Polymers & Hydrogels in bioengineering

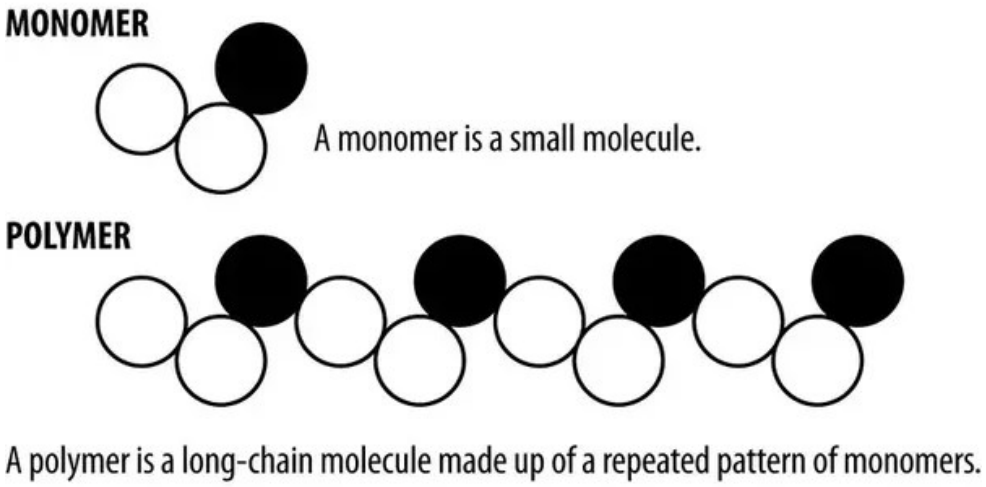
Corso Materiali intelligenti e Biomimetici
16/04/2020

ludovica.cacopardo@ing.unipi.it

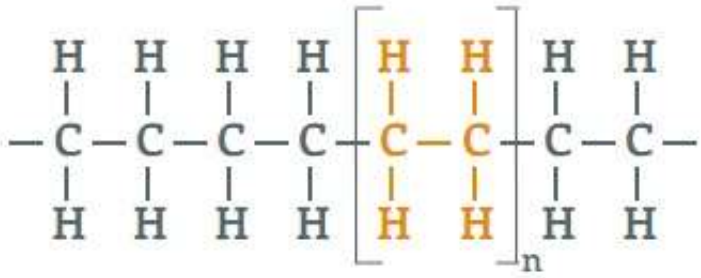
Polymers

A **polymer** molecule consists of a *chemical entity (repeat unit)* which is covalently bonded to its identical neighbors forming a polymeric chain. The repeat unit is usually a called **monomer**.

How individual polymer molecules move when supplied with thermal or mechanical energy and how groups of molecules interact and entangle can explain the *observed behavior*.



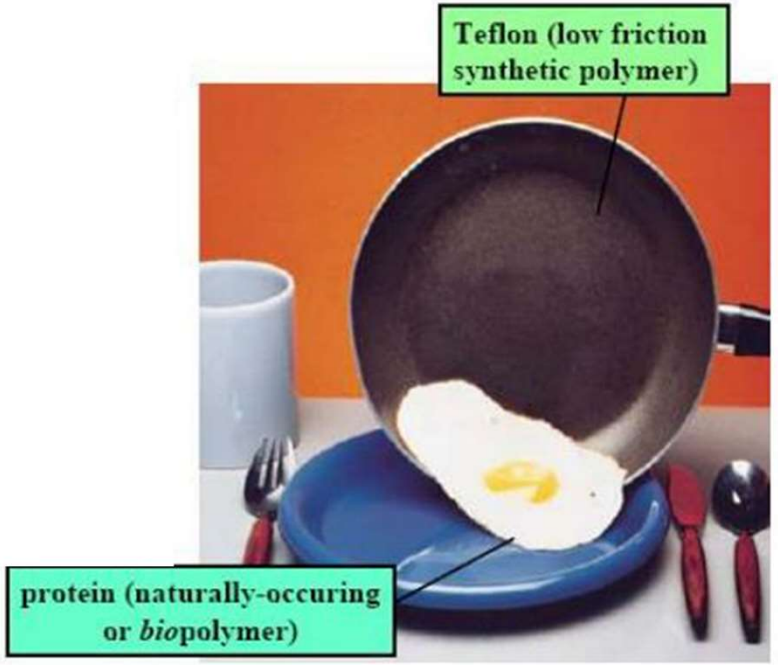
Example: PE - $(C_2H_4)_n$



Natural and Synthetic Polymers

Polymers are abundant in nature, found in all living systems and **organic materials** such as wood, paper, leather, natural fibers have found extensive use. Today **synthetic materials** are mostly used.

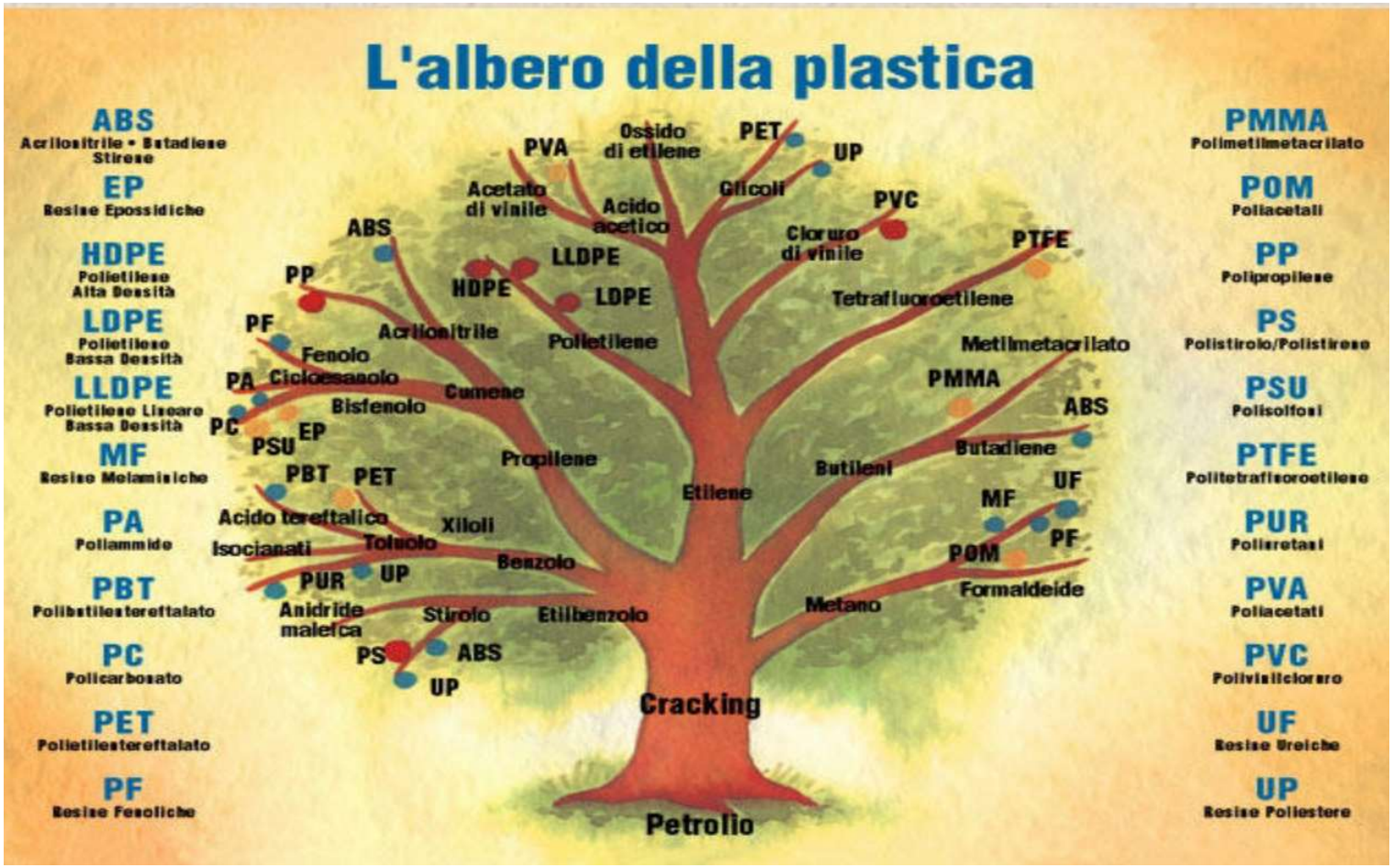
CLASS OF BIOMOLECULE	THE MONOMER	THE POLYMER
Carbohydrates	Monosaccharides	Polysaccharides
Proteins	Amino Acids	Polypeptides
Lipids	Fatty Acids	Fats, Oils, Steroids, Waxes
Nucleic Acids	Nucleotides	DNA, RNA, ATP



Synthetic Polymers

The first man-made polymers, formed by **chemical modification of natural materials,** were produced in the second half of the *19th century*.
e.g.: *Celluloid (*from nitrocellulose and camphor*)*

Fully **synthetic polymers** were developed in the 20th century, most in the period 1950–1970s driven by chemical industry expansion.
e.g.: *Bakelite* (formed from a condensation reaction of phenol with formaldehyde)



Fully synthetic polymers are the so-called **plastics,** which derive from **petrochemical** processes. *Cracking* is the process that breaks down complex organic molecules such long-chain hydrocarbons into simpler molecules such as light hydrocarbons.

