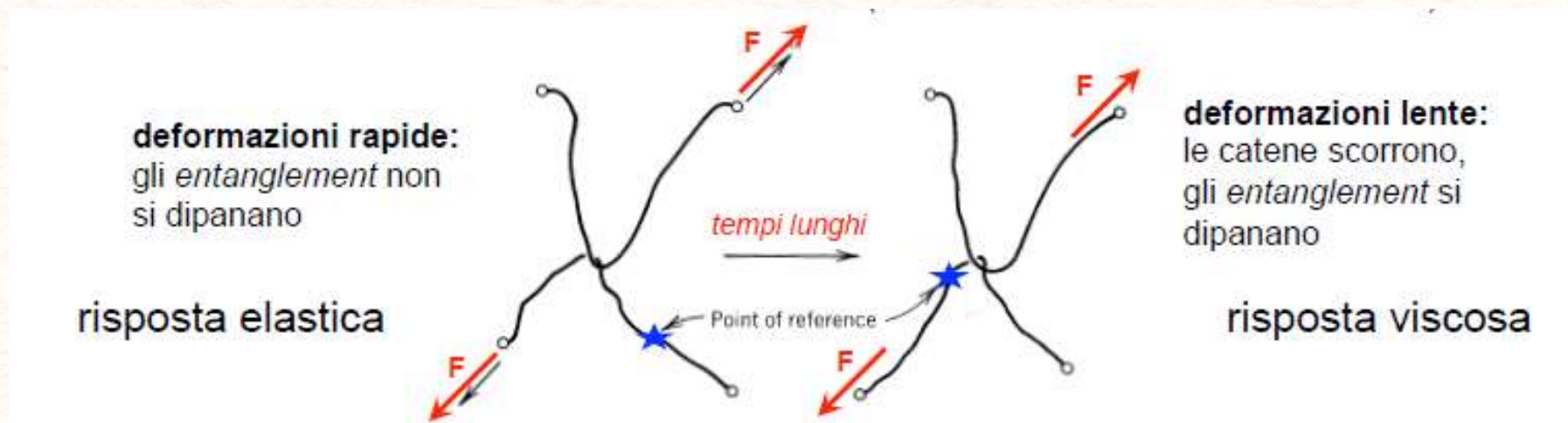
Mechanical Properties

- Elastic modulus: slope of the stress-strain curve in the linear region

- Viscoelastic properties:
  Eist= E1+ E2
  Eeq= E1
   $\tau = \eta/E2$
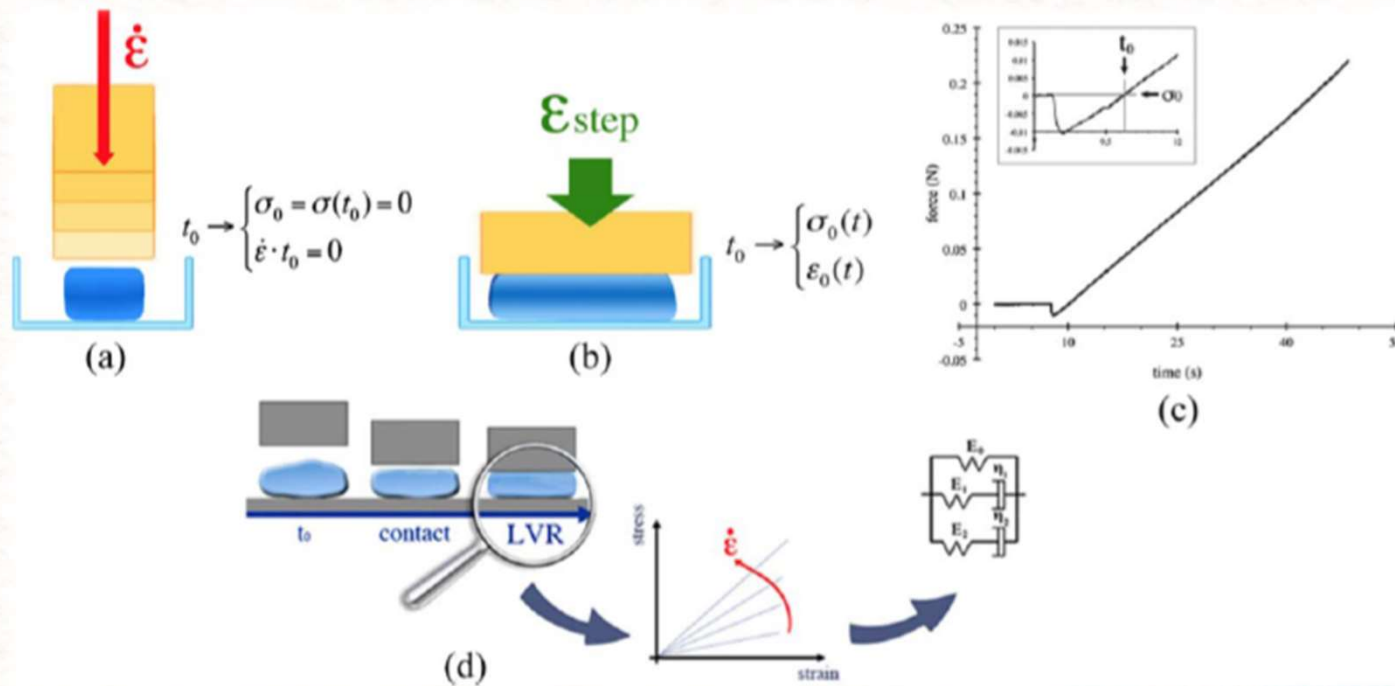

