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Abstract

The article deals with the description of the most relevant Gelatin/Hyaluronic acid biomimetic hydrogels systems covering some specific methodologies of preparation and their clinical applications. Some authors´ results related with the topic are included. The final remarks reflect the future perspectives and the excellent properties of these hydrogels.

Keywords	Hydrogels; Gelatin; Hyaluronic acid; In vivo applications; Photopolymerization; covalent crosslinking

Corresponding Author	Ana Mora Boza
Corresponding Author's Institution	Spanish National Research Council (CSIC)
Order of Authors	Ana Mora Boza, María Puertas Bartolomé, Blanca Vázquez-Lasa, Julio San Roman, Antonio Pérez-Caballer, Marta Olmeda-Lozano

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Authors: Ana Mora-Boza, María Puertas-Bartolomé, Blanca Vázquez-Lasa, Julio San Román, Antonio Pérez-Caballer, Marta Olmeda-Lozano

Corresponding authors: Ana Mora-Boza, María Puertas-Bartolomé

Recommended Reviewers:

- Francisco Goycoolea
  University of Leeds
  F.M.Goycoolea@leeds.ac.uk
- Sanjukta Deb
  Dental School, King College, U. London
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- Joao Mano
  University of Aveiro
  Jmano@ua.pt
- Assunta Borzachiello
  U. Naples Federico II
  bassunta@unina.it
- Waldo Argüelles
  CIAD, Hermosillo, Mexico
  waldo@ciad.mx

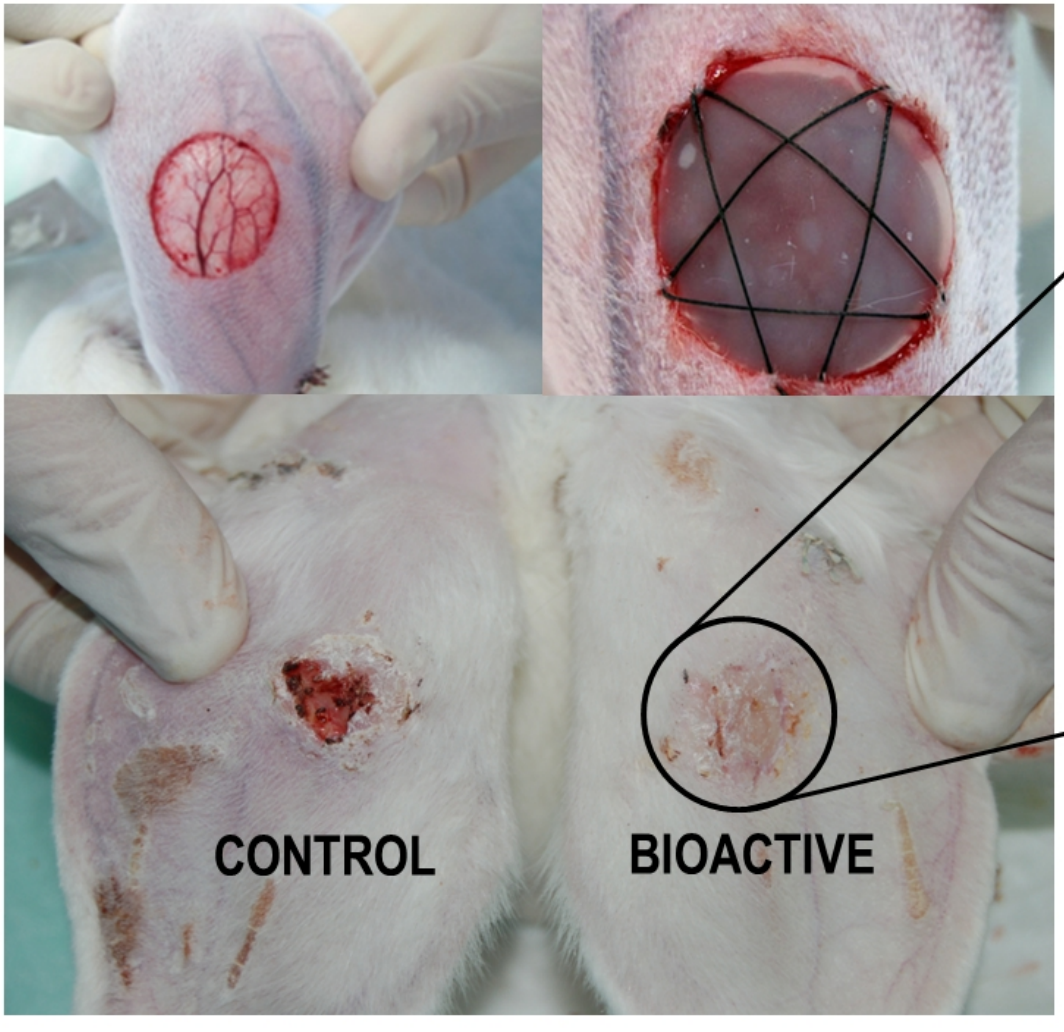
Dear G. J. Vancso:

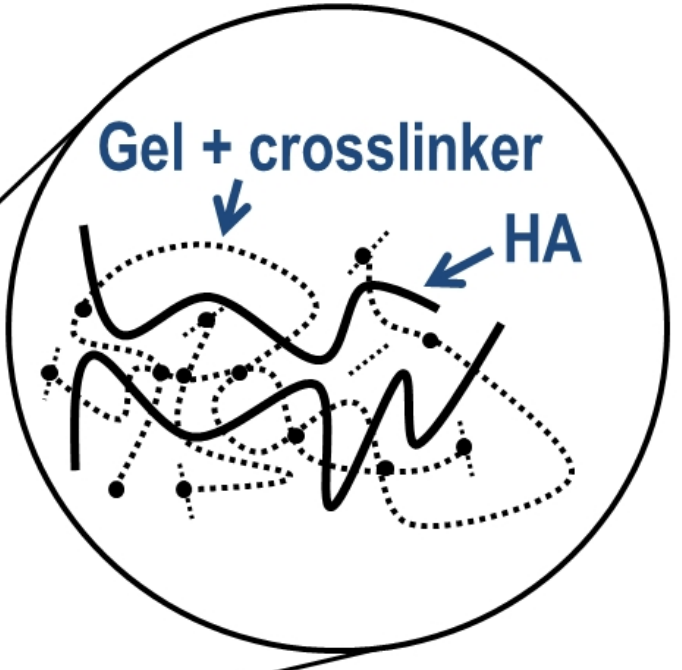
We are sending our manuscript that was written by letter of invitation of Alejandro Müller to Prof San Román, to be considered for publication in European Polymer Journal as a feature article.

The authors confirm that the manuscript has been read and approved its submission.

Thank you in advanced for your consideration,

Ana Mora-Boza.





- Biomimetic
- Bioactive
- Biodegradable
- Viscoelastic properties

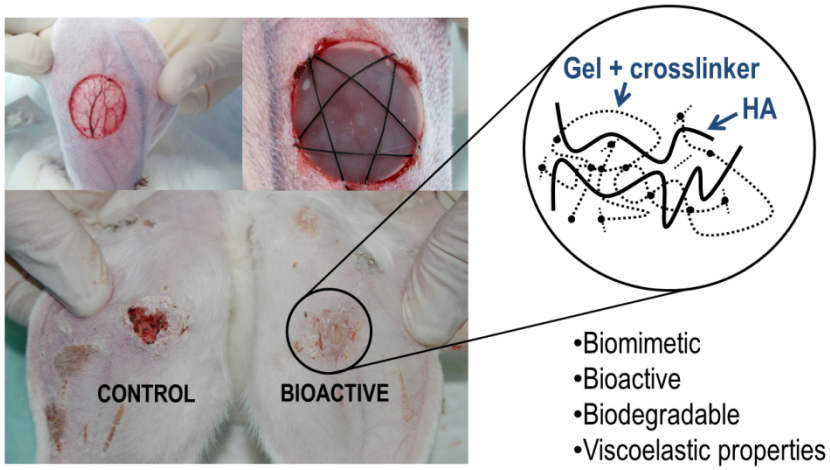
Contribution of bioactive hyaluronic acid and gelatin to Regenerative Medicine. Methodologies of gels preparation and advanced applications.

Ana Mora-Boza* [1, 2], María Puertas-Bartolomé* [1, 2], Blanca Vázquez-Lasa[1, 2], Julio San Román[1, 2], Antonio Pérez-Caballer[3], Marta Olmeda-Lozano[4]

[1]*Institute of Polymer Science and Technology-ICTP-CSIC, C/ Juan de la Cierva 3, 28006 Madrid, Spain*
[2]*CIBER-BNN, Health Institute Carlos III, C/ Monforte de Lemos 3-5, Pabellón 11, 28029 Madrid, Spain*
[3]*Faculty of Health Science, Francisco de Vitoria University, Ctra. Pozuelo-Mahadahonda Km.1800 28223 Pozuelo de Alarcón, Madrid, Spain*
[4]*University Hospital Infanta Elena, Av. de los Reyes Católicos, 21, 28342 Valdemoro, Madrid, Spain*

**Corresponding Authors:*
Ana Mora-Boza: *ana.mora@ictp.csic.es*
María Puertas-Bartolomé*: mpuertas@ictp.csic.es*

Graphical abstract



Contents

1. Introduction

The evolution of the nature in the design of materials and fabrication of human and animal tissues, has clearly demonstrated the enormous importance of relatively single macromolecules in the composition of the main tissues and organs of the body. It is clear that the "cell" is the protagonist of the whole activity of the living organisms, but the different kind of cells, from stem cells to differentiated fibroblasts, endothelial and dermal cells, chondrocytes, osteoblasts, etc. need the appropriate medium for growing, differentiation and proliferation. This is the extracellular matrix (ECM), that can be considered as a well-defined "hydrogel" generated by the own cells and with specific characteristics like capacity of absorption of water or body fluids, adequate medium for cells diffusion and interaction, and a dynamic system that can be replaced permanently by processes of resorption (biodegradation) and production by the cells. In this equilibrium, mainly three macromolecular components play an important role: proteins (Collagen, elastin, fibronectin), polysaccharides (hyaluronic acid, heparin sulfate, heparin) and in small amounts, growth factor (hormones) and nucleic acids (DNA, RNA). From a biomechanical point of view, the ECM is the scaffold secreted by cells to form the connective tissue or medium to facilitate the cell-cell interaction, cell proliferation and differentiation. Of course, the specific characteristics of ECM depend on the function of each tissue. This means that the ECM of bone tissue has not the same components as those of epithelial tissue or vascular system. According to the properties, for example the ECM of bone tissue has to present excellent stiffness and toughness, which is achieved by the incorporation of

important amount of mineral components (hydroxyapatite) , whereas in the composition of the vascular system (blood vessels) and ligaments in joints, the elastin in conjugation with collagen are fundamental to bring the adequate elasticity required. [1-12]

The scheme of Fig 1 shows the complex but organized structure and morphology of the ECM. It is clear that the coexistence of components based on proteins (collagen) and carbohydrates (hyaluronic acid, heparin sulfate, proteoglycans, agregan) are very important to contribute to the organization of the matrix and the adequate medium for the coexistence of cells (fibroblasts, endothelial, etc..) and for the optimum function of the ECM and cells proliferation to form well defined structured tissues, with the specific vascularization (blood vessels) and functionality.

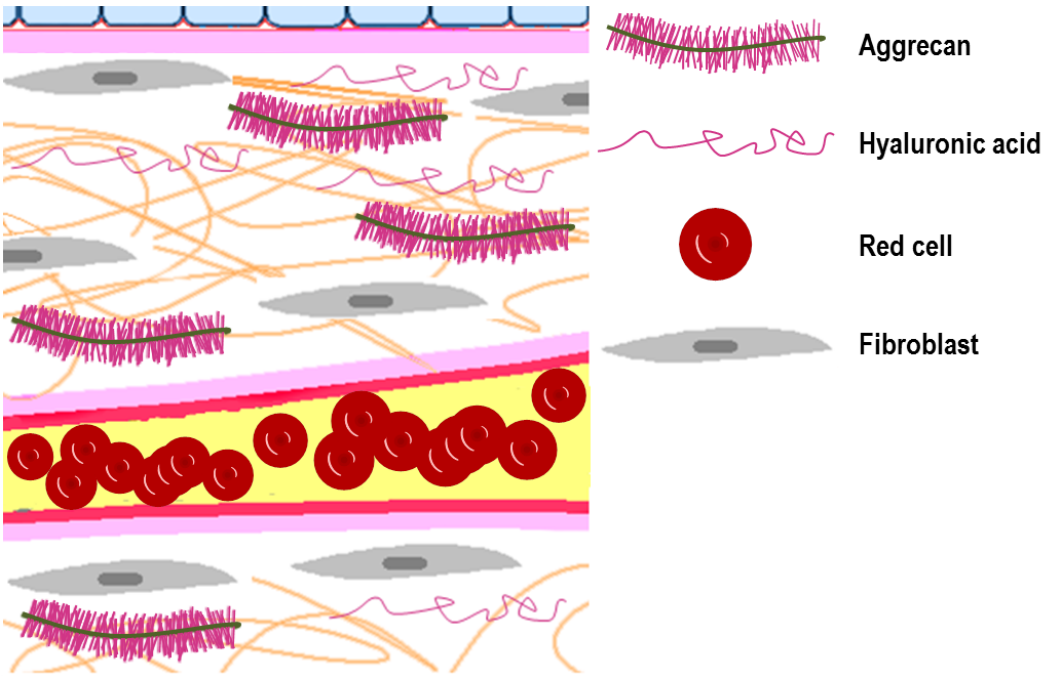


Fig 1. Schematic representation of the structure and morphology of extracellular matrix ECM in human tissues.