TIPI DI POLIMERI TERMOPLASTICI

• **POLIETILENE (PE)** ***1935***

usato per flaconi per liquidi, **tubi, giocattoli, pellicole, sacchi per la spazzatura, cassette**.

Dal PE riciclato si producono nuovamente sacchi per la spazzatura, flaconi e nastri adesivi, tappi.
È una delle materie plastiche più diffuse per il basso costo e la versatilità.

• **POLIPROPILENE(PP)**

utilizzato per la produzione di **contenitori per alimenti, oggetti di arredo, flaconi per detersivi e per l'igiene personale, giocattoli, moquette, mobili da giardino, tubi .**

• **POLIETILENTEREFTALATO (PET)**

usato per produrre bottiglie per bevande. Dal suo riciclo si ottengono alcuni tessuti come il pile, interni ed accessori per auto, contenitori, fibre per imbottiture

• **ACRILONITRILE-BUTADIENE-STIRENE (ABS)**

usato per produrre telefonini, tower pc molto resistente agli Urti.

• **POLICARBONATO(PC)**

utilizzato per produrre caschi protettivi e parti di automobili presenta una buona resilienza e durezza.

• **POLIVINILCLORURO (PVC)**

usato per vaschette e contenitori di alimenti, per finestre e porte, per piastrelle, per i tesserini magnetici (BANCOMAT), per i nastri magnetici delle videocassette e pellicole cinematografiche. Come per il PE, il basso costo e la versatilità rendono il PVC uno dei polimeri più diffusi nel panorama delle plastiche.
Dal PVC riciclato si producono tubi usati in edilizia e negli impianti fognari.

• **POLISTIROLO o polistirene (PS)**

usato per produrre vaschette per alimenti, posate, piatti, tappi, pannelli isolanti per l'edilizia, imballaggi. **NON RICICLABILE.**

• **POLIAMMIDE (PA) (NYLON)**

E' stata la prima fibra tessile sintetica (1940). Calze da donna, paracaduti, pellicola trasparente.....

Durante guerra le donne si disegnavano 1 riga nera sulla gamba per fingere di indossare le calze di nylon

TIPI DI POLIMERI TERMOINDURENTI

NON RICICLABILI (tramite calore)

- **BACHELITE (resine Fenoliche)**

La prima materia prima sintetica (1909 belga Leo Baekeland). Usata per spine, prese di corrente, telefoni, articoli casalinghi.




- **FÒRMICA (resine Melaminiche)**

Resistono bene all'umidità e alle alte temperature, sono altamente colorabili




EP – Resine epossidiche.

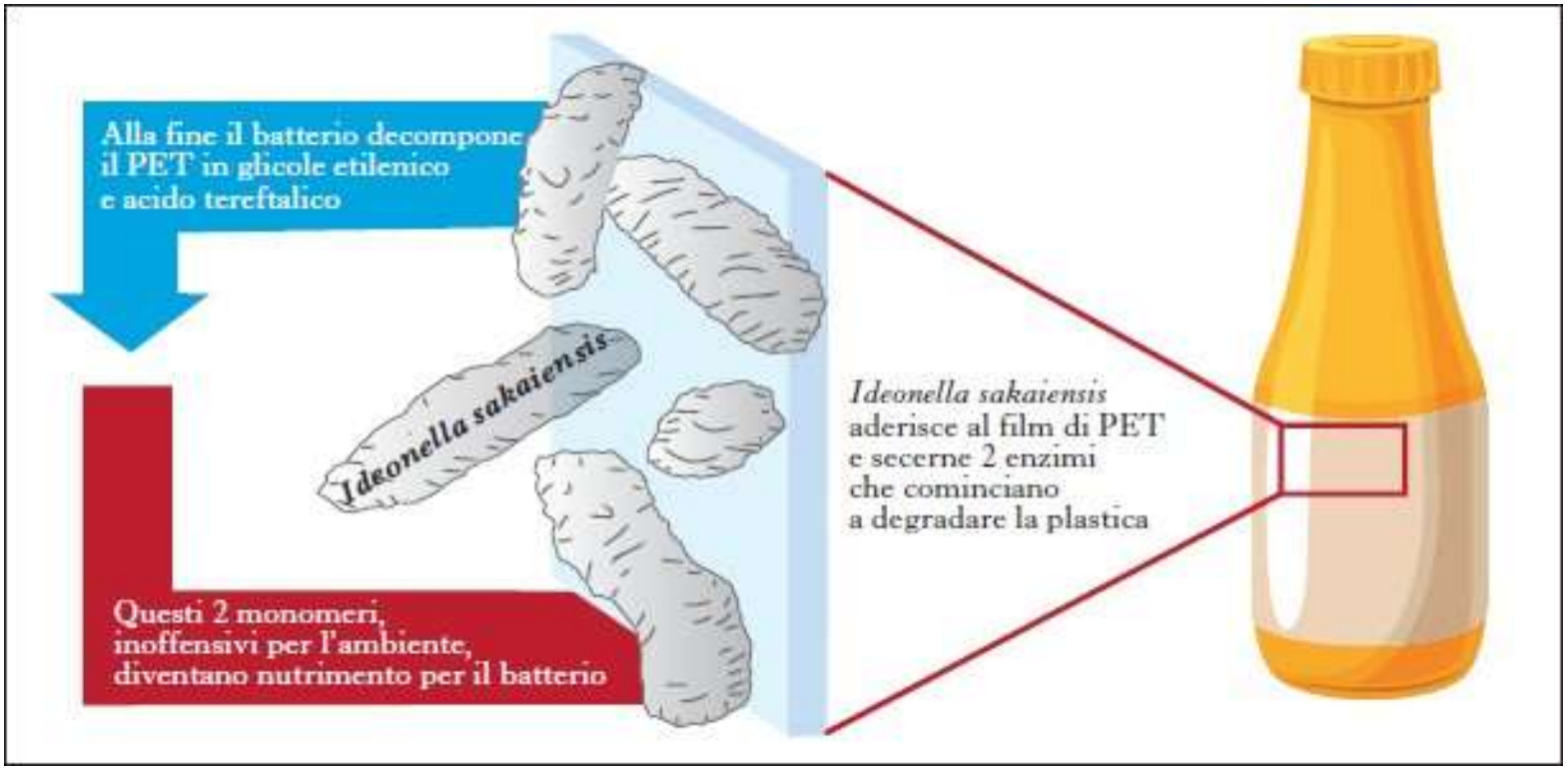
Utilizzate nella produzione di vernici, adesivi, laminati, isolatori elettronici. Si usano anche rinforzate con fibre di vetro, di carbonio e altre resine

PU – Poliuretani.

Utilizzati nella fabbricazione di finte pelli, suole e tacchi da scarpe, film per isolamento elettrico, articoli per lo sport, per uso tecnico e nel settore medicale

Batteri mangia-plastica:

Scoperto in alcuni siti di riciclaggio giapponesi, *Ideonella sakaiensis* è un batterio in grado di degradare completamente il PET.



'The PET film was damaged extensively and almost completely degraded after 6 weeks at 30°C'
Yoshida et al., *Science* 2016- https://science.sciencemag.org/content/351/6278/1196.long

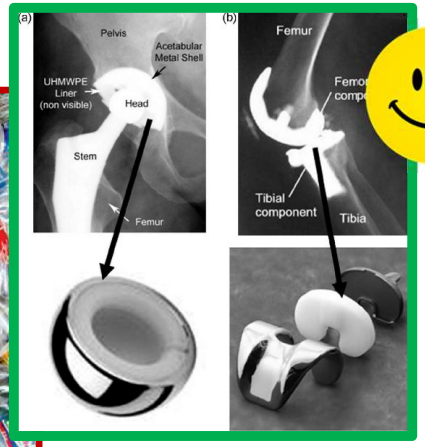
Plastic pollution vs. enginineering application

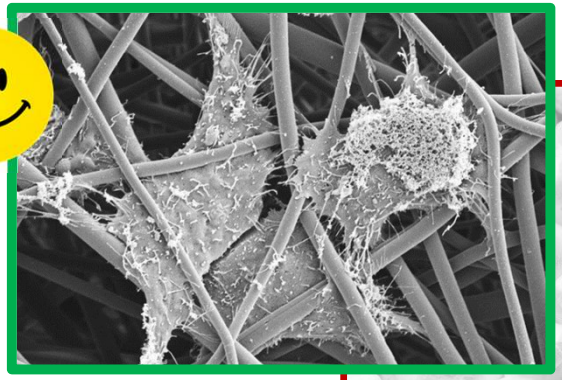
Table 1
Classes of plastics that are commonly encountered in the marine environment.