SLS (standard linear solid) model

Mechanical Characterization (2) – test types

Elastic properties:

- Unconfined compression

- Tension

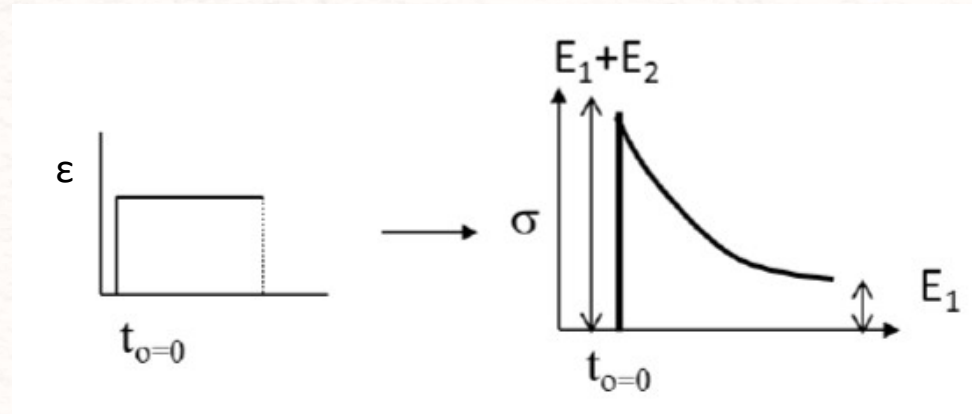**Viscoelastic Properties:**

- SR (stress relaxation), creep
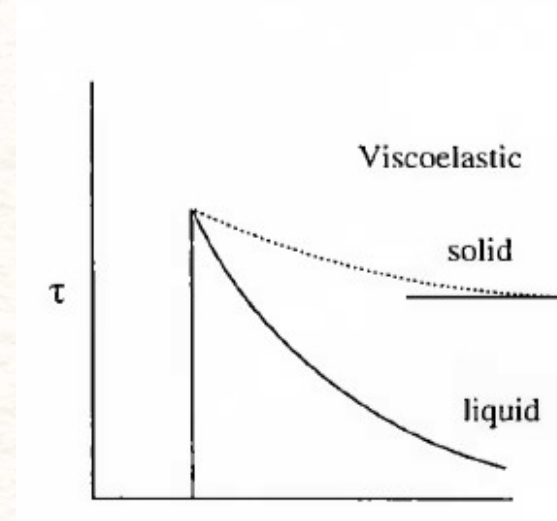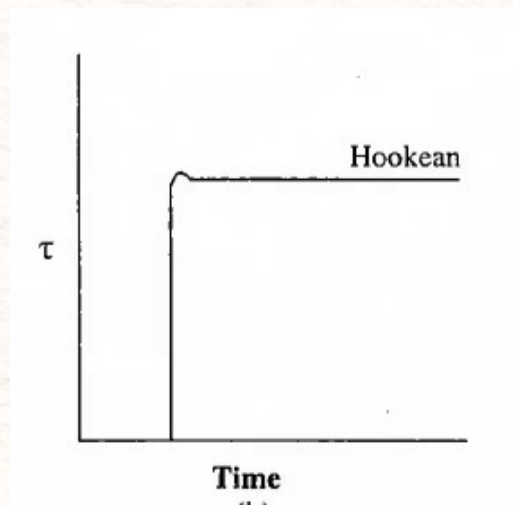
- Epsilon-dot & nano-epsilon-dot

Mechanical Characterization (3) – test types

- SR for SLS

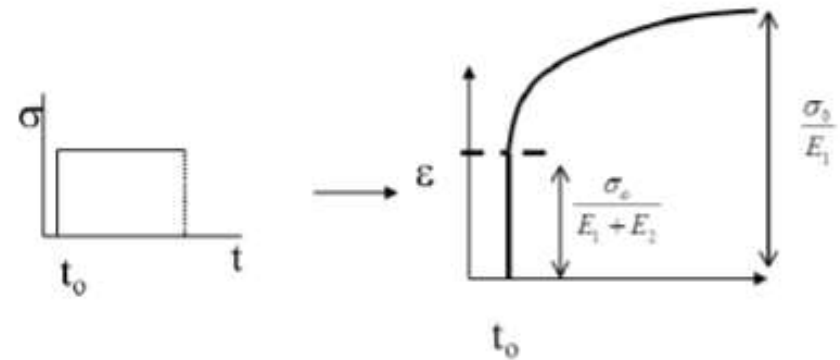$$\sigma(t) = \varepsilon_0 \left(E_1 + E_2 e^{-\left(\frac{E_2}{\eta_2}\right)t} \right)$$