The direct interaction between the specific proteins and polysaccharides (glycosaminoglycans) is of great importance to obtain a scaffold with the appropriate characteristics for connective tissue and cells life. In this sense, the chemical composition and microstructure of the macromolecular components as well as the methods for the preparation biomimetic systems, are crucial to obtain

supports that could perform as temporal substitutes of the ECM, not only from a basic point of view, but also considering the applications in the modern concepts of regenerative medicine.

One common characteristic in all the ECM in body tissues is the equilibrium between hydrophilic and hydrophobic components as well as the crosslinked nature of the complex systems (proteoglycans), which provides enough stability and flexibility to facilitate the diffusion of cells and their function and connections. This is the main reason for the intense dedication of research groups to offer macromolecular systems that could present the most optimal properties for a specific application.

The relevance of gelatin and hyaluronic acid biopolymers in the field of tissue engineering has been already clearly demonstrated. Here, it is presented an overview of the most relevant crosslinking processes and agents that are being developed for regenerative medicine, including crosslinking methodologies as well as different hydrogel modifications and several interesting and advanced applications.

2. Crosslinking methods for Gel/HA hydrogels

2.1. Photopolymerization

The conversion of Gelatin (Gel) and Hyaluronic Acid (HA) into photocrosslinkable precursors through the chemical addition of methacryloyl groups (MA) to its amine groups has been widely used during the last few years. The synthesis of Gel-MA/HA-MA hydrogels by ultraviolet (UV) or visible (VIS) light irradiation in the presence of some photoinitiator (PI) molecules such as Irgacure 2959 [13], lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) or VA-086 [6, 12, 14] has been described by many authors for different applications like drug delivery, tissue engineering and gene therapy [6, 12, 15, 16]. The main advantage of photocrosslinking compared to other crosslinking methods resides in the exceptional spatiotemporal control over the polymerization, which enables the tunability of the final properties of the crosslinked matrix. In fact, the modulation of the time, intensity and wavelength of the irradiation plays a crucial role in the control of the cellular behavior (cell adhesion, differentiation, proliferation, etc). This tunability is fairly demonstrated in the work of Chan et al. where the UV

intensity, PI concentration and UV exposure modulation considerably affects the microparticle size and stiffness [17]. Moreover, photocrosslinking is a low-cost technique that can be carried out at ambient conditions [5, 6, 11, 12, 14, 17-22].

However, photocuring also shows some drawbacks due mainly to the cytotoxicity and inflammation reactions that are provoked by the generation of free radicals by UV exposure that can damage DNA and cellular components [5, 6, 22]. For this reason, activated PI under visible or A-UV light are being extensively used during the last years [6, 14]. Although many authors have demonstrated in their studies that a proper adjustment of the UV irradiation time, intensity and wavelength, could ensure cell viability [4, 15, 19, 23-25], in our opinion, the use of these PI would allow the encapsulation of cells assuring cellular viability.

Although Gel-MA hydrogels possess suitable properties for biomedical engineering applications, they are usually combined with other natural glycosaminoglycans (GAG) such as, chondroitin sulfate (CS), chitosan (Ch) or hyaluronic acid (HA) [6, 9, 14-16, 23, 24, 26]. Levett et al. concluded that the use of hybrid hydrogels provides the different advantages of each component thanks to a comparative study between the behaviour of chondrocytes face to different single-type hydrogels. In case of Gel-MA hydrogels for example, they found that the development of extracellular matrix (ECM) with functional properties were confirmed, but the phenotype of chondrocytes was not homogeneous. On the other hand, HA-MA hydrogels promote a better chondrogenic distribution, but they did not present such high stability as Gel-MA, which reinforces the idea of the combined use of both [24]. Applying this knowledge, in other of their studies, Levett et al. developed a biomimetic ECM for cartilage tissue engineering based on photocrosslinkable Gel-MA, HA-MA and CS-MA. In this work, they used Irgacure 2959 as PI and the hydrogels were crosslinked for 15 min exposure to 365 nm light at 2.6 mW/cm2. The addition of HA-MA to Gel-MA-based hydrogels not only enhances chondrogenesis but also improves mechanical properties of the hydrogel, making them promising candidates for cartilage regeneration as it is shown in Fig. 2 [9, 15, 27]. For these reasons, Gel-MA/HA-MA hydrogels are being increasingly used for cartilage tissue engineering during the last few years [9, 15, 23, 24, 27, 28]. For example, O'Connell et al. developed an easy-handle device for medical surgery

named “biopen” in an attempt of bringing together 3D printing technology and surgical processes. The tool was able to print Gel-MA/HA-MA hydrogels, which were photocrosslinked instantaneously by using VA-086 molecule as PI. Photocuring was carried out with a UV source at 130 mW / cm2 for 60 s, and the process was compatible with the deposition of adipose stem cells at chondral wound side because of the low cytotoxicity demonstrated by the protocol [23]. Other example of chondrogesis boarded from Gel-MA/HA-MA matrices was performed by Bartnikowski et al. who developed 3D printed hydrogels for 3D encapsulation of human articular chondrocyte to promote osteochondrogenesis by the incorporation of hydroxyapatite and the formation of a zone of calcified cartilage. The UV photocrosslinking was carried out using Irgacure 2959 at 365 nm at 2.3 mW/cm2 for 11 min [28].

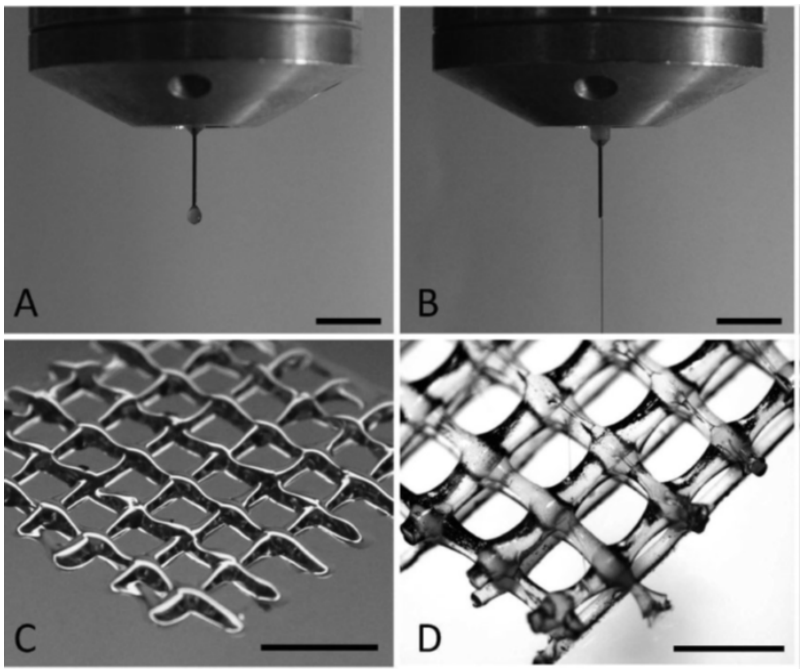


Fig. 2: Bioprinting of Gel-MA without (A, C) and with HA (B, D). When 2.4% HA was added, strands could be deposited from the nozzle (B), resulting in construct of four layers (D). The scale bars in A–C represent 5 mm; the scale bar in D is 2 mm. Reproduced with permission from [9]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

Apart from chondrogenic regeneration, Gel-MA/HA-MA scaffolds application for cardiac tissue engineering has acquired much attention in the last few years [12, 20, 25, 26, 29] . Hjortnaes et al. demonstrated that these hybrid hydrogels promote

the maintenance of direct valvular interstitial cells (VIC) phenotype against myofibroblast differentiation, which is crucial for the study of valve diseases. The photocuring was carried out with Irgacure 2959 as PI and UV light exposition at 2.5 mW/cm^2 for 30 s. These Gel-MA/HA-MA platforms served as a model for heart cell environment *in vivo* [29]. In similar studies, Duan et al. developed Gel-MA and HA-MA hydrogels, being this last component oxidized at different degrees, confirming the capacity of these platforms to maintain a quiescent VIC fibroblastic phenotype. In this case, the photocrosslinking was carried out at 365 nm UV light for 5 min, using also Irgacure 2959. Moreover, they demonstrated that the addition of Gel-MA promoted cell-hydrogel interactions and GAG secretion, and therefore cell spreading and proliferation [20]. In other work, they also studied the stiffness and viscosity hydrogel modulation varying Gel and HA final concentrations, which also affects cell behavior. As reported previously, the increase of Gel-MA concentration resulted in a stiffness decrease, which increases cell adhesion, enhancing therefore HA-VIC phenotype maintenance [25].

On the other hand, not every Gel/HA hydrogel photopolymerizations make use of methacryloyl modification, but other chemical functionalization based on the step-growth thiol-ene photopolymerization which consists of the reaction between a thiol and a vinyl group, known also as thiol-ene click chemistry [7, 14, 22, 30-33]. The main advantages of these photocuring protocols are that they are not oxygen-inhibited and the small necessary amount of photoinitiator to induce the reaction [7, 14, 21, 22], being even not necessary in some cases [34]. Hynes et al. in an innovative study developed a live-cell-based reactive oxygen species (ROS) sensor making use of 3D Bioprinting and the thiol-ene click chemistry. They used thiolated hyaluronan/thiolated gelatin to couple the thiol groups to the alkene groups of 4-arm polyethylene glycol (PEG)-norbornene molecules, obtaining a highly crosslinking matrix to monitor redox activity *in vitro* [22]. In another work, Zhang et al. developed a novel *in situ* phototriggered-imine-crosslink (PIC) where HA was grafted with an o-nitrobenzyl (NB) derivative. This HA-NB formulation was combined with gelatin and hydroxyapatite nanoparticles for osteogenesis. In this case, the photocuring process was induced by 10 min at 365 nm light irradiation (20 mW/cm^2) without the necessity of adding a photoinitiator [34]. In other study, Nemeth et al. took advantage of photopolymerization for the

development of nanopatterned hydrogels by capillary force lithography (CFL), demonstrating the influence of nanotopography and HA addition for enhancing cartilage tissue engineering [32].

Finally, as it has been indicated before, photopolymerization crosslinking can be also compatible with cell encapsulation [4, 14, 15, 18, 22, 23, 25, 28, 29, 31, 33, 34]. Although UV-based photocuring is being extensively disputed because it could trigger cellular damage, a myriad of authors claim its safe use [4, 5, 22, 23]. In this context, Camci-Unal et al. amply demonstrated the possibility of 2D and 3D cell culture within photocrosslinkable hydrogels. Moreover, they studied different compositions of HA-MA and Gel-MA hybrid hydrogels for the encapsulation of human umbilical cord vein endothelial cells (HUVECs) [4]. Nevertheless, currently more and more authors put in place the alternative use of VIS-activated PIs [7, 31, 34]. In addition, many of these studies based on VIS-photopolymerization do not make use of acrylic modification, but of the click chemistry explained before [14, 31, 34]. Shih et al. for example, developed a bioactive and biomimetic hybrid hydrogel including norbornene functionalized Gelatin (GelNB) and thiolated Hyaluronic Acid (THA). The photocuring was carried out using Eosin-Y as PI and visible light irradiation at 400-700 nm during 5 min [31]. Table 1 summarizes the main photocrosslinkable systems recently reported.

Table 1: Summary of Gel/HA systems synthetized by photocrosslinking

Modification	Additional components	Photoinitiator	Polymerization conditions	Application	Ref.
Acrylic addition	Chondroitin Sulfate	Irgacure 2959	365 nm 2.6 mW/cm^2 15 min	Chondrogenesis	[9, 15, 24, 27]
Acrylic addition	-	VA-086	UV-A 130 mW/cm^2 60 s	3D printing chondral wound repair	[23]
Acrylic addition	Human Umbilical Vein Endothelial Cells	Irgacure 2959	UV-A 2.5 mW/cm^2 30-120 s	Cell encapsulation	[4]
Acrylic addition	Hydroxyapatite	Irgacure 2959	365 nm 2.3 mW/cm^2 11 min	Osteochondral regeneration	[28]
Acrylic addition	-	Irgacure 2959	UV-A 2.5 mW/cm^2 30 s	Cardiac regeneration	[29]
Acrylic addition	-	Irgacure 2959	365 nm 5 min	Cardiac	[20]

and oxidation				regeneration	
Acrylic addition	-	Irgacure 2959	365 nm; 2 mW/cm^2 5 min	Valve disease investigation	[25]
Grafting reaction	Hydroxyapatite nanoparticles	No	365 nm 2.0 mW/cm^2 10 min	Osteogenesis	[34]
Thiol-ene reaction	Thiolated PVA PEG-dinorbornene	Eosin-Y	VIS light 5 min	Artificial tumor niche	[31]
Thiol addition	PEG-norbornene	Irgacure 2959	365 nm 2 min	ROS biosensor	[22]
Acrylic addition for Gel	-	Irgacure 2959	VIS light 360-480 6.9 mW/cm^2 2 min	Prevention of abdominal adhesion	[35]
Acrylic addition for Gel	PEG	2-Hydroxy-2-Methylpropiophenone	365 nm	Chondrogenic differentiation	[32]
Acrylic addition for Gel	Laminin-411	Irgacure 2959	365 nm 2.7 mW/cm^2 10 min	3D cancer culture	[18]

2.2. Michael type addition

The well-known Michael addition consists on the addition of a nucleophile like thiols or amine to an α,β-unsaturated carbonyl compound. For preparing *in situ* injectable hydrogels containing gelatin and hyaluronic acid, thiols are predominantly preferred as the reaction is thermodynamically favorable under physiological conditions, so both polymers are thiol-modified. These thiol groups are conjugated with multi acrylated poly(ethylene glycol) (PEG) as nucleophile acceptor to form an injectable hydrogel system. The gelling time can be varied depending on the molecular weight, thiol-substitution degree and concentrations.