Plastic Class		Specific Gravity	Percentage production[#]	Products and typical origin
Low-density polyethylene	LDPE LLDPE	0.91–0.93	21%	Plastic bags, six-pack rings, bottles
High-density polyethylene	HDPE	0.94	17%	Milk and juice jugs
Polypropylene	PP	0.85–0.83	24%	Rope, bottle caps, netting
Polystyrene	PS	1.05	6%	Plastic utensils, food containers
Foamed Polystyrene				Floats, bait boxes, foam cups
Nylon	PA		<3%	Netting and traps
Thermoplastic Polyester	PET	1.37	7%	Plastic beverage bottles
Poly(vinyl chloride)	PVC	1.38	19%	Plastic film, bottles, cups
Cellulose Acetate	CA			Cigarette filters

[#] Fraction of the global plastics production in 2007 after (Brien, 2007).

PET

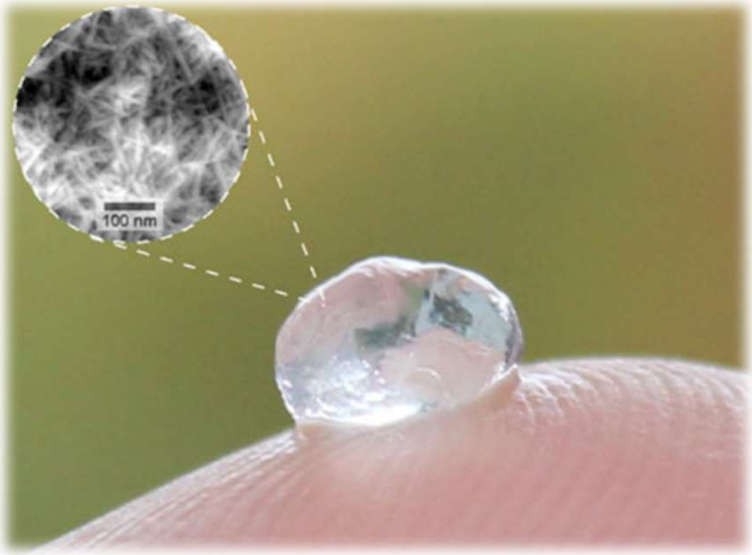




PS

Different use of ‘plastics’

Hydrogels



Hydrogels constitute a group of **polymeric materials**, characterized by **the hydrophilic structure** of which renders them capable of holding large amounts of water in their three-dimensional networks (10–20% up to thousands of times their dry weight in water).
Crosslinks have to be present to avoid dissolution of the hydrophilic polymer chains/segments into the aqueous phase.

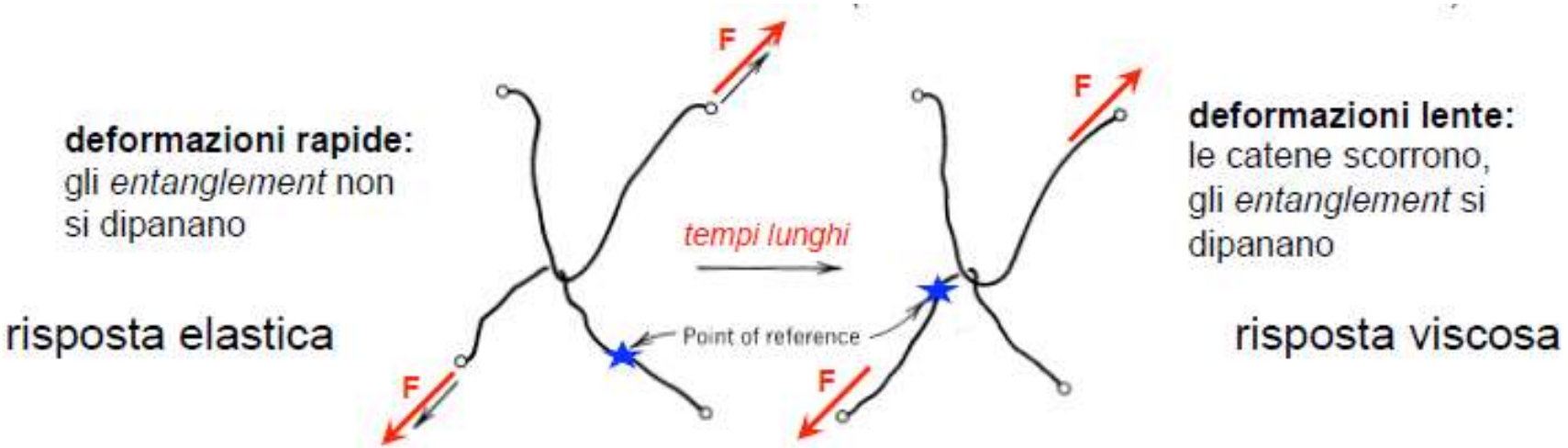
Biphasic Structure

- Solid network → Elastic Properties
- Aqueous solution → Viscous Properties

→ **Viscoelastic properties**

Hydrogel viscoelasticity

- Biphasic structure (solid/aqueous phase)
- Intermolecular and intramolecular forces
- Entanglements behaviours

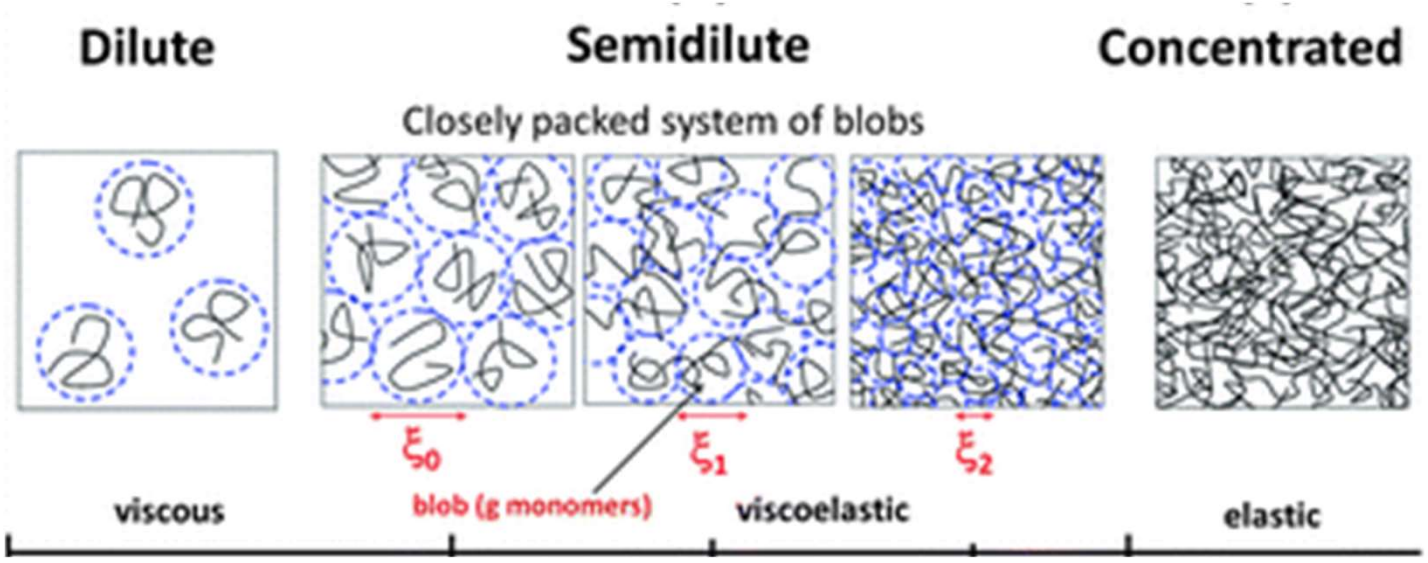


Hydrogels (2)

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

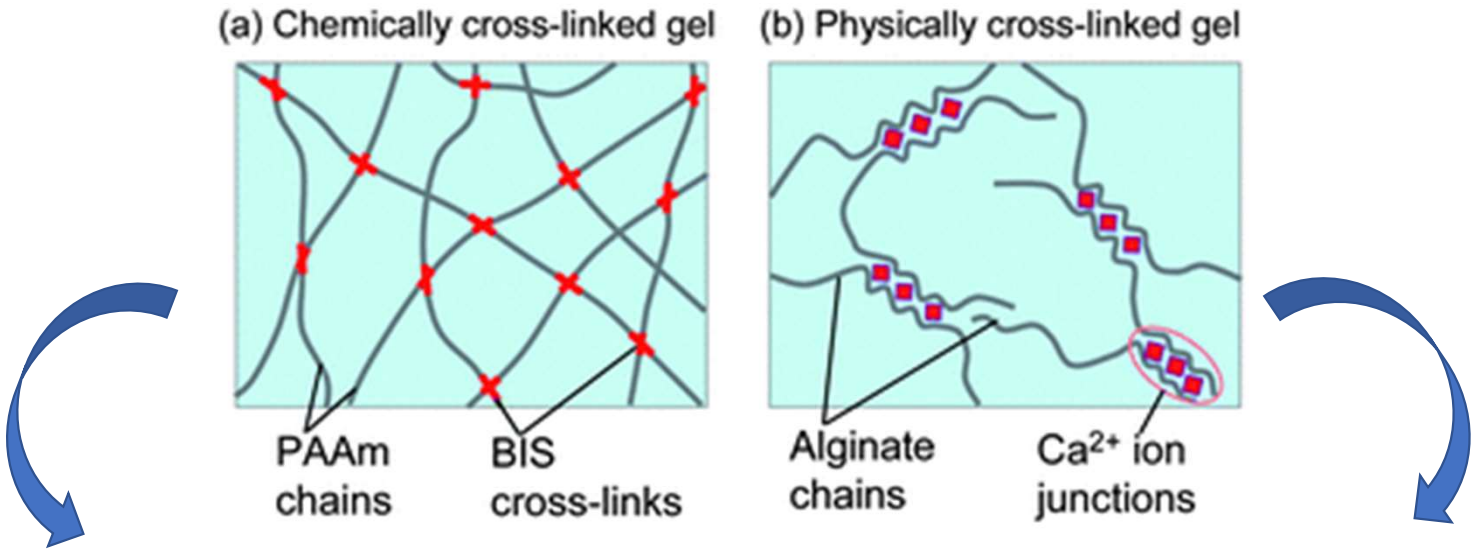
Aqueous solutions of hydrophilic polymers at low or moderate concentrations, where **no substantial entanglement of chains occurs,** normally show **Newtonian behavior**.