The Hookean solid shows no stress relaxation

Mechanical Characterization (3) – test types

- Creep for SLS

$$\varepsilon(t) = \sigma_o\left(\frac{1}{E_1} - e^{-\frac{E_{series}}{\eta_2}t}\left(\frac{E_2}{E_1(E_1+E_2)}\right)\right)$$

- Espilon dot for SLS

$$\sigma(t) = \dot{\varepsilon}E_1 t + \dot{\varepsilon}\eta_2\left(1 - e^{-\left(\frac{E_2}{\eta_2}\right)t}\right)$$

$$\frac{d\varepsilon}{dt} = \dot{\varepsilon} = \text{costant}$$
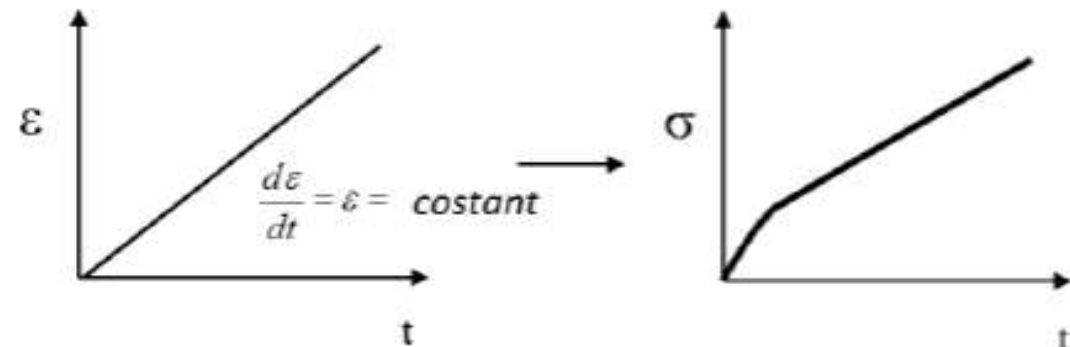
Mechanical Characterization (1) – Measurement techniques

	Bulk Measurement	Local Measurement
Surface properties	-	+
Volumetric properties	+	-
Reproducibility	+	? (very dependent on material homogeneity)
Robustness	+	-

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Bibliography:

1. Hoffman, A. S. (2012). Hydrogels for biomedical applications. *Advanced drug delivery reviews*, *64*, 18-23.
2. Gibas, I., & Janik, H. (2010). Review: synthetic polymer hydrogels for biomedical applications.
3. Drury, J. L., & Mooney, D. J. (2003). Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials*, *24*(24), 4337-4351.
4. Bigi, A., Cojazzi, G., Panzavolta, S., Rubini, K., & Roveri, N. (2001). Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking. *Biomaterials*, *22*(8), 763-768.
5. Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., & Rubini, K. (2002). Stabilization of gelatin films by crosslinking with genipin. *Biomaterials*, *23*(24), 4827-4832.
6. Yung, C. W., Wu, L. Q., Tullman, J. A., Payne, G. F., Bentley, W. E., & Barbari, T. A. (2007). Transglutaminase crosslinked gelatin as a tissue engineering scaffold. *Journal of Biomedical Materials Research Part A*, *83*(4), 1039-1046.
7. Sakai, S., Hashimoto, I., & Kawakami, K. (2007). Synthesis of an agarose-gelatin conjugate for use as a tissue engineering scaffold. *Journal of bioscience and bioengineering*, *103*(1), 22-26.
8. Hennink, W. E., & Van Nostrum, C. (2012). Novel crosslinking methods to design hydrogels. *Advanced drug delivery reviews*, *64*, 223-236.
9. McDermott, M. K., Chen, T., Williams, C. M., Markley, K. M., & Payne, G. F. (2004). Mechanical properties of biomimetic tissue adhesive based on the microbial transglutaminase-catalyzed crosslinking of gelatin. *Biomacromolecules*, *5*(4), 1270-1279.
10. http://www.polymertechnology.it/bacheca/NanocompositeForm/page6/files/Viscoelasticity.pdf
11. Tirella, A., Mattei, G., & Ahluwalia, A. (2014). Strain rate viscoelastic analysis of soft and highly hydrated biomaterials. *Journal of Biomedical Materials Research Part A*, *102*(10), 3352-3360.
12. Mattei, G., Gruca, G., Rijnveld, N., & Ahluwalia, A. (2015). The nano-epsilon dot method for strain rate viscoelastic characterisation of soft biomaterials by spherical nano-indentation. *Journal of the mechanical behavior of biomedical materials*, *50*, 150-159.