HA and collagen are two important extracellular matrix (ECM) components of the human vocal fold lamina propia (VFLP). Crosslinked Gel-S/HA-S networks have been used as synthetic extracellular matrix hydrogels to create injectable vocal fold biomaterials. In 2015 Kazemirad et al. measured the frequency-dependent viscoelastic properties of the hydrogels trough a Raileigh wave propagation method, showing that varying the composition of HA, Gel and crosslinker had viscoelastic properties similar to those of human vocal fold tissue [36]. Then, in 2016 this research group developed Gel-S/HA-S microgels and human vocal fold fibroblast (hVFF) cells behavior was analyzed in 2D and 3D culture conditions. The study showed that microgels promote cell viability adhesion, spreading and

migration, so these systems would thus offer a potential therapeutic avenue in vocal fold tissue engineering [37]. Heffernan et al. also developed the system of HA-S and Gel-S chemically crosslinked with thiol reactive PEG polymers, including PEG diacrylate (PEGDA) and PEG divinyl sulfone (PEGDVS) [38]. This study describes an interesting method for quantitative measurement of malignant glioblastoma (GBM) cells movement in a customizable 3D tumor micro environment. HA is the most abundant component of brain ECM and Gel provides cellular adhesion sites. Furthermore, crosslinking improves the enzymatic stability of hydrogels, so this platform is of particular interest to study the proliferation and invasion of GBM cells in a 3D culture. Malinen et al. use the hydrogel system of Gel-S/HA-S crosslinked by PEGDA and wood-derived nanofibrillar cellulose (NFC) as extracellular matrix mimicking biomaterials to create a 3D culture environment for HepaRG liver progenitor cells. Results showed that these hydrogels are a good supporting material to expedite the hepatic differentiation of HepaRG liver progenitor cells [39]. Chan et al. reported a method to prepare soft, injectable and functionalizable hydrogel microparticles (MP) of natural polymers for applications as soft-tissue fillers [17]. Spherical MP are synthesized through suspension photopolymerization and semi-interpenetrating network (semi-IPN) of HA, Gel and acrylated derivatives of PEG. HA and Gel were chosen because of their biocompatibility and their use in commercial dermal fillers and PEG was used because of its use in FDA-approved products. Furthermore, photocrosslinking was carried out because its soft conditions, efficiency and control over the crosslinking (described in photocrosslinking section). So this system and method allow preparation of MP of independently tunable size and stiffnes suitable for use as biomedical fillers.

Several authors have applied composite Gel/HA hydrogels as mimicking scaffolds for improving stem cell survival in regenerative medicine [32, 40-43]. Thiol modified HA and Gel scaffolds provide a supportive 3D tissue mimicking microenviroment to improve stem cell engraftment. These biomaterials have a great biocompatibility, ease of injection and tunable properties, creating a stem cell niche with similar characteristics to the natural niche in the tissue. Adjusting biochemical and biomechanical properties of the scaffold is a powerful tool in

controlling stem cell function, fate and trajectory of stem cell differentiation. A potential tool to monitor *in vivo* the disintegration over time of these hydrogels has been analyzed by Liang et al. For this purpose they used a chemical exchange saturation transfer magnetic resonance imaging (CEST MRI) to follow dynamic changes in the stem cell scaffold, which is very useful for the further use of biomaterials in regenerative medicine with stem cells [41]. Li et al. created a stem cell niche to provide a regeneration microenvironment for transplanted neural stem cells (NSCs) to survive, to promote central nervous system (CNS) regeneration. Optimal conditions of this microenvironment are studied by varying the crosslinking density, the amount of crosslinker and the concentration of gelatin, for the survival proliferation and neuronal differentiation [40]. Mesenchymal stromal/cells (MSCs) are also combined with Gel/HA scaffolds to modulate inflammatory response restoring tissue architecture. Here, King et al. study the interaction between MSCs encapsulated in the hydrogels and differentiating macrophages by analyzing ECM gene expression and cytokine and growth factor concentrations [42]. Nemeth et al. have examined the effects of surface nanotopography and the scaffold on *in vitro* chondrogenesis of dental pulp stem cells (DPSCs). It is demonstrated that DPSCs form spheroids in these scaffolds providing an appropriate environment for *in vitro* chondrogenic differentiation [32]. In the study of Zhao et al. the stiffness of the hydrogel is controlled by varying the crossliking density, and hMSCs differentiate into adipogenic and osteogenic cells. So it is also demonstrated that scaffold mechanical properties modulate cell phenotype [43].

2.3. Schiff base formation

Various injectable hydrogel Gel/HA systems have been reported using Schiff base crosslinking. In this crosslinking amine groups of gelatin reacts with aldehyde groups. Recently, to overcome the disadvantages of glutaraldehyde (GTA), an aggressive crosslinker commonly used in the past, more biocompatible crosslinkers have emerged based on oxidized polysaccharides. In 2016, Khorshidi et al. developed a self-crosslinking *in situ* forming hydrogel based on oxidized hyaluronic acid (HA-ox), oxidized alginate (Alg-ox) and gelatin for cartilage tissue engineering. Reaction of free amino groups of lysine residuals of gelatin with

available aldehyde functions of partially oxidized polysaccharides via covalent bonding can undergo self-crosslinking without using any extraneous crosslinker, creating a highly stable and physiologically degradable network. Physical and mechanical properties of the hydrogel were greatly influenced by the concentration of the components. This tri-component self-crosslinkable hydrogel is a promising biocompatible material with tunable physico-chemical properties for cartilage tissues regeneration (51).

Our research group is developing injectable hydrogels based on gelatin and hyaluronic acid using oxidized dextran (Dex-ox) because of the stability and biocompatibility of this crosslinker. Aldehyde groups of the Dex-ox react with amine groups of gelatin, giving a semi-interpentrating polymer network (semi-IPN) with hyaluronic acid as shown in Fig. 3.

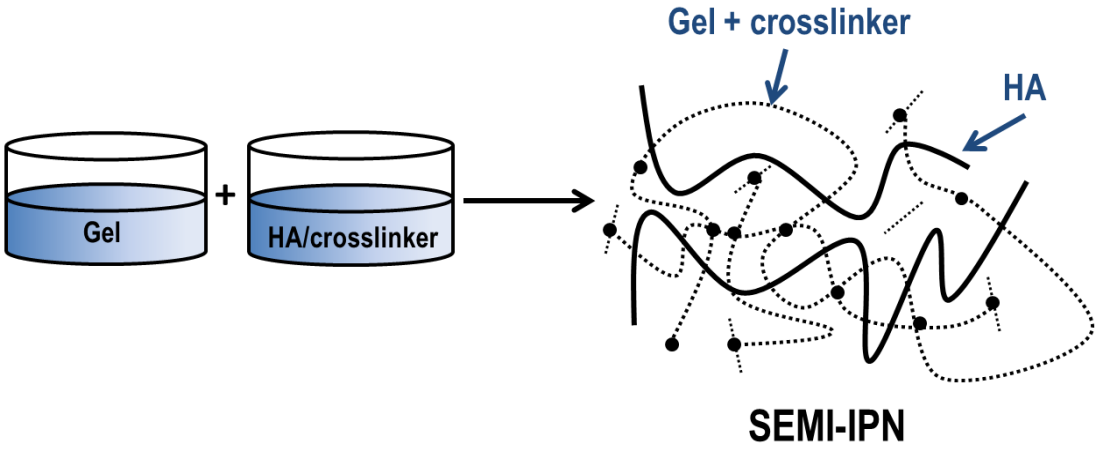


Fig 3. Preparation of semi-IPNs hydrogels based on Gel/HA crosslinked with Dex-ox.

The dextran (Dex) is oxidized in water with $NaIO_4$ under conditions shown in Table 2.

Table 2. Oxidation conditions of Dex[a] along with oxidation degree (OD) and molecular weight results of Dex-ox.

Medium	Dex: $NaIO_4$ ratio (mol/mol)	Reaction time (h)	OD[b] (mol %)	M_w[c] (kDa)
Distilled water	0.15	4	20	48.5
Distilled water	0.3	4	40	40

a: Dextran (M_w 70 kDa) supplied by Pharmacosmos.
b: OD = oxidation degree determined by the hydroxylamine method [44]
c: M_w = weight average molecular weight determined by size exclusion chromatography (SEC) using pullulan standards (708 kDa-108 Da).

OD of Dex-ox increases with the content of oxidasing agent whereas M_w decreases indicating the cleavage of some polysaccharide backbone during oxidation as it is reported elsewhere [45]. Dex-ox of 40% OD is selected to prepare Gel/HA hydrogels (Table 3) so that, its cytotoxicity is initially tested using human dermal fibroblasts and MTT assay as reported by the authors [46]. Results are shown in Fig. 4a. Cell viability of fibroblasts in presence of different dilutions of Dex-ox remains around 100 % at concentrations of 10 mg/mL or lower. Accordingly, concentration range in which Dex-ox are not cytotoxic [47] is taking into consideration to prepare the polysaccharide hydrogels. Other conditions applied in their formulation are described in Table 3.

Table 3. Formulation of Gel[a]/HA[b] hydrogels using Dex-ox of 40% oxidation degree.

Gel/Dex-ox ratio (wt/wt)	1/1
Gel concentration in the final mixture(mg/ml)	50
Gel/HA ratio (wt/wt)	53/47
Medium	PBS of pH 7.4

a: Gel of pharma grade supplied by Reinert-Gruppe
b: HA with M_w = 200 kDa, supplied by BIOIBERICA
c: PBS = Phosphate buffer solution, 0.01 M Dulbecoo's, supplied by Sigma-Aldrich

Cytotoxicity of the hydrogels is tested using a standard Alamar Blue test [46] Fig. 4b shows that cell viability in presence of extracts of hydrogels taken between 1 and 21 days oscillates around 100 %, demonstrating that the materials do not release toxic compounds derived from Dex-ox.

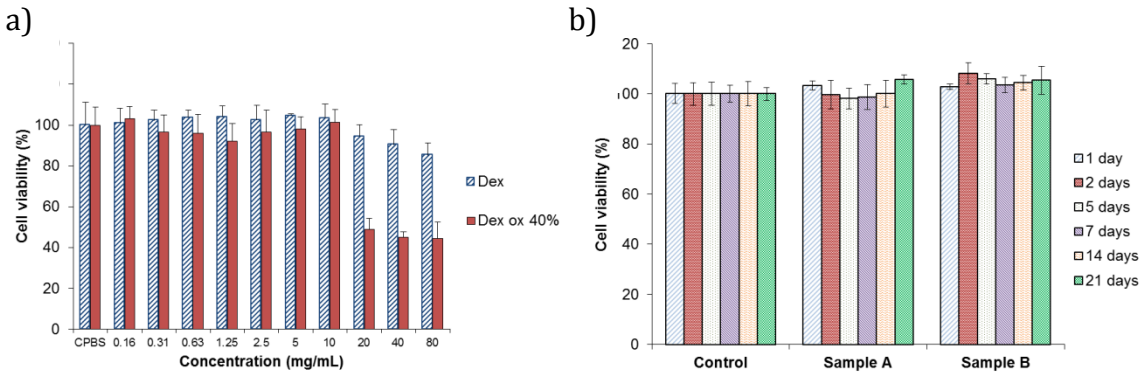


Fig. 4. a) MTT results of Dex-ox of 40% OD at different concentrations. b) Alamar Blue results of Gel-HA hydrogels prepared using conditions shown in Table 3. Letters *A* and *B* refer to replicates of the sample described in Table 3.

These Gel/HA hydrogels that seem promising as drug delivery systems are being used to encapsulate anti-inflammatory drugs to treat osteochondral lesions. Preliminary *in vivo* results are reported in the following section.

2.4. Amidation reactions

This crosslinking reaction between carboxyl groups and amine groups of lysine and hydroxylysine of gelatin is frequently carried out with the water-soluble 1-(3-dimethyl-aminopropyl)-3-ethyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling system.

A new bone substitute material was developed by Nguyen et al. with a hydrogel of Gel and HA loaded with calcium phosphate. In this study an excellent hybrid scaffold with micro and macroporous orientation is obtained to enhance bone healing for bone regeneration applications [48]. Then, they used this system to encapsulate and immobilize plattelet rich plasma (PRP), improving the mechanical properties and biocompatibility of the scaffold. Both systems are crosslinked with EDC coupling agent creating scaffolds with covalent unions for enhanced bone regeneration [49]. Ouyang et al. fabricated a novel Gel/HA/sodium alginate porous scaffold by freezing-drying method crosslinked with EDC that produced suitable materials for cell adhesion and growth for tissue engineering applications [50]. Similar scaffolds were created by Zhou et al. with interconnected pores as potential wound dressing materials [51]. Peng et al. also developed these

tricomponent systems, investigating the process variables on the physical properties of the composite hydrogels. The study found that the concentration of crosslinker, crosslinking time and pH significantly change the morphology, swelling ratio and compressive strength of the hydrogels. Therefore, these composite hydrogels are potential materials for cartilage tissue engineering [52].

As an alternative to chemical crosslinkers, the naturally occurring crosslinker genipin is chosen due to its good biocompatibility [53] and efficiency in producing the covalent coupling of natural polymers [54, 55]. The crosslinking mechanism involves the formation of some intermediate structures [56] and the reaction of the ester groups of genipin with amino groups to form new secondary amide linkages [57-59]. Additionally, the resulting network is stabilized by ionic interactions [55]. Our research group has recently prepared biodegradable Gel/HA hydrogels crosslinked with genipin and loaded with the proangiogenic and anti-inflammatory N-terminal 20 peptide proadrenomedullin (PAMP). This highly hydrophilic hydrogel constituted the internal layer of a bilayered dressing designed for application in the healing of compromised wounds [60]. The external layer was a biodegradable polyurethane derived from poly(ε-caprolactone) and pluronic L61, loaded with resorbable nanoparticles of bemiparin, that promotes the activation of growth factors. Design and preparation of the bilayered system is shown in Fig. 5.

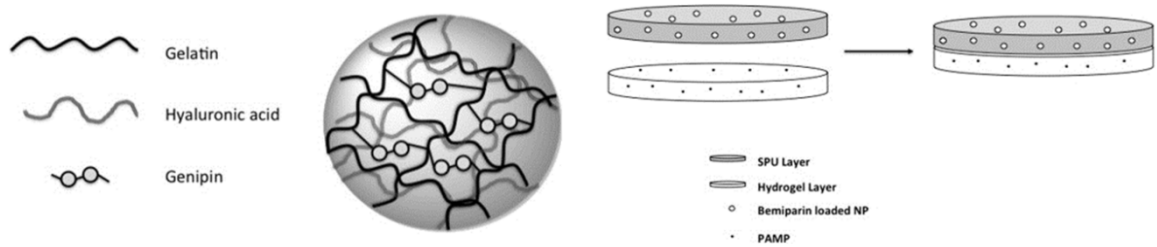


Fig. 5. Preparation and design of bilayered dressing [60]

Hydrogel formation is produced by the natural crosslinker genipin which forms covalent bonds with amine groups of gelatin, leading to a chemically crosslinked network containing hyaluronic acid stabilized by Van der Waals interactions. Genipin crosslinking reaction was followed by FTIR spectroscopy (Fig. 6).

Crosslinking produced a decrease of the band intensities of the functional groups (υ_{OH}=3480 cm^{-1}, υ_{NH2}=3400 cm^{-1} and υ_{CO} =1660 cm^{-1}) caused by the formation of hydrogen bonds between the OH, COOH, and NH_2 groups. Furthermore, the new amide bond formed between the free amine groups of gelatin and the carboxylic groups of genipin caused the appearance of a new carbonyl amide band at 1620 cm^{-1}.

This research suggests that this crosslinker is suitable to create biodegradable and resorbable hydrogels for fabrication of bio-functional bilayered systems that can be a new approach to treat compromised wounds.

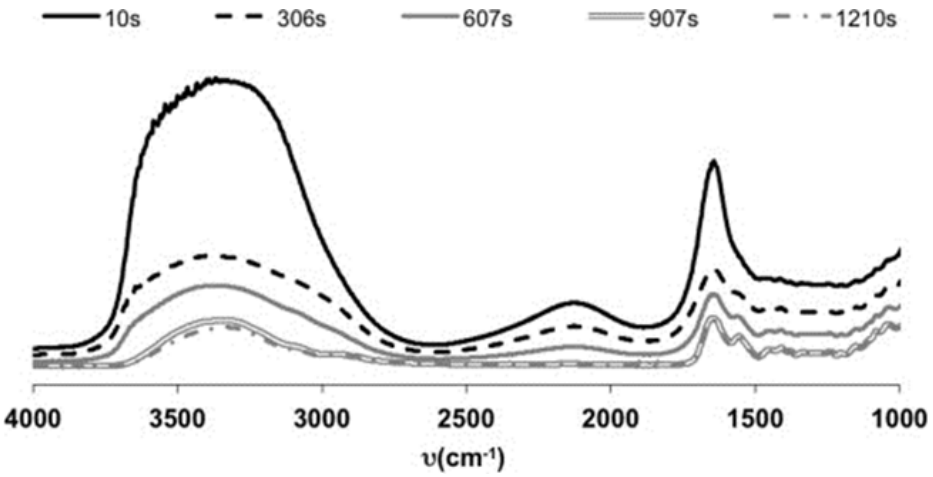


Fig 6. Normalized ATR-FTIR spectra of 5% genipin crosslinked Gel/HA hydrogel at different reaction times.

Table 4 collects a resume of Gel/HA systems obtained using different approaches of functional groups reactions.

Table 4: Summary of Gel/HA systems synthetized by Michael type addition, Schiff base formation and amidation reactions.

Crosslinker system	Hydrogel composition	Application	Reference
Acrylated PEG	Gel-S/HA-S	Vocal fold, glioblastoma, stem cell survival	[27, 31-33, 35-38]
	Gel-S/HA-S/Nanofibrillar cellulose	Liver progenitor cells	[34]
Acrylated PEG + Photopolymerization	Gel-MA/HA-MA	Soft-tissue fillers	[7]
Self-crosslinking	Gel/HA-ox/Alg-ox	Cartilage tissue	[51]

EDC/NHS	Gel/HA/Calcium phosphate	Bone regeneration	[39, 40]
	Gel/HA/Sodium alginate	Tissue engineering, wound dressing, cartilage regeneration	[41-43]
Genipin	Gel/HA	Compromised wounds	[44]

2.5. Enzymatic crosslinking

In the last years, enzyme-catalysed crosslinking has attracted attention as a promising methodology for the preparation of stable hydrogel networks [61]. The main advantages of using enzymatic crosslinking among other crosslinking processes are its mild conditions of reaction, the non-generation of toxic derivative products, as well as its highly tunability of the final properties of the scaffolds [61-63]. In addition, enzymes possess high selectivity and energy-efficiency. Although several kinds of enzymes have been used for the crosslinking of hydrogels, if we focus on gelatin and hyaluronic acid networks, horseradish peroxidase (HRP) [8, 61-64] and transglutaminase (MTGase) are the most commonly used ones [62, 65].

After the work of Kurisawa et al. [66], the HPR-mediated crosslinking process has been extensively studied. For this protocol, it is necessary the presence of phenolic groups, which has been boarded through the functionalization of Gel and HA with tyramine (Tyr) residues, and hydrogen peroxide (H_2O_2) as oxidant substrate [61, 63]. For example, Sanmartin-Masia et al. obtained Gel-Tyr and HA-Tyr derivatives through the reaction of carboxylic groups of Gel and HA with amine groups of Tyramine [8]. Poveda-Reyes et al. who carried out the same functionalization, used the crosslinked matrices for the study of skeletal muscle and soft tissue engineering [64]. In another interesting work, Khanmohammadi et al. studied the immobilization of low molecular weight HA (LMWHA) derivative within gelatin-based hydrogel. The LMWHA was functionalized with phenolic moieties as in previous works and the immobilization was catalyzed by HRP in presence of H_2O_2. In this case, they concluded that these hybrid networks promoted endothelial cells migrations and arise as a promising approach for vascularized dense tissue engineering [63].

On the other hand, the MTGase-mediated crosslinking is based on the formation of covalent N-ε-(γ-glutamyl) lysine amide bonds, which means that HA has to be modified since it does not carry the necessary primary amine groups. M. de Colli et al. modified HA with the dipeptide glycine-lysine (GK) to develop gelatin-GAG hybrid hydrogels to study the hepatocytes functions *in vitro* [65]. Finally, we would like to highlight another interesting work by Fan et al. where they used a bienzymatically crosslinking procedure combining both enzymes: HRP, for HA-Tyr and MTGase, for gelatin. They demonstrated the excellent biocompatibility of the scaffolds to be used for tissue engineering, wound repair, as well as drug delivery applications. Gel/HA systems obtained by mediated enzyme reactions are showed in Table 5.

Table 5: Summary of Gel/HA systems synthetized by enzymatic crosslinking.

Gel-HA chemical modification	Enzyme	Application	Reference
Tyramine functionalization	HRP + H_2O_2 MTGase	Tissue Engineering Wound repair Drug delivery	[62]
Tyramine functionalization	HRP + H_2O_2	Vascularized dense tissue engineering	[63]
Tyramine functionalization	HRP + H_2O_2	Injectable hydrogels	[8]
Tyramine functionalization	HRP + H_2O_2	Skeletal muscle	[64]
GK modification for HA	MTGase	Hepatocytes *in vitro*	[65]

2.6. Others

Innovative systems have been recently prepared in order to improve the architecture of the Gel/HA hydrogels, using different crosslinkers depending on the application they are created for.

Zarembinski et al. developed a new crosslinking system based on thioldisulfide exchange reaction of oxidized glutathione (GSSG) (a dimer of two disulfide crosslinked glutathione molecules), with the multiple thiol groups of the HA-S and Gel-S. GSSG is a biocompatible crosslinker with a history of safe in humans that can

cause gelation in less than 5 min at room temperature with no additional crosslinking molecule. This research suggests that this system could be a suitable reactant for cell-based therapeutics in the eye and local ocular delivery [67]. In 2015 Bas et al. developed a hybrid system combining highly oriented poly(ε-caprolactone) (PCL) fibers fabricated by Melt Electrospinning Writing (MEW) with Gel-MA/HAMA hydrogels to achieve extracellular matrix (ECM) like structures with improved mechanical properties. The crosslinker used in this case is a reduction–oxidation system (ammonium persulphate/tetramethylethylenediamine). Then, hydrogel is reinforced by microfibers providing a significant increase in compressive modulus and bringing together the advantages of Gel and HA [68]. Tsaryk et al. developed a semiinterpenetrating network by promoting collagen fibrillogenesis in the presence of HA loaded with gelatin microspheres. The ECM- based hydrogel is a promising suitable material for tissue engineering of the inner part of the intervertebral disc combining important rheological and biological properties [69].

3. Clinical Applications of Gel/HA Hydrogels

The use of Gel/HA for tissue engineering applications has acquired much attention in the recent years from the clinical point of view. In this section, we will discuss the main applications where the benefits of these hybrid hydrogels have been demonstrated.

3.1. Musculoskeletal tissue

The limited self-healing potential of articular cartilage makes necessary the implementation of new approaches for the promotion of cartilage regeneration. The main current therapeutic methods include marrow stimulation and autologous chondrocyte implantation. However, these methods have several drawbacks such as size limitation, limited donor cell availability and donor site morbidity. For this reason, HA hydrogels are being used as vehicles of other substances capable of promoting cartilage regeneration [9, 15, 23, 24, 27, 70-73]. In one of their works, Levett et al. studied the mechanical properties of Gel-MA/HA-MA hydrogels using *in vitro* culture models of human chondrocytes from

osteoarthritis patients. Their results demonstrated that the encapsulated chondrocytes maintained their phenotype showing an adequate morphology and ECM secretion which increased the compressive modulus by up to 3-fold after 8 weeks of culture. Interestingly, these results were observed in every cell lines independently of the patient precedence [27].

Osteoarthritis (OA) is another common pathology related to musculoskeletal tissue. Patients who suffer from OA usually experience variable degrees of inflammation and degeneration of the articular cartilage, resulting in the exposure of the underlying bone, pain, and disability. Hyaluronic Acid hydrogels loaded with dexamethasone (DMS) have been employed to treat knee OA at clinical practice due to its chondroprotective and antiinflamatory effects, respectively. In their work, Zhang et al. demonstrated that the knee joint treated with HA-DMS hydrogel was wider than those treated with HA hydrogel alone. In addition, the group treated with HA-DMS formulation presented less cartilage fibrillation and less cartilage damage [74]. In our group, we are currently performing an *in vivo* study about the effect of Gel/HA hydrogels loaded with DMS or naproxen in a rabbit model knee inducing osteoarthrosis by intraarticular infiltration of collagenase. In the preliminary results of the histological study, the non-treated knees with OA presented greater evolution of OA with deep chondral fissures and greater fragmentation of the articular cartilage. As for the knees treated with OA and naproxen, as with HA, the evolution of OA was slower, with some articular cartilage repair. Those treated with DMS presented superficial chondral fissures and a cell arrangement in columns, slightly irregular. These results are encouraging in the use of Gel/HA hydrogels in the treatment of osteoarthrosis. The whole *in vivo* study will be the object of a further paper.

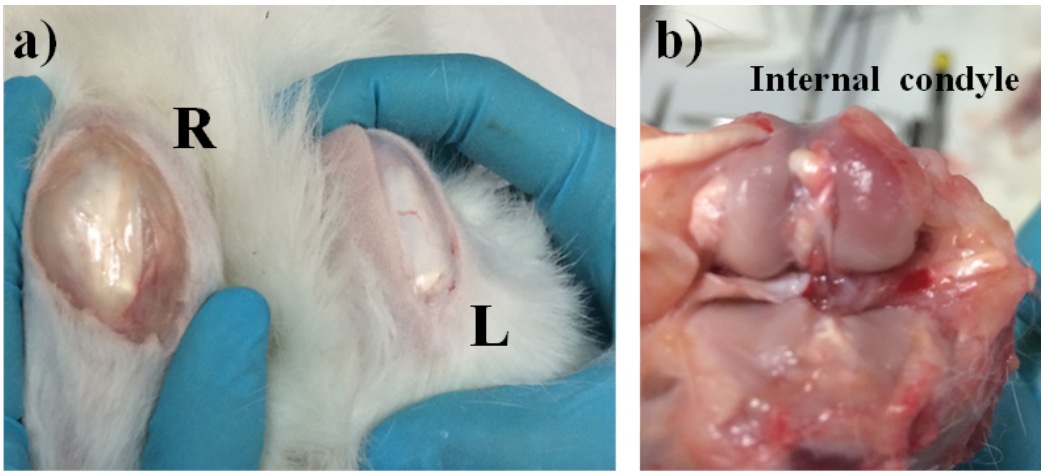


Fig 7: a) Right knee (R) with inflammation and joint effusion compared to left knee (L), b) Right knee: OA showing erythema and fibrillation in internal femoral condyle.

Actually, regarding to bone repair, the main approach to promote it still remains to be autograft bone. In the last years, several authors have proposed Gel/HA hybrid gels as a promising alternative for bone repairing and osteogenesis. One interesting study was carried out by Yeom et al. who developed a osteoconductive bone graft consisting of Gel/HA hydrogels in combination with bioactive MegaGen synthetic bone (MGSB), whose effect was assessed in the skull of rabbits demonstrating a well resorbability and a partial substitution of the lamellar bone after its implantation for 8 weeks [75]. In other study explained below carried out by Zhang et al. based on HA, Gel and hydroxyapatite nanoparticles, the matrices were studied in the calvarial defect model to examine the bone regeneration. They demonstrated that a significant increasing of newly formed bone was observed when using the three-component hydrogel, which was mainly due to the osteoinductive properties of the embebed hydroxyapatite nanoparticles [34]. Other interesting study which consisted also of a three-component system was carried out by Nguyen et al. In this case, the Gel/HA hydrogel was loaded into a biphasic calcium phosphate (BCP) ceramic. The *in vivo* study was assessed through a hole in the parietal part of the femur rabbit, which was filled with the scaffolds. Their histological and immunochemical results highly confirmed the formation of a new bone matrix and a high degree of collagen mineralization [48]. In a similar study, Son et al. took advantage of Gel/HA loaded within a BCP scaffolds with

inmobilized platelet-rich plasma (PRP), which provided mechanical and biocompatible properties to the matrices. *In vitro* studies using preosteoblast cells demonstrated that cell proliferation and survival was enhanced in the PRP-loaded scaffolds. Interestingly, *in vivo* studies assessed in a calvarial defect of rat models did not show an improvement for the new bone formation for PRP-loaded matrices, while Gel/HA/BCP scaffolds demonstrated better osteogenesis after 8 weeks [49].

3.2. Intervertebral discs

The degenerative discopathy of the spine, especially lumbar spine has a palliative treatment in most cases, being one of the main pathologies boarded from tissue bioengineering perspective. The intervertebral disc (IVD) consists of an outer part, the annulus fibrosus (AF), and an inner part, the nucleus pulposus (NP), which is a gel-like tissue containing type II collagen and GAGs that form the ECM. Fig 8 shows an schematic representation of the morphology and distribution of the main components of the intervertebral discs and the role of this important devices in the human backbone.

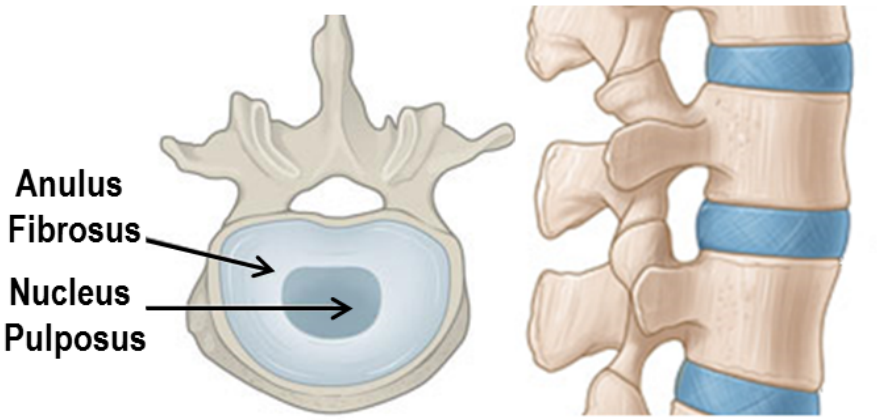


Fig 8. Schematic view of the structure and morphology of the main components of the intervertebral discs in the human body.

The degeneration of IVD remains currently as one of the main causes for the pain syndrome, and the use of Gel/HA hydrogels become a promising approach for this pathology due mainly to their similarities with ECM, adequate mechanical strength after polymerization and high biocompatibility. One example is the work of Tsaryk

et al. who developed a hybrid collagen/LMWHA loaded with gelatin microspheres as a substitute material for NP. They also encapsulated bone marrow-derived mesenchymal cells in the hydrogels and implanted them in subcutaneous pockets performed in the subscapular area of the mice. These *in vivo* experiments showed the production of GAGs and the formation of cartilage-like lacunae around the cells. In addition, they supported the growth and chondrogenic differentiation of mesenchymal stem cells [69].

3.3. Wound healing

Wound healing is a complex remodeling tissue process that needs from equilibrium between inflammatory and vascular activity between connective tissue and epithelial cells. Hybrid hydrogel matrices consisting of Gel/HA can be considered an interesting approach for challenging compromised epidermal regenerative process. As mentioned above, we developed a bilayered system based on an internal layer of Gel/HA and an external polyurethane layer responsible for permeability of the dressing. In addition, the internal layer was loaded with proadrenomedullin N-terminal 20 peptide (PAMP), an antimicrobial and proangiogenic peptide widely used for wound healing applications, and the external layer with resorbable nanoparticles of bemiparin (a fractionated low molecular weight heparin) which promotes the activation of growth factors. The *in vivo* study was carried out in the dorsal dermis of genetically modified mice expressing green fluorescent protein (GFP) and New Zealand white rabbits (male). Our results demonstrated a clear modulation of the inflammatory response for dressings that contained bemiparin and PAMP and a similar mean closure rate, which made these scaffolds suitable for epidermal regenerative processes [76].

Fig 9 shows the excellent regenerative behavior of the bilayered membranes obtained in the ear of rabbits after three weeks of implantation. The left side corresponds to the natural healing without application of the bioactive membrane (control) whereas the right side clearly shows the result obtained by the application of the bioactive membrane.

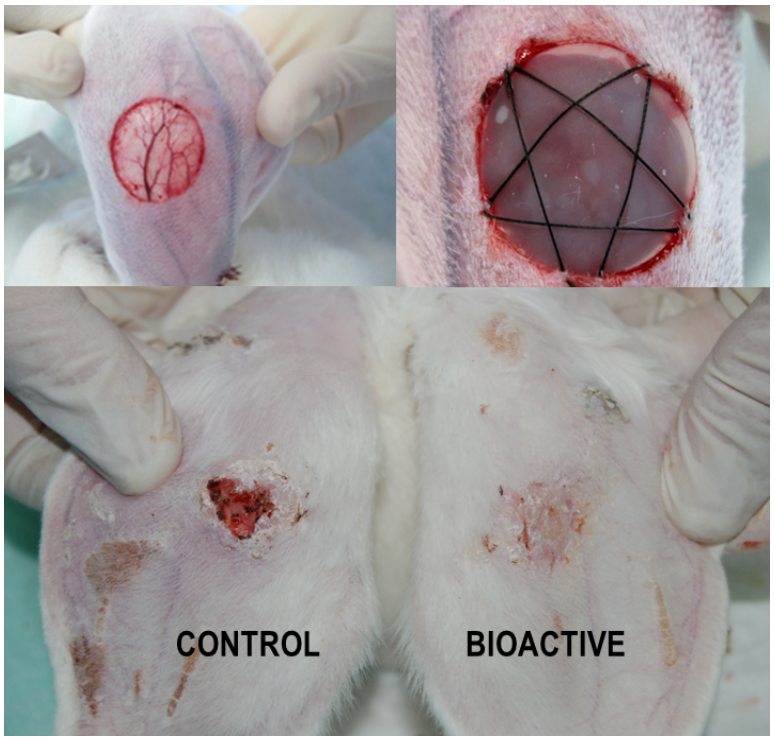


Fig 9. Microscopic photographs showing the experimental application of bioactive bilayered membranes (upper) and the healing of the compromised wound in the ear of rabbits (bottom), for the control (left) and for the system based on hyaluronic acid and gelatin loaded with bemiparin and proadenomedulin PAMP (right) after 3 weeks of operation.

Hyaluronic acid (HA) has been a natural polymer commonly used for the treatment of vocal fold scarring, due mainly to its beneficial properties such as the promotion of fibroblast migration, its role in wound healing processes and the reduction of wound contracture in later stages [77]. One example of the use of HA hydrogels for this purpose was carried out by Coppoolse et al., who investigated the local injection of a hierarchically microstructured Gel/HA hydrogel for the treatment of acute vocal fold injury using a rat model. Their results suggested that Gel/HA hydrogel was biocompatible and did not induce adverse response, reducing inflammation, and thereby promoting the repair of damaged tissue by the host tissue [78]. In other innovative study, Kazemirad et al. used Gel/HA matrix as synthetic ECM hydrogels and tested them on rabbit vocal folds that underwent biopsy bilaterally. Their results demonstrated that the viscoelastic properties of the designed scaffolds were in the order than values of those of original vocal fold tissue, being powerful candidates for the use as injectable hydrogels in vocal fold

tissue engineering [36].

3.4. Myocardial Infarction

The use of cardiosphere-derived cells (CDCs) encapsulated in Gel/HA hydrogels has been explored by Ruckdeschel et al. in order to treat patients with myocardial infarction. CDCs have been under clinical development since 2009 and they are currently being tested in a clinical trial. Combination of hydrogel and CDCs therapy has been tested in mouse models giving significant improvements so it could be a new generation therapy for this pathology [79].

3.5. Peritoneal adhesions

Post-operative peritoneal adhesions are regarded as inevitable and serious complications in surgeries such as cholecystectomy, gastrectomy, appendectomy, hysterectomy, colostomy, abdominoperineal resection, and abdominal vascular procedures. In this context, injectable Gel/MA hydrogels could provide beneficial properties for preventing peritoneal adhesions. In the study explained before carried out by Wu et al., Gel-MA/HA-MA hydrogels were used for this purpose. *In vivo* studies did not show inflammation signs and the specimens injected with the hydrogels experienced a considerably reduction in adhesion formation in comparison to the control group, being lower while the proportion of Gel-MA increased [35].

3.6. Ocular Pathology

In the study of Zarembinski et al. hydrogels of Gel/HA crosslinked with GSSG are injected intracutaneously on the back of adult rabbits showing the biocompatibility of this system for local dermal applications. They also evaluated the hydrogel behaviour in the intracutaneous area of the skin and in the subconjunctival space of the eye of the animal for two weeks resulting in a high biocompatibility and an excellent level of in-life tolerability. Thereby, these Gel/HA/GSSG hydrogels are suitable biocompatible formulations for using in dermal and ocular applications, and could be applied into other soft HA-rich tissues [67].

3.7. Oncology

Physical and chemical properties of the ECM have a strongly influence over cancer cells behavior, growth and survival. In the study of Kaemmerer et al., biomimetic Gel-MA/HA-MA hydrogels have been created with tunable physical and chemical characteristics, obtaining a suitable cancer cell platform. These spheroid-based hydrogels are used as *in vitro* and *in vivo* models for ovarian cancer to investigate the interactions between the cancer cells and the ECM-like materials. The *in vivo* study by intraperitoneal implantation in mice showed that this system was an excellent matrix for 3D cancer cultures and could help us to understand the behavior and progression of cancer cells in the nature, being a key tool in cancer research [18].

4. Final Remarks

The functionality and reactivity of polysaccharides and in particular hyaluronic acid in combination with proteins like gelatin, collagen and many others offer very interesting opportunities for the new trends in regenerative medicine. The biomimetic approaches for the application in regenerative processes and tissue engineering have enormous possibilities because of the excellent response of these biomimetic materials and their bioactive and biodegradative character in the human body. These characteristics together with the possibility of application by minimally invasive surgery offer very interesting applications in regeneration of tissues and organs, as well as in targeting and release of bioactive compounds applications as drug delivery systems. In addition, the growing clinical applications of these macromolecular components as assemblies open new and advanced opportunities in regenerative medicine and drug delivery.

Acknowledgments

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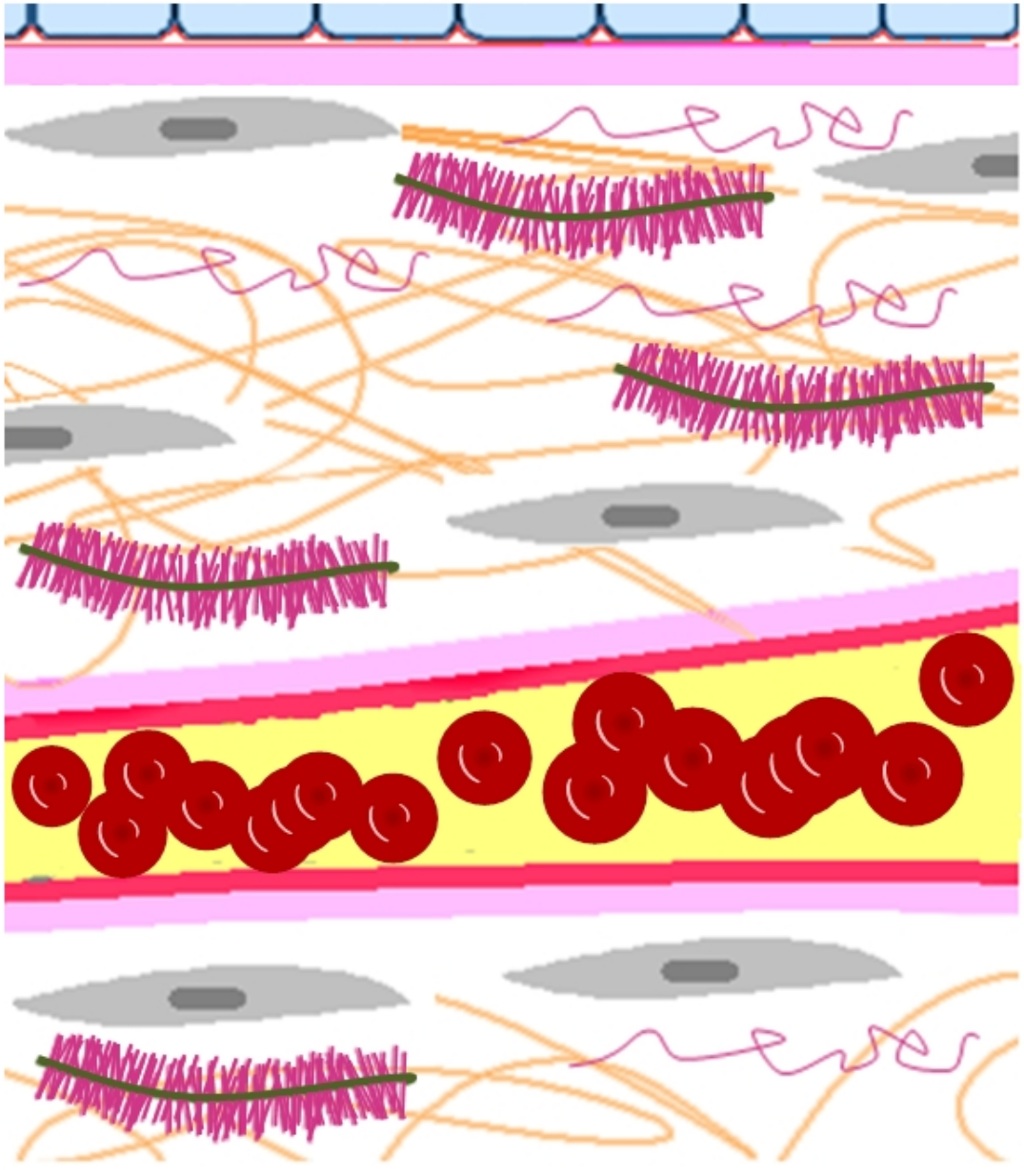
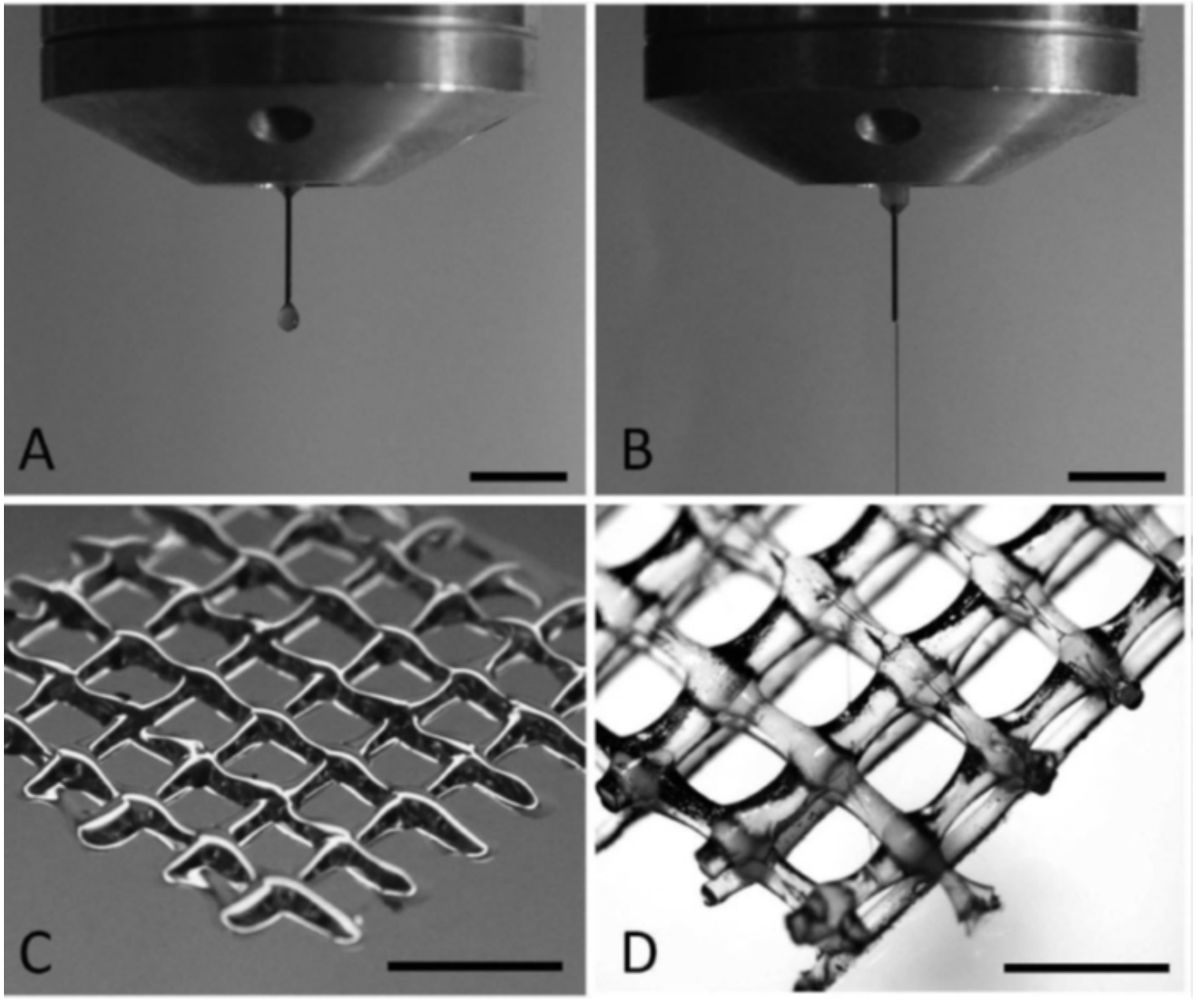


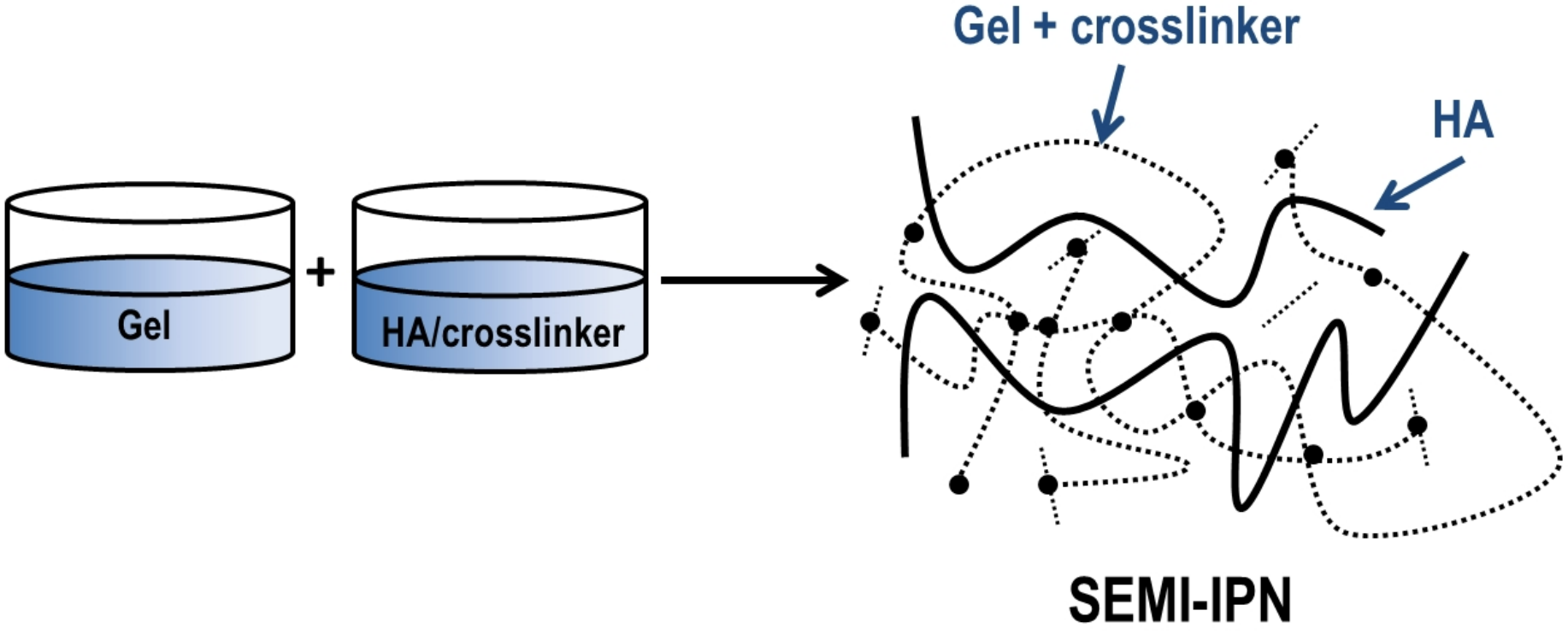
Aggrecan

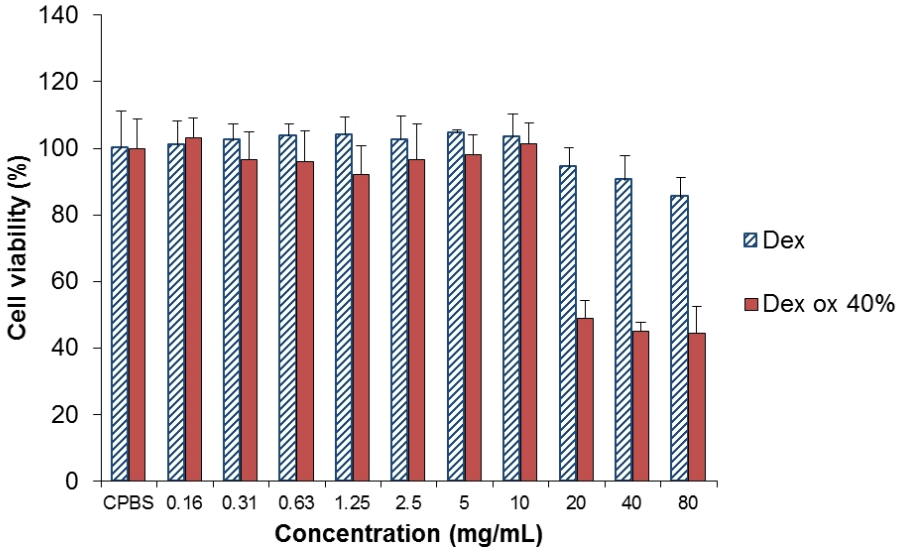
Hyaluronic acid

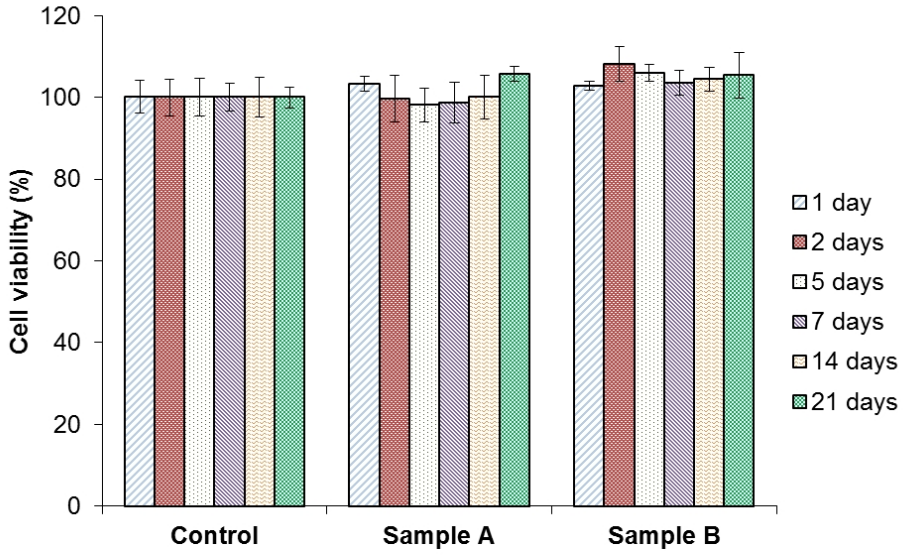
Red cell

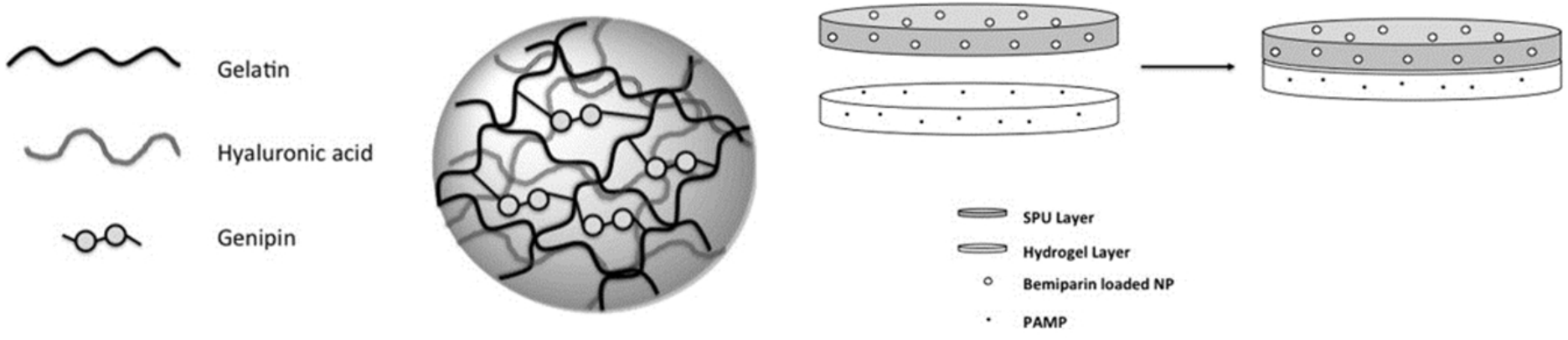
Fibroblast

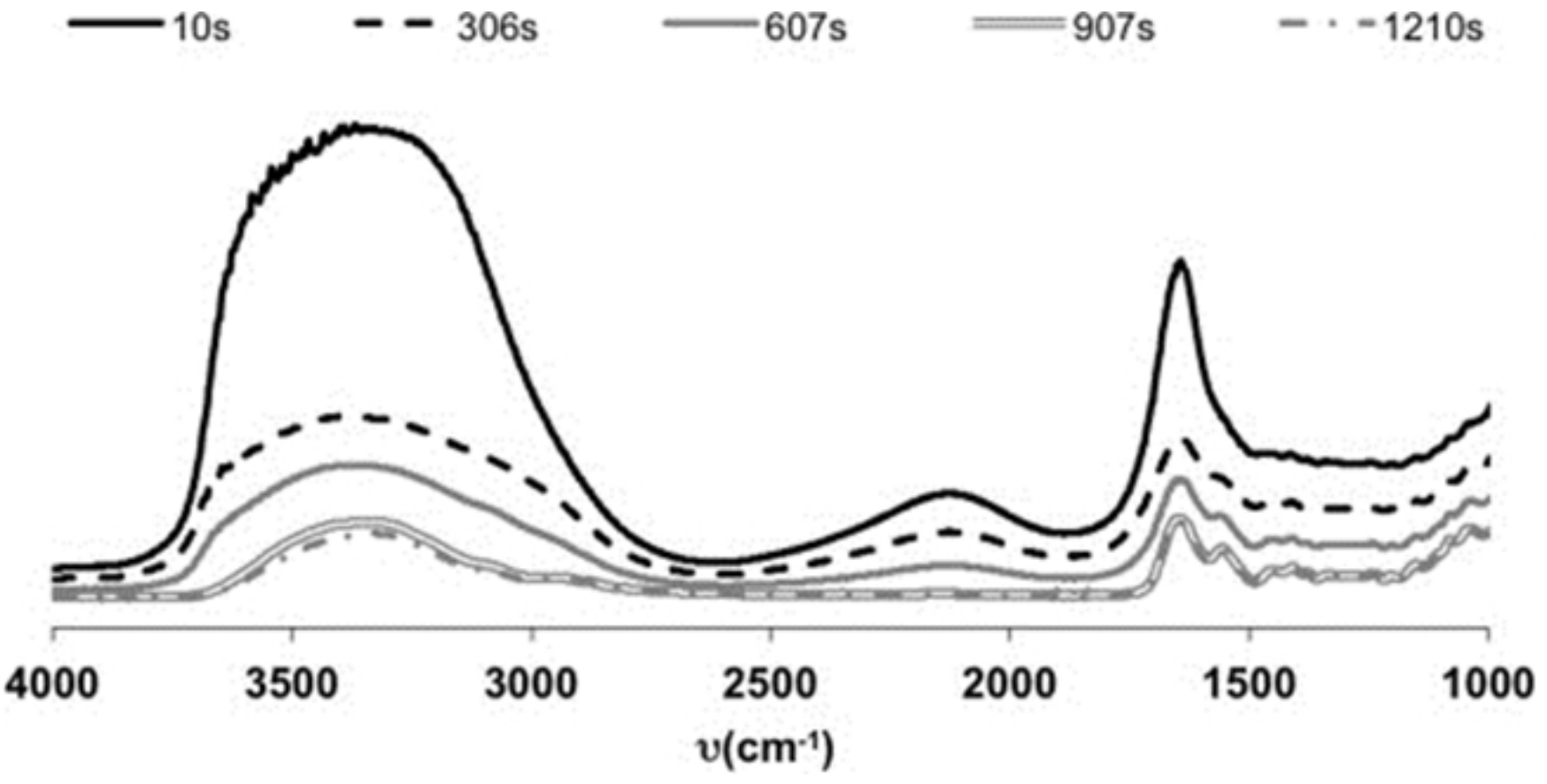

A
B
C
D


Gel + crosslinker
HA
Gel
HA/crosslinker
SEMI-IPN


Cell viability (%)
140
120
100
80
60
40
20
0
CPBS
0.16
0.31
0.63
1.25
2.5
5
10
20
40
80
Concentration (mg/mL)
Dex
Dex ox 40%


Cell viability (%)
120
100
80
60
40
20
0
Control
Sample A
Sample B
1 day
2 days
5 days
7 days
14 days
21 days


Gelatin
Hyaluronic acid
Genipin
SPU Layer
Hydrogel Layer
Bemiparin loaded NP
PAMP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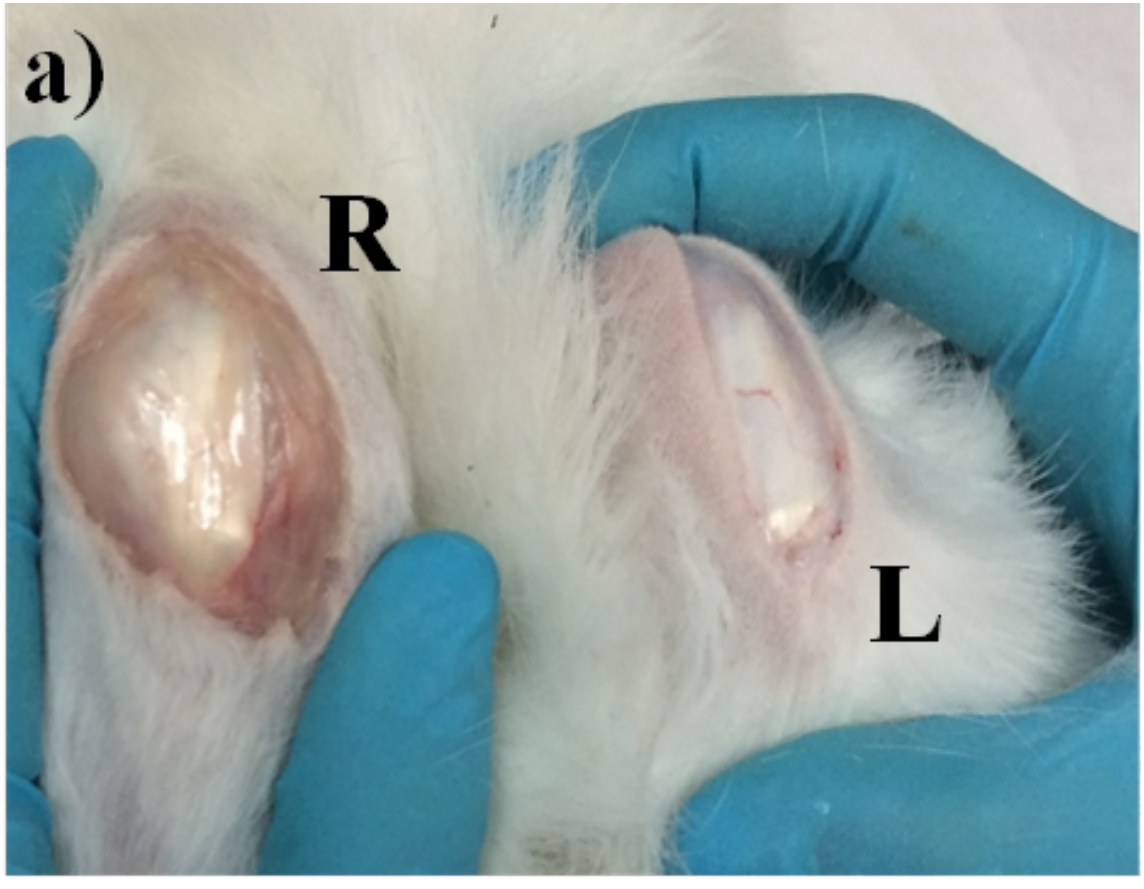
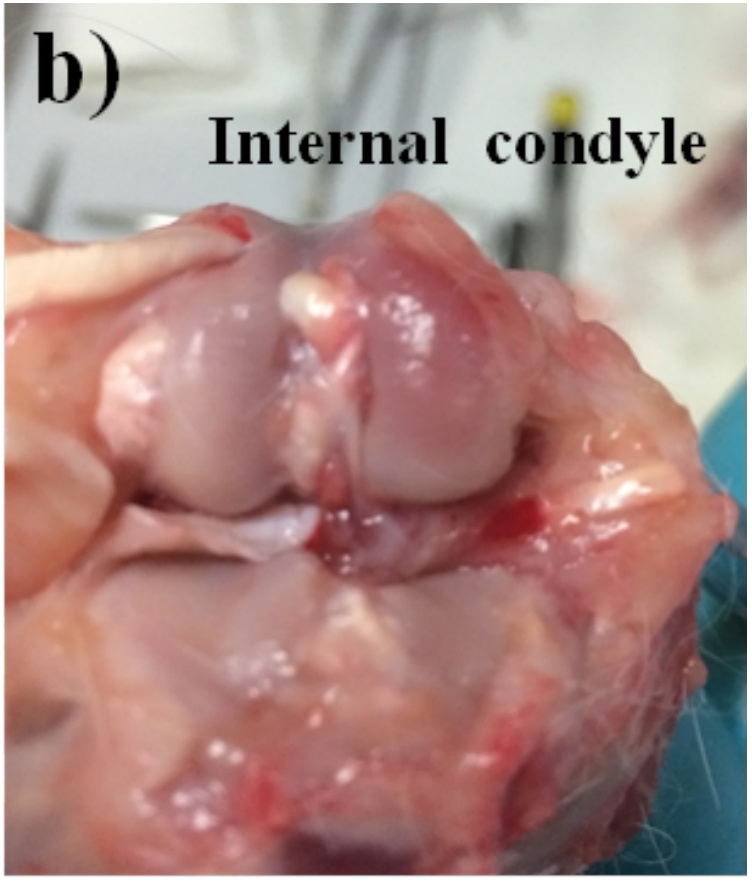
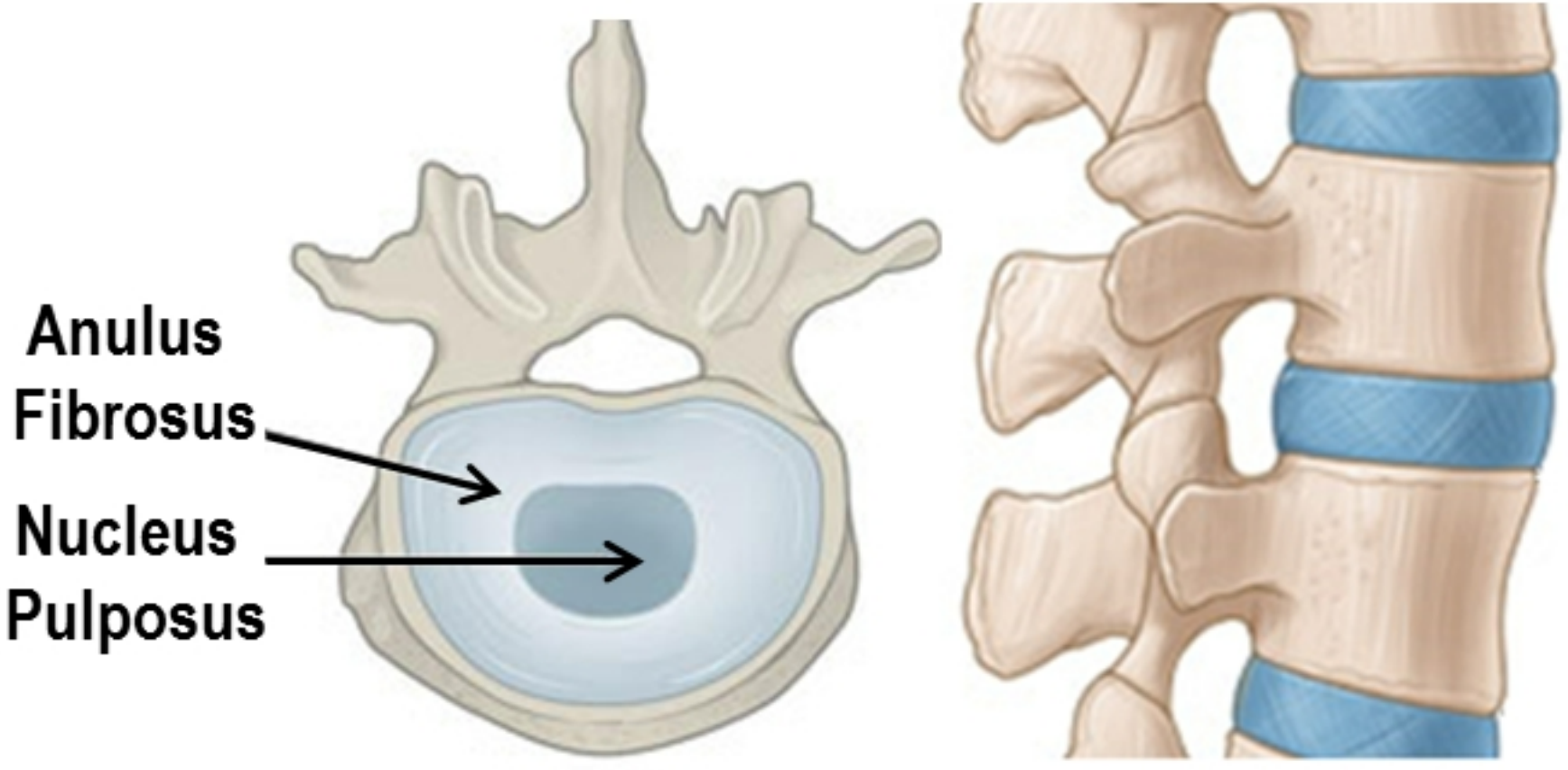
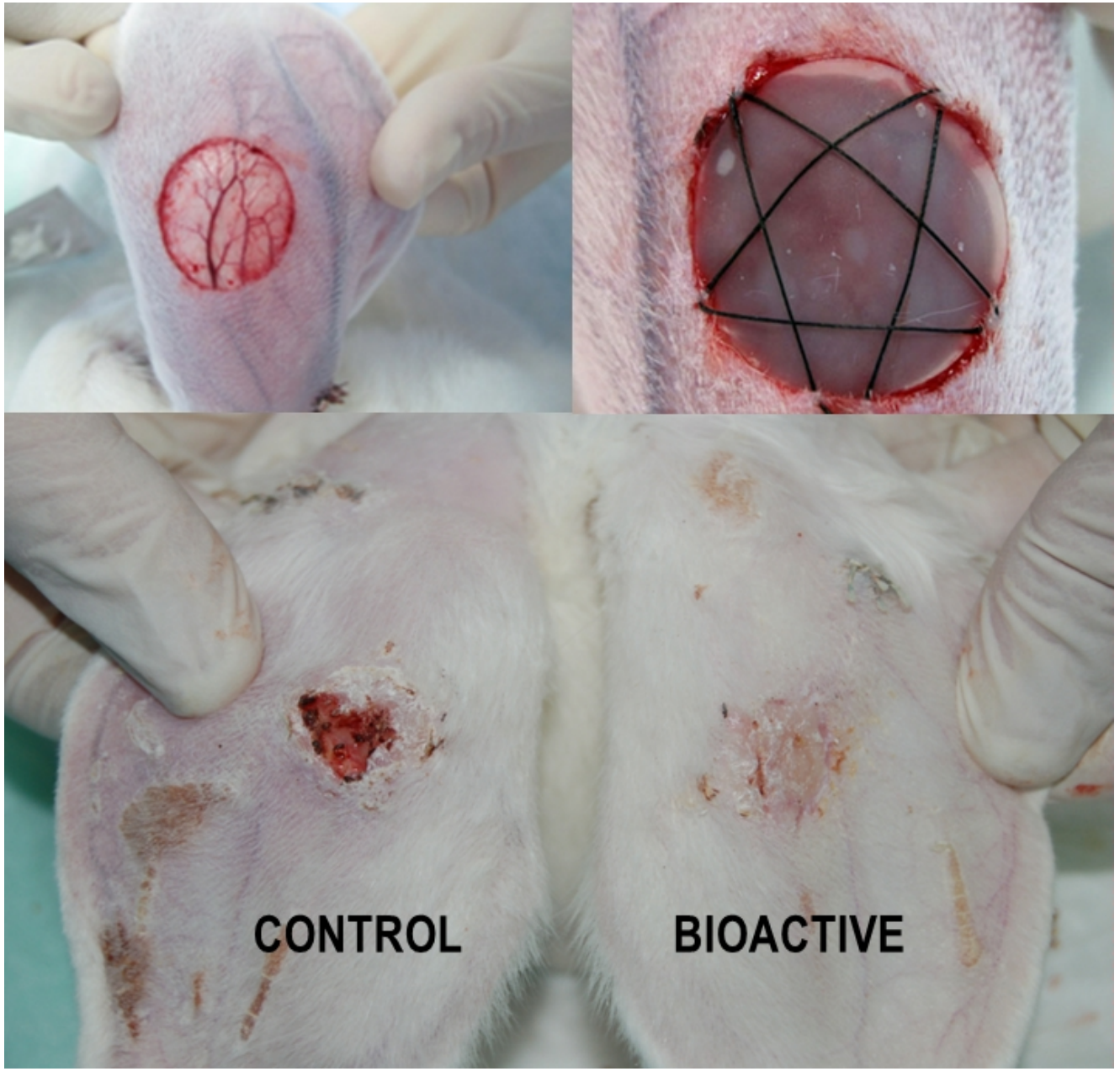
υ(cm-1)


a)
R
L


b)
Internal condyle


Anulus
Fibrosus
Nucleus
Pulposus


CONTROL
BIOACTIVE

Ana Mora-Boza is a graduate student and currently pursuing her Ph.D. in Materials Science and Engineering from Polymer Science and Technology Institute, Spanish National Research Council (CSIC), Spain. She received her degree in Biotechnology from Pablo de Olavide University of Seville in 2014 and completed master studies in Science and Technology of New Materials in Seville University in 2015. She is co-author of 3 international publications, 1 patent and 1 book chapter. Currently she is working on the area of polymeric materials for tissue regeneration. Her research areas of interests are polymeric materials for tissue regeneration, bioprinting techniques and advanced materials for biomedical applications.

María Puertas-Bartolomé is a graduate student and currently pursuing her Ph.D. in Chemistry of Synthesis, Catalysis and Advanced materials from Polymer Science and Technology Institute, Spanish National Research Council (CSIC), Spain. She received her High Specialization Master's Degree in Plastics and Rubber from the International University Menéndez Pelayo, Spain in 2015 and her Bachelor's degree in Chemistry from University of Valladolid, Spain in 2014. She has 7 publications in conference proceedings. Currently she is working on the area of bioactive and bioinspired polymeric scaffolds for tissue regeneration. Her research areas of interests are bioadhesive and thermosensitive polymers, tissue engineering scaffolds and polymeric materials for regenerative medicine.

Blanca Vázquez-Lasa, (Ph.D.University of Basque Country, Spain, 1991) is a Scientific Researcher at the Institute of Polymer Science and Technology (Madrid), Spanish National Research Council (CSIC), Spain. The main research lines in which she is involved are the preparation of injectable and bioactive systems for surgery and regenerative medicine, preparation of polymeric drugs and controlled delivery systems of bioactive principles, development of polymeric systems with antimicrobial activity and the preparation of implant devices for ophthalmological applications. She has participated in 50 National and European research projects, including networks of excellence of the EU, has contributed to the redaction of 20 chapters in scientific books, in more than 100 papers of international scientific journals in the area of Polymer Science and Biomaterials, is coauthor of 13 patents related with the orthopedics and ophthalmologic fields and has been supervisor of 7 doctoral thesis.

Julio San Román (Ph.D. Complutense University of Madrid, Spain, 1975) is a full Research Professor at the Institute of Polymer Science and Technology (Madrid), Spanish National Research Council (CSIC), Spain, and active member of the CIBER-BBN, specialized in the design, preparation and application of biofunctionalized polymers for biomedical applications, including polymeric systems for drug delivery, tissue engineering and regenerative medicine. He is associated professor of the University of the Basque Country, invited professor of

the University of Havana (Cuba) and member of the cathedra UNESCO of Biomaterials at the University of Havana. He has published more than 430 articles in journals SCI, 35 chapters of specialized books, and coeditor of 3 books. He has participated as plenary speaker in more than 250 international congresses and has 25 patents and has supervised 33 doctoral thesis. He is president of the group of polymers of the Spanish Royal Society of Chemistry and Physics, and fellow of the international societies of biomaterials, and has been president of the European Polymer Federation in the period 2010-2011, and member of the European Society of Biomaterials since 1987, being in the council of the society during the period 2010 – 2014.

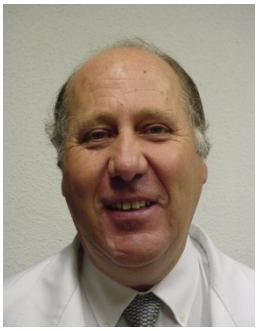
Antonio Perez-Caballer (MD, Ph.D. Complutense University of Madrid, 1993) is Head of Orthopedic Surgery Team at Ruber Hospital in Madrid and Associate Dean for International Affairs and Professor of Orthopaedic Surgery at School of Medicine, University Francisco Vitoria in Madrid. He was a former visiting fellow in Sports Medicine at Florida University (Gainesville, Fl, USA) and he achieved the National Prize of the Spanish Royal Academy of Medicine and Surgery and the Prize of Investigation of the Spanish Society of Orthopaedic Surgery. His scientific activities and investigations are focussed on the study of different ways of surgical regeneration of cartilage with the use of platelet rich plasma (PRP) and adult mesenchymal stem cells from the bone marrow and adipose tissue. Besides, he is also centered in the field of biomaterials in orhopaedic surgery. He has published more than 100 articles in specialized journals in different surgical areas. He has also contributed with more than 30 chapters in books of surgical techniques and surgical investigations in orthopaedics. He has been invited to present lectures in more than 50 national and international meetings. He is an active member of the American Academy of Orthopedic Surgeons (AAOS) and the European Federation of Orthopedics (EFORT).

Marta Olmeda-Lozano is currently pursuing her Ph.D. in Biomedical Sciences at the Francisco de Vitoria University with the collaboration of the Institute of Polymer Science and Technology (CSIC), Spain. She received her degree in Medicine from the Complutense University of Madrid in 2011 andShe is a Specialist Physician in Orthopedic Surgery and Traumatology at Infanta Elena University Hospital in Madrid in 2016. She has published a chapter in a book. Currently, her work is focused on Hand and Knee Surgery as well as collaborating with the University Francisco de Vitoria in teaching with medical students. Her research areas are mainly in Biomedicine (treatment of osteochondral lesions) and Biotechnology.

Ana Mora-Boza

María Puertas-Bartolomé

Blanca Vázquez-Lasa



Julio San Román

Antonio Perez-Caballer

Marta Olmeda-Lozano