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



Crosslinking types

Considering inter-molecular chains:



- **covalently-crosslinked** networks

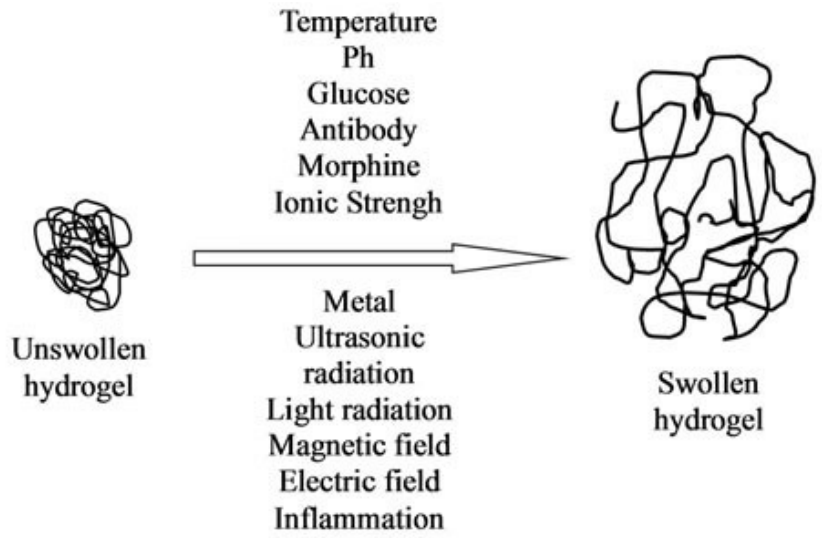
- molecular **entanglements**
- **secondary forces** (ionic, H-bonding or hydrophobic forces)

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

Hydrogel Swelling

In the crosslinked state, **crosslinked hydrogels reach an equilibrium swelling level** in aqueous solutions which depends mainly on the crosslink density, but also from environment conditions (pH, T, etc)

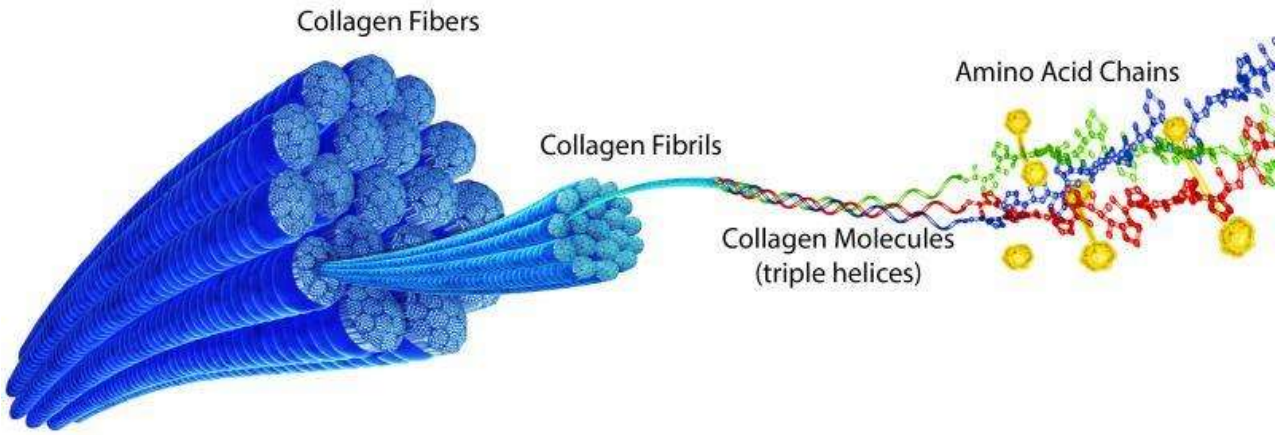
Equilibrium Water Content: $$EWC = \frac{W_w}{W_t}$$



Hydrophilic Polymers used to synthesize hydrogels				
Natural Polymers	**Anionic polymers:** HA, alginic acid, pectin	**Cationic polymers**: chitosan, poly-lysine	**Amphipathic polymers**: collagen (and gelatin), fibrin	**Neutral polymers**: dextran, agarose
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** (extra-cellular matrix).



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**.
Collagen strands can self aggregate to form stable fibers.

Collagen solutions form **physical gels passing from 4°C to 37°C** at physiologic pH (7.4).

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).

Example 2 - Gelatin

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

Gelatin aqueous solutions **(50°C)** form **physical 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,** generally *extracted from red seaweed*, which is known to form **thermo-reversible 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 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.

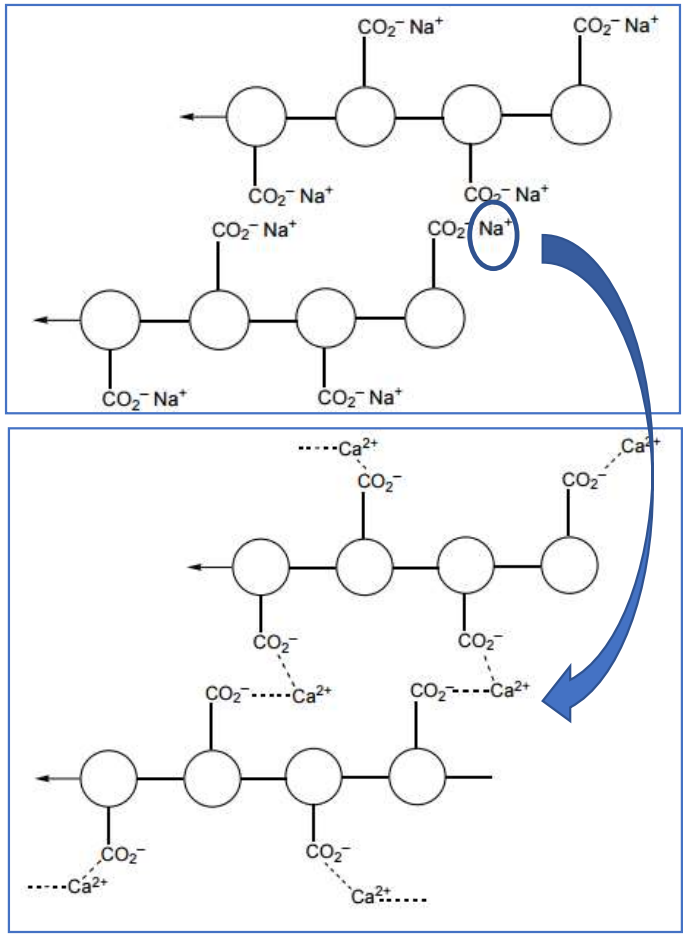
Example 4 - Alginate

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**.

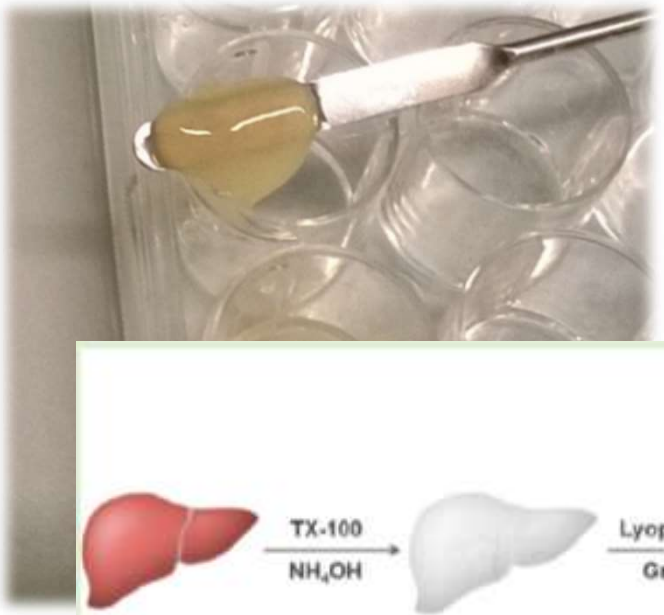
Ionically crosslinked alginate hydrogels do not specifically degrade but undergo **slow uncontrolled dissolution**. Mass is lost through ion exchange of calcium followed by dissociation of individual chains, which results in loss of mechanical stiffness over time.

$$\{\text{Chelator-Ca}\}_{\text{complex}} \text{ <===> Chelator + } Ca^{2+}$$



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

Example 5 – decellularised ECM



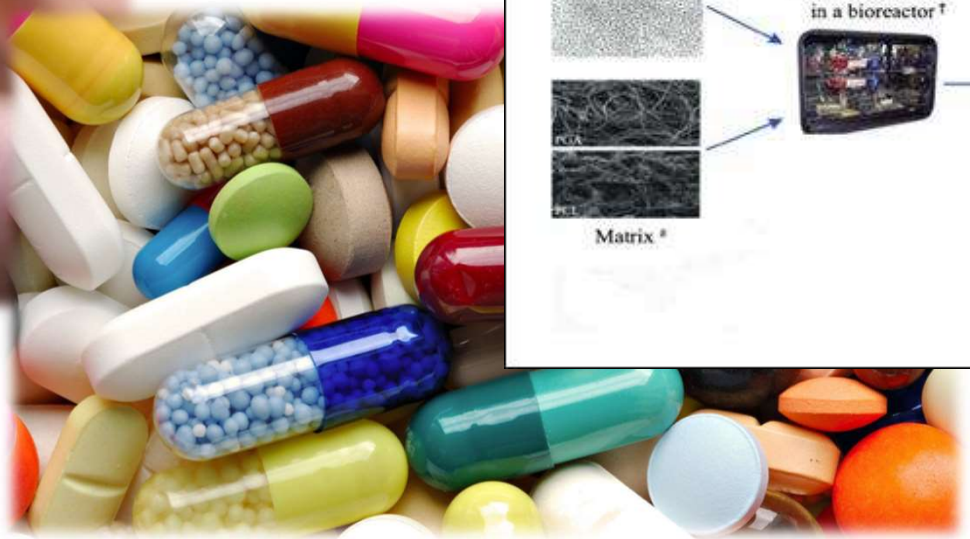
Decellularization maintains **microstructures of native extracellular matrices and its biochemical compositions**, providing tissue-specific microenvironments for efficient tissue regeneration.

Digestion**,** its necessary to solubilize decellularized ECM (i.e. breaks down proteins into smaller peptides).

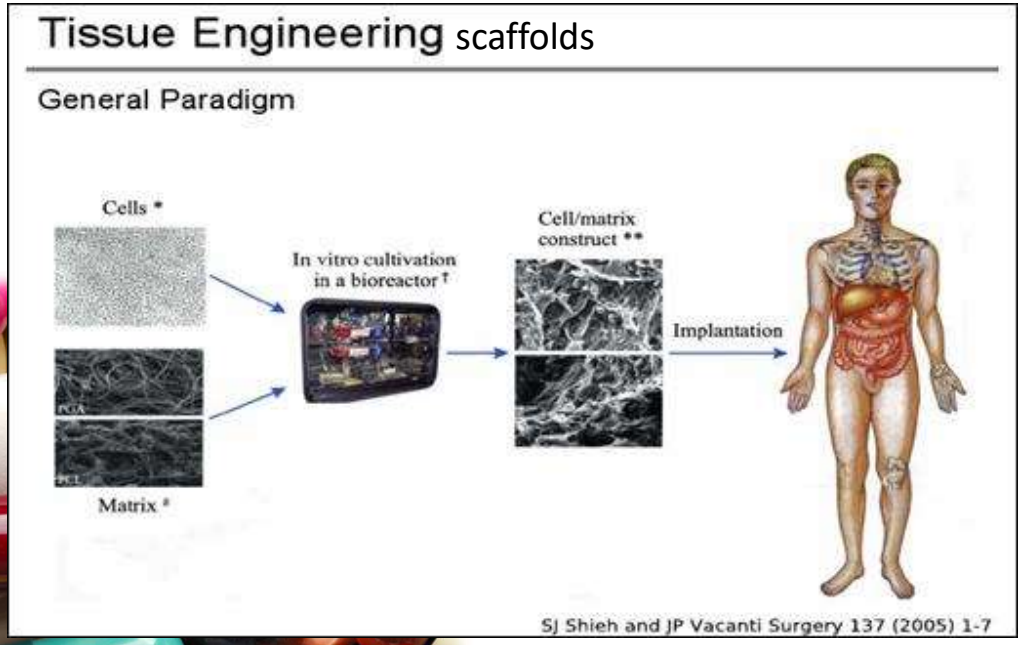
The digested ECM solution is brought **from 4°C to 37°C to form hydrogels**.

Applications

soft contact lenses

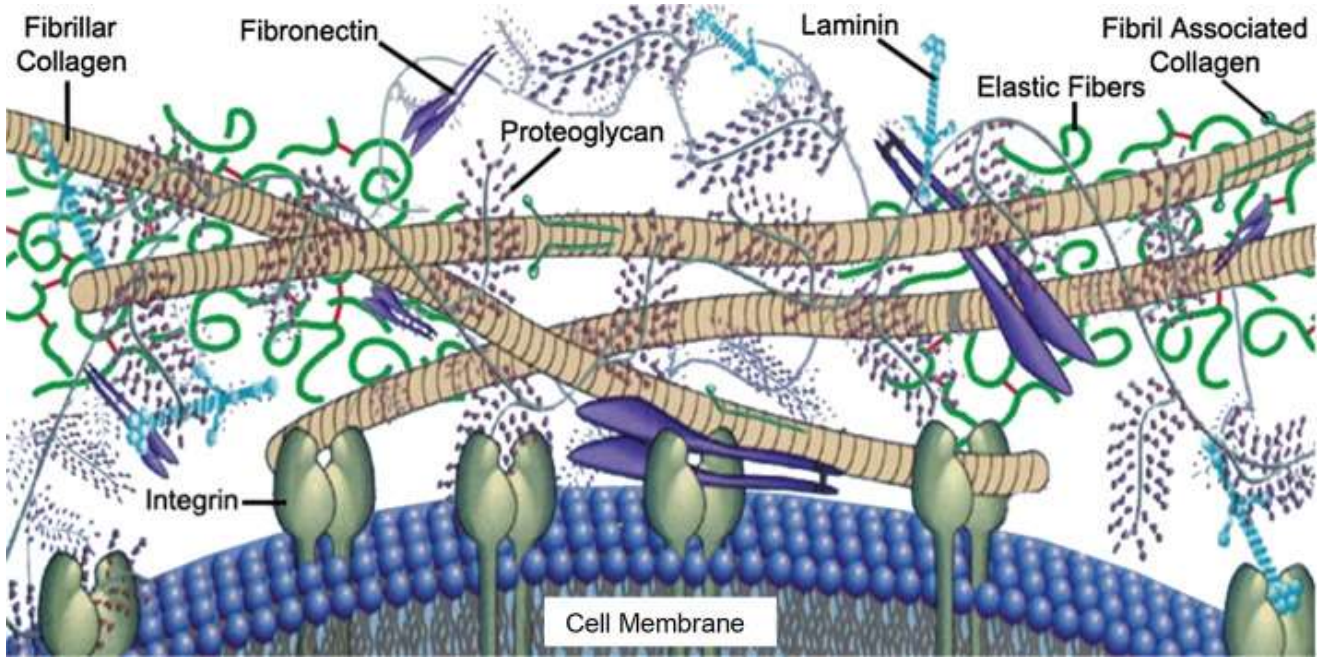


pills or capsules for oral ingestion



Adequate 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).

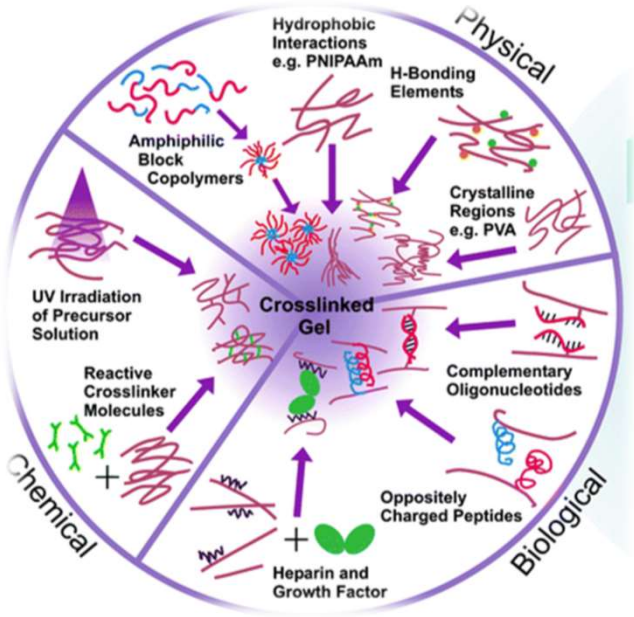
Healthy TE


Fibrillar Collagen
Fibronectin
Laminin
Fibril Associated Collagen
Elastic Fibers
Proteoglycan
Integrin
Cell Membrane

Methods for synthesizing physical and chemical hydrogels

Physical gels

- Warm a polymer solution to form a gel (e.g. collagen)
- Cool a polymer solution to form a gel (e.g., agarose or gelatin)
- 'Crosslink' a polymer in aqueous solution, using freeze–thaw cycles to form polymer microcrystals
- Lower pH to form an H-bonded gel between two different polymers in the same aqueous solution
- Adding ions in solution (e.g. alginate)
- 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:

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

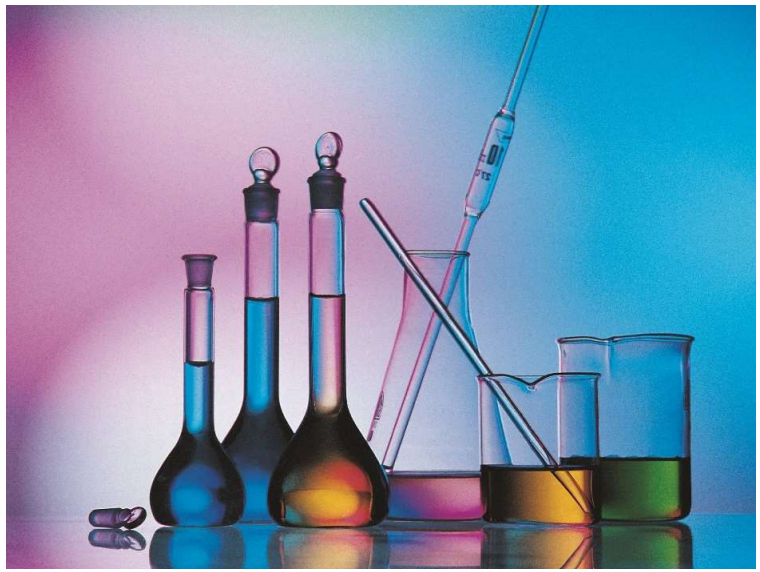
Crosslinker types

- 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: Glutaraldehyde (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*

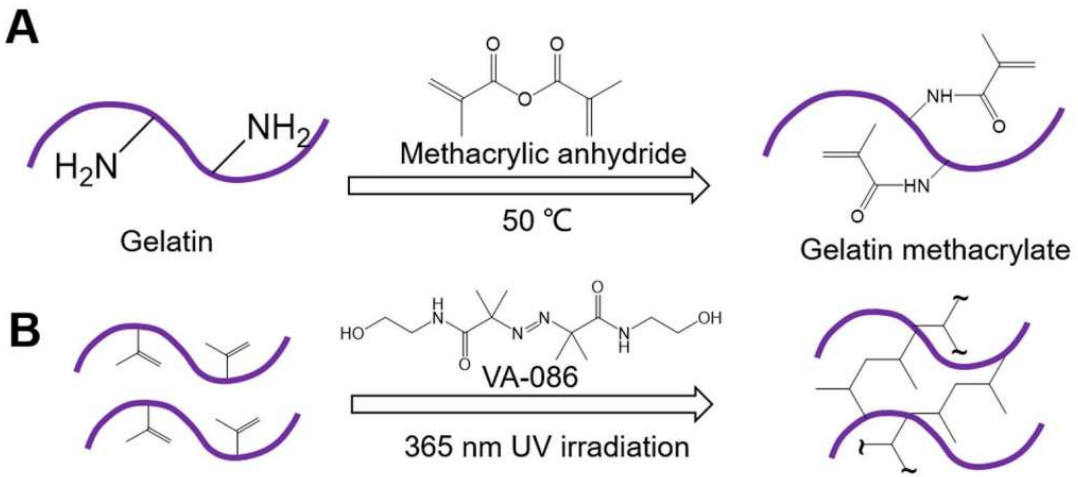
Since glutaraldehyde is a **toxic** compound that even at low concentration shows cell-growth inhibition, hydrogels need to be careful washed before use.

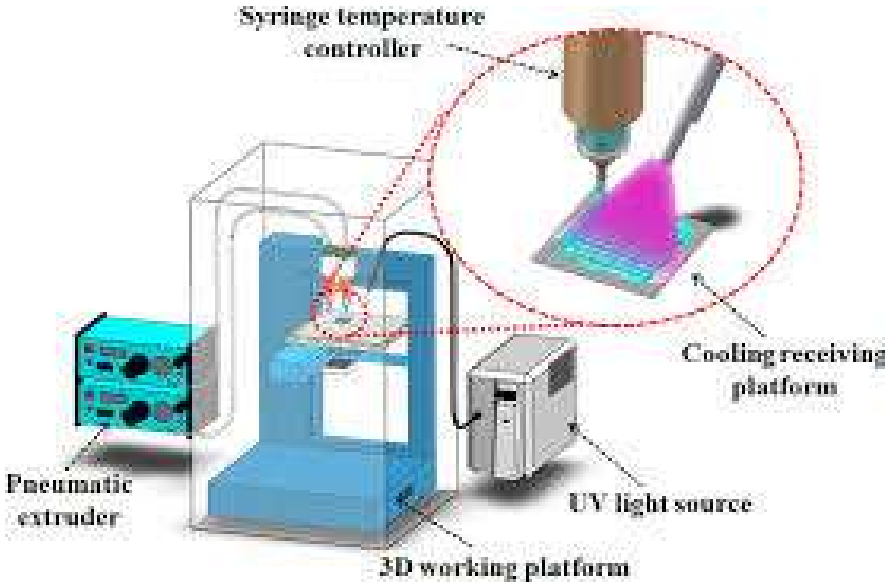
UV crosslinking

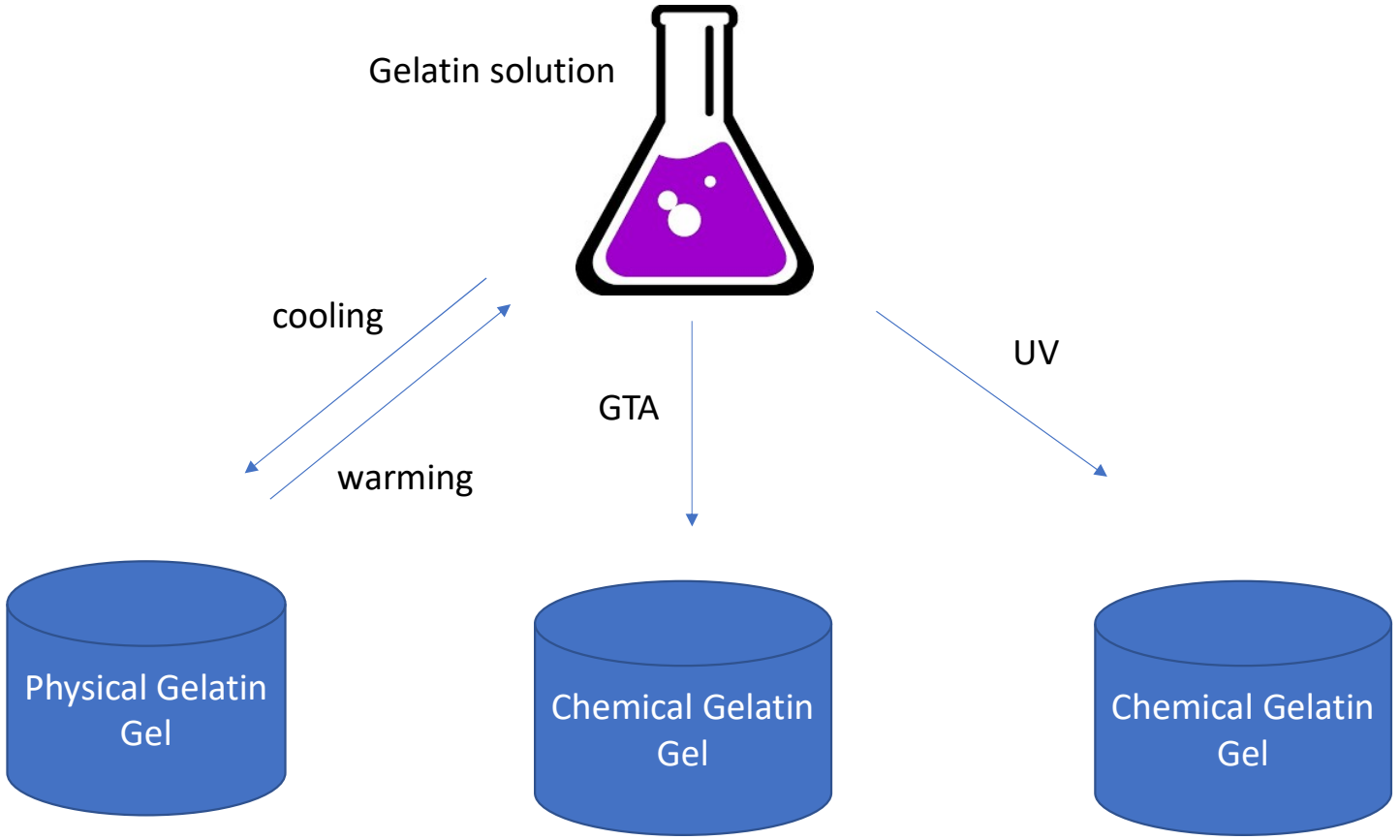
Polymers need to be modified with **reactive chemical groups** or mixed with **photoinitiators**.

Example: Gelatin methacrylate (GelMA) is a widely used natural hydrogel for biofabrication because of cost-effectiveness, the ease of synthesis and photocrosslinking, as well as the great biocompatibility to allow cell adhesion and proliferation.

GelMA has been employed as the bioinks for stereolithography bioprinting or laser-based bioprinting. Building blocks for bioassembly, such as microdroplets and microfibers, can be also fabricated using the GelMA.





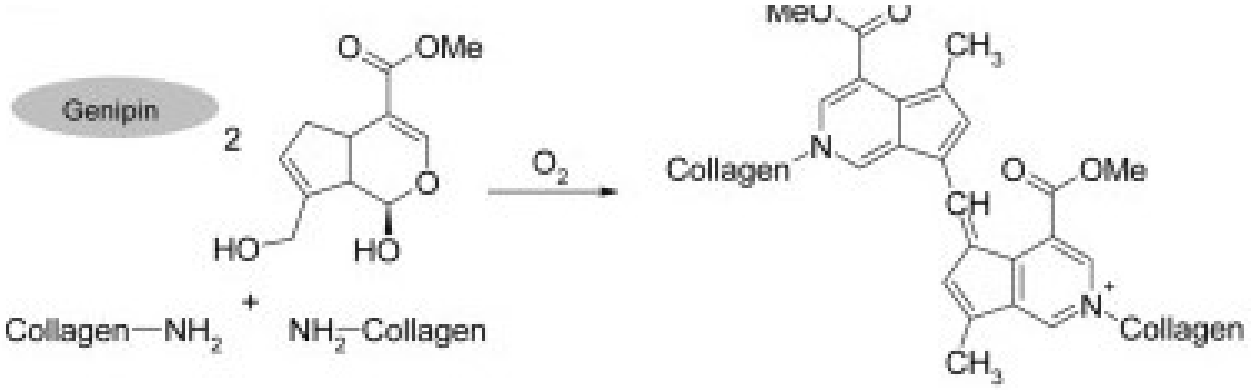

Gelatin solution
cooling
warming
GTA
UV
Physical Gelatin Gel
Chemical Gelatin Gel
Chemical Gelatin Gel

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.



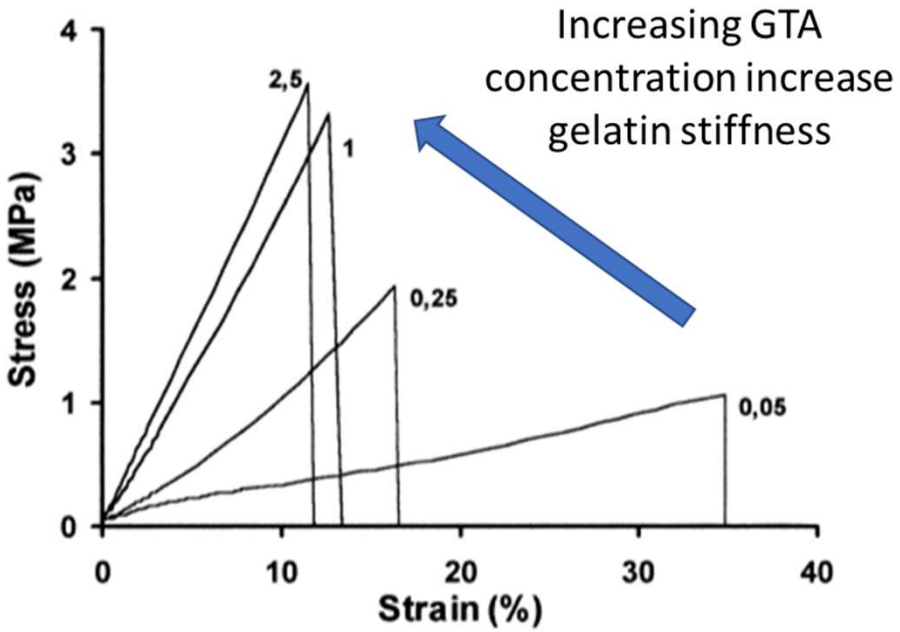


Fig. 1. Typical stress–strain curves recorded from gelatin films cross-linked with GTA. The numbers near the curves indicate the concentration of GTA, expressed as wt%.

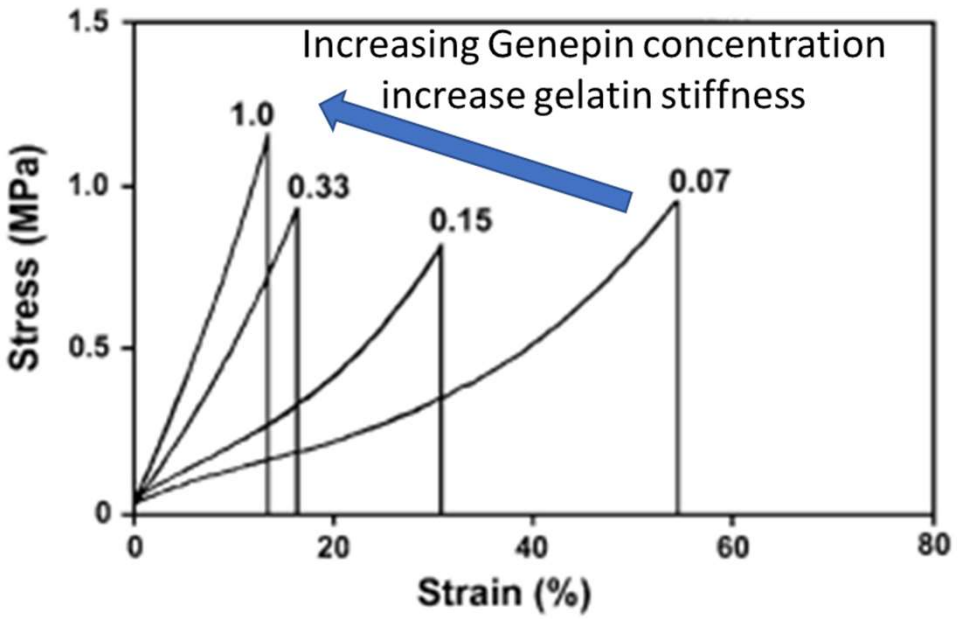
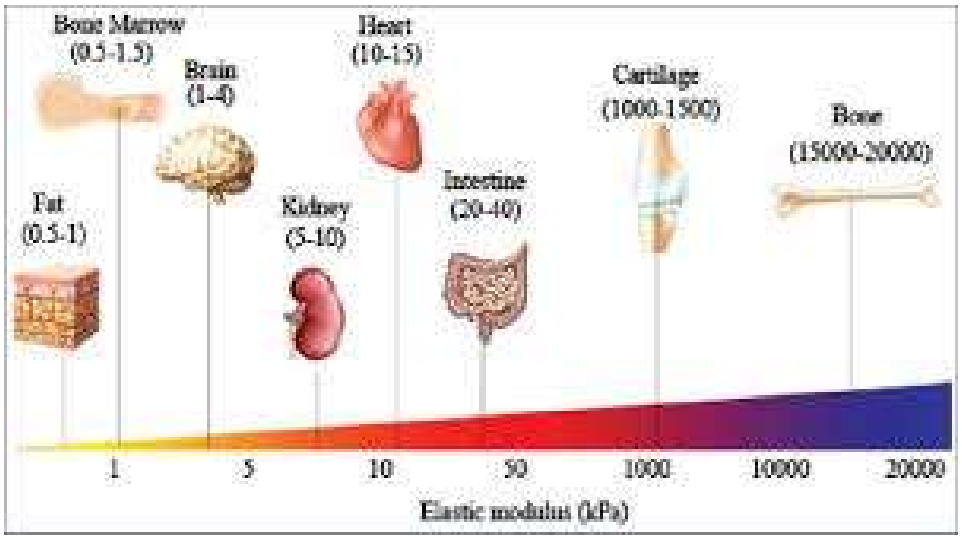


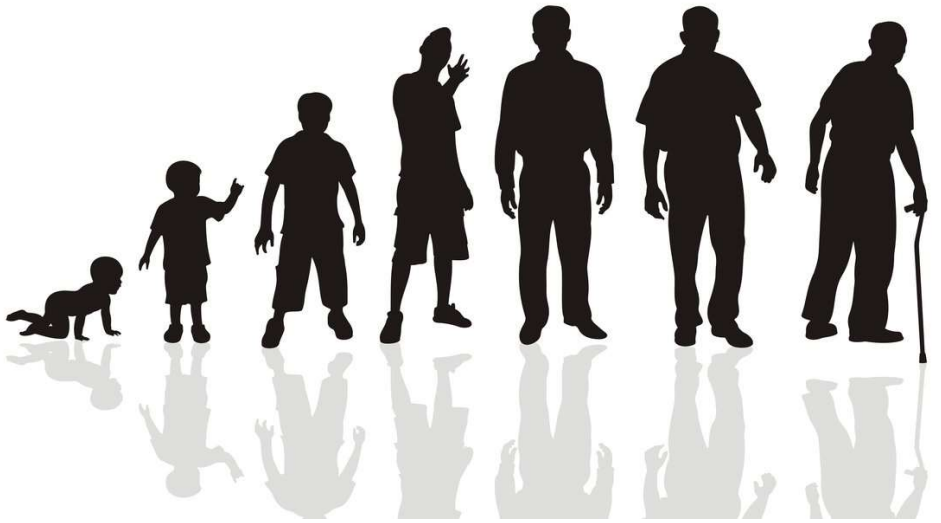
Fig. 1. Typical stress–strain curves recorded from gelatin films cross-linked with genipin. The numbers near the curves indicate the concentration of genipin, expressed as wt%.

Why crosslinking?

- Stabilize hydrogels/Enhance mechanical properties
- Modulate Mechanical Properties (TE & IVMs)



Hydrogel mechanical properties can varied to **match tissue ecm properties**: initial polymer/crosslinker concentration



Hydrogel mechanical properties can be **modulated over space/time** to mimic *pathophysiological process (foetal growth, ageing, fibrosis)*

TIME-variant mechanical properties

Table 1. Summary of cell culture substrate mechanical properties and their time dependence (or viscoelasticity) and time evolution (or change in properties with time).

	Change in Properties with Time	Viscoelasticity	Parameter(s):	Examples of Typical Substrates	Refs
Elastic	no	no	E	GTA or genepin crosslinked gelatin, Ha/Gel, PAAm	[8–11]
Viscoelastic	no	yes	E_i, η_i	PAAm, alginate, agarose	[22–25]
Dynamic/time-evolving Elastic	yes	no	$E(t)$	PEG-thiol, HA/PEGDA, Ca^{2+}-liposome loaded alginate	[12–14,18]
Dynamic/time-evolving Viscoelastic	yes	yes	$E_i(t)$, $\eta_i(t)$	Magneto active PAAm, HA-based gels	[26–29]

Enzymatic stiffening: mTG

A naturally occurring protein crosslinking enzyme, **microbial 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).

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.

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.

materials

MDPI

Article

Engineering Gels with Time-Evolving Viscoelasticity

Giorgio Mattei [1,†], Ludovica Cacopardo [2,†,*], and Arti Ahluwalia [1,2]

[1] Department of Information Engineering, University of Pisa, Via Girolamo Caruso 16, 56122 Pisa, Italy; g.mattei86@gmail.com

[2] Research Centre "E. Piaggio", University of Pisa, Largo Lucio Lazzarino 1, 56122 Pisa, Italy; arti.ahluwalia@unipi.it

* Correspondence: ludovica.cacopardo@ing.unipi.it; Tel.: +39-050-2218255

† These authors contributed equally to the work.

Received: 18 November 2019; Accepted: 14 January 2020; Published: 16 January 2020

Abstract: From a mechanical point of view, a native extracellular matrix (ECM) is viscoelastic. It also possesses time-evolving or dynamic behaviour, since pathophysiological processes such as ageing alter their mechanical properties over time. On the other hand, biomaterial research on mechanobiology has focused mainly on the development of substrates with varying stiffness, with a few recent contributions on time- or space-dependent substrate mechanics. This work reports on a new method for engineering dynamic viscoelastic substrates, i.e., substrates in which viscoelastic parameters can change or evolve with time, providing a tool for investigating cell response to the mechanical microenvironment. In particular, a two-step (chemical and enzymatic) crosslinking strategy was implemented to modulate the viscoelastic properties of gelatin hydrogels. First, gels with different glutaraldehyde concentrations were developed to mimic a wide range of soft tissue viscoelastic behaviours. Then their mechanical behaviour was modulated over time using microbial transglutaminase. Typically, enzymatically induced mechanical alterations occurred within the first 24 h of reaction and then the characteristic time constant decreased although the elastic properties were maintained almost constant for up to seven days. Preliminary cell culture tests showed that cells adhered to the gels, and their viability was similar to that of controls. Thus, the strategy proposed in this work is suitable for studying cell response and adaptation to temporal variations of substrate mechanics during culture.

Keywords: viscoelasticity; dynamic mechanical properties; transglutaminase; ageing

Journal of the Mechanical Behavior of Biomedical Materials 89 (2019) 162–167

Contents lists available at ScienceDirect

Journal of the Mechanical Behavior of Biomedical Materials

journal homepage: www.elsevier.com/locate/jmbbm

Engineering hydrogel viscoelasticity

Ludovica Cacopardo[a,b], Nicole Guazzelli[a], Roberta Nossa[a,b], Giorgio Mattei[b,c,d], Arti Ahluwalia[a,b,*]

[a] Research Centre "E. Piaggio", University of Pisa, Largo Lucio Lazzarino 1, 56122 Pisa, Italy
[b] Department of Information Engineering, University of Pisa, Via Girolamo Caruso 16, 56122 Pisa, Italy
[c] Optics11 B.V., De Boelelaan 1081, 1081 HV Amsterdam, the Netherlands
[d] Biophotonics & Medical Imaging and LaserLaB, VU University Amsterdam, De Boelelaan 1105, 1081 HV Amsterdam, the Netherlands

ARTICLE INFO

Keywords:
Viscoelasticity
Damping component modulation
Agarose
Polyacrylamide
Mechanobiology

ABSTRACT

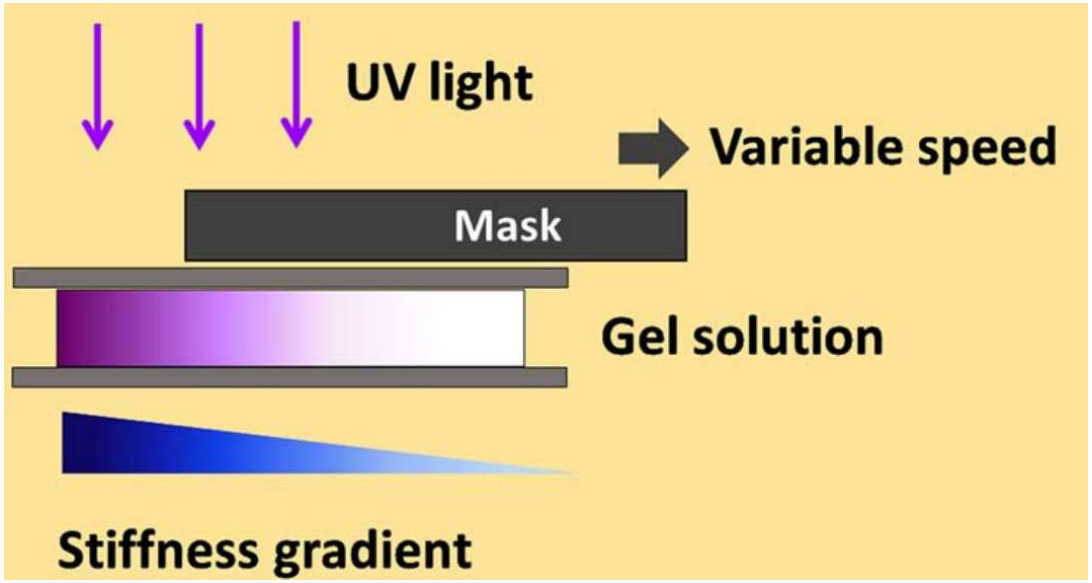
The aim of this study was to identify a method for modifying the time-dependent viscoelastic properties of gels without altering the elastic component. To this end, two hydrogels commonly used in biomedical applications, agarose and acrylamide, were prepared in aqueous solutions of dextran with increasing concentrations (0%, 2% and 5% w/v) and hence increasing viscosities. Commercial polyurethane sponges soaked in the same solutions were used as controls, since, unlike in hydrogels, the liquid in these sponge systems is poorly bound to the polymer network. Sample viscoelastic properties were characterised using the epsilon-dot method, based on compression tests at different constant strain-rates. Experimental data were fitted to a standard linear solid model. While increasing the liquid viscosity in the controls resulted in a significant increase of the characteristic relaxation time (τ), both the instantaneous (E_{inst}) and the equilibrium (E_{eq}) elastic moduli remained almost constant.

However, in the hydrogels a significant reduction of both E_{inst} and τ was observed. On the other hand, as expected, E_{eq} – an indicator of the equilibrium elastic behaviour after the occurrence of viscoelastic relaxation dynamics – was found to be independent of the liquid phase viscosity.

Therefore, although the elastic and viscous components of hydrogels cannot be completely decoupled due to the interaction of the liquid and solid phases, we show that their viscoelastic behaviour can be modulated by varying the viscosity of the aqueous phase. This simple-yet-effective strategy could be useful in the field of mechanobiology, particularly for studying cell response to substrate viscoelasticity while keeping the elastic cue (i.e. equilibrium modulus, or quasi-static stiffness) constant.

SPACE-variant mechanical properties

Within the body, tissues do not present an uniform stiffness but complex **stiffness gradients,** which can be found at the interface within the tissue, between different tissues or in pathologic conditions.



Methods to generate mechanical gradients:

- Sliding mask
- Mask with an opacity gradient
- Crosslinker diffusion

In sintesi

- Polimeri: naturali/sintetici
- Hydrogel: fisici/chimici
- Crosslinker: chimici/UV, biologici, enzimatici
- Modificare proprietà iniziali hydrogel:

  - variando concentrazione iniziale monomero/cross-linker
- Modularle nel tempo:
  - stiffening enzimatico
- Modularle nello spazio:
  - gradienti diffusione crosslinker, gradient/sliding mask
- Modulare la componente viscosa: consente di modificare le proprietà tempo-dipendenti del substrato senza alterare quelle elastiche all'equilibrio

Polymeric solution-gel transition