CHAPTER x

Animal protein-based soft materials for tissue engineering applications

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Abstract

Proteins, have long been used in coatings for cell culture plates and to supplement cell culture media. Due to their unmatched biocompatibility, biodegradability, bioactivity and immune-privilege, the interest in proteins rapidly advanced for the design and engineering of more complex substrates for biomedical applications.

In this chapter, the proteins typically used in the design and fabrication of biomedical devices are presented and discussed, with particular focus in human based platforms.

Still, restrictions in the use of protein-derived materials are associated with their limited processability and stability, to overcome that, multiple bioconjugation techniques have been described and are herein presented.

An overview of current protein-based materials that have found clinical application and are in commercialization is also provided.

1. Introduction

Typically, biomedical applications involve the integration of an engineered material with a living organism. The success of the biomedical device is highly dependent on the material used, that should be noncytotoxic, nonimmunogenic and minimally proinflammatory[1,2]. Ranging from synthetic to natural polymers, countless materials have been reported for biomedical uses[3 4].

The extracellular matrix (ECM) is the natural 3D microenvironment in which cells reside. It is a dynamic and complex milieu, composed of multiple proteins, glycosaminoglycans and soluble factors. Accordingly, the ECM, its components, and more particularly, proteins are highly interesting for soft materials design and fabrication, especially when aiming for implant or close contact with cells. Human proteins are markedly appealing under the assumption that, materials made from these biomolecules would be considered as less foreign to the body and, therefore, less immunogenic[5].

This chapter gives a brief description of the most widely used and most promising proteins to engineer materials for biomedical applications.

Proteins are perhaps the most common targets for modification or conjugation techniques, this chapter reviews advances in chemical modification of proteins to enhance cross-reactivity. In designing and manufacturing 3D protein based structures, multiple techniques have been explored, here is also presented the state-of-the-art in the application of micro- and nanotechnologies for the development of soft materials with controlled micro and nano-scale features.

Once idealized and developed a new biomaterial should be affordable and commercially available with appropriate regulatory guidelines. Protein derived materials and in particular human derived are now available in the market in the form of multiple products, that are also presented and discussed in the final part of this chapter.

2. Human proteins as soft materials

2.1 Collagen and gelatin

Collagen is the most abundant ECM protein in mammalian body, constituting approximately one third of the total protein content. As a fundamental component of connective tissues, this structural glycoprotein plays a pivotal role in maintaining the biological integrity and mechanical resilience of tissues[6]. The fibrillar structure of collagen that provides great tensile strength to biomaterials combined with its low antigenicity, biodegradability and water uptake, are attractive properties for its use biomedical applications[6,7]. Although collagen has been historically extracted from animal sources, cadaveric and discarded human tissues (e.g. adipose tissue, placenta), and recombinant human collagen, offer alternate sources that overcome immunogenicity issues[8–10]. Collagen modification with crosslinking agents and ECM components has been explored to increase the mechanical performance of scaffolds, allowing the control of crosslinking conditions and degradation rate[6,11] (section 3). For example, the combination of collagen with other polymers and inorganic ECM components is recognized as one of the best strategies for bone, cartilage and tendon engineering owing the improved mechanical strength and biofunctionality[12–16]. Studies on corneal regeneration have reported the successful construction of optically transparent and mechanical suitable hydrogels, supporting the expression of specific corneal markers and hampering *in vivo* edema and immune rejection[17,18].

Gelatin, an hydrolyzed form of collagen, offers lower antigenicity and less batch-to-batch variation due to the denaturation process. Owing to its excellent biocompatibility, availability and low cost, gelatin has been extensively used in tissue engineering (TE) and pharmaceutical applications. The rapid biodegradation and low stability of gelatin scaffolds motivated researchers to develop new crosslinking mechanisms and blended composites to prepare gelatin based 3D structures that can be easily processed[19,20]. This have

expanded the application of gelatin materials for multiple applications, ranging from bone and ocular TE to disease modelling[21–23].

2.2 Blood plasma proteins

2.2.1 Serum albumin

Albumin, a non-glycosylated protein, is the major constituent of blood plasma in mammals, making serum albumin (SA) an attractive and ready available biomaterial. Its physiological stability, low immunogenicity, and interaction with exogenous substances (e.g. therapeutic agents) makes SA relevant for biomedical science[24,25].

Albumin-based hydrogel formation have commonly been induced by non-physiological pH and/or temperature conditions, which promotes albumin self-assembling through hydrophobic interactions, resulting in mechanically stable and biocompatible hydrogels[26,27]. An important consideration on the use of these methods is the denaturation and aggregation of albumin, which have implications in its interaction with cells. The chemical modification and conjugation with other materials improved the biophysical and have prompted the use of albumin-based hydrogels for biomedical purposes. Apart of that, tissue regeneration is a promising field for albumin materials application. The ability to support osteoblast mineralization, chondrocyte proliferation and angiogenesis makes albumin hydrogels good candidates for bone[28] and cartilage[29] regeneration and chronic wound healing[30]. Moreover, the hydrogel elasticity combined with the assembly of aligned albumin fibres can improve the functionality of cardiac engineered scaffolds[27,31].

2.2.2 Fibrin

Fibrin is one of the oldest blood plasma-derived proteins used in the biomedical field. Synthesized from the cleavage of fibrinogen monomers by activated thrombin, fibrin is involved in the coagulation cascade

where an insoluble fibrin network is formed[32]. The biological importance of fibrin matrix to support cell adhesion, migration, and molecular signaling, and its successful application as a sealant after surgeries over a century, has incited its use in biomedical applications[33,34].

The properties and orientation of single fibrin fibres in fibrin-based hydrogels are strongly correlated with material elastic modulus, tensile and adhesive strength and fibrinolytic stability[35,36]. However, fibrinogen/thrombin produces mechanically poor hydrogels with high degradability, accordingly, biochemical and mechanical improvements have been proposed by the incorporation of other macromolecules or inorganic components[37,38]. Inspired by its safe and benefic clinical use, fibrin hydrogels have been largely explored for wound healing[39], cartilage and bone regeneration[37,38], and to improve tissue vascularization[40]. The fibrin-mediated controlled release of growth factors (GF) is also a promising approach to enhance cell-based tissue repair[41,42]. Bone[17,43], cartilage[37,44], skeletal muscle[45], skin[42] and cardiovascular[46] engineering are some applications of fibrin-based platforms. However, the most notable application concerns the development of vascular grafts[9,47,48].

2.2.3. Platelet-Rich Plasma

Blood is rich in a high variety of proteins, GF, cytokines, chemokines and multiple bioactive molecules that are stored into circulating non-cellular reservoirs, the platelets[49]. Upon activation, platelets initiate the coagulation cascade, promoting tissue repair and revascularization[32,49]. The natural ability of activated platelets to release a pool of bioactive molecules and recruit host stem cells has been exploited for therapeutic applications as a platelet concentrate, named platelet-rich plasma (PRP)[50]. Recognized as an autologous source of GF, PRP is clinically applied to enhance the regenerative process after surgical procedures and treat chronic nonhealing wounds, musculoskeletal conditions, and peripheral neuropathies.

Owing its allogenic origin and biological activity, PRP has been widely explored for TE purposes[50]. For example, PRP activation has been used to develop PRP-based hydrogels to study their angiogenic effects[51] or potential to enhance cartilage regeneration[52]. The poor mechanical properties of the obtained hydrogels limit their application. Although PRP polymerization with other materials has already been reported[52], the incorporation or coating of hydrogels or fibres with activated PRP has been the main approach to stimulate tissue vascularization[53] and bone tissue formation[54] .

2.2.4. Platelet lysates

Platelet lysates (PL), the soluble content of PRP are a pool of proteins, GF and other bioactive molecules[50]. This cost-effective protein cocktail is a great source of signaling molecules involved in cell recruitment, morphogenesis and angiogenesis. Noteworthy, human polled PL have been successfully explored as an alternative to animal-derived serum for *in vitro* cell culture[55,56].

In the field of 3D cell culture, several studies have evidenced the benefits of using PL[57,58]. PL-derived hydrogels have been an interesting approach exploring the self-assemble ability of proteins in the presence of a coagulation factor[59]. However, their poor mechanical properties and limited stability *in vitro* and *in vivo* have stimulated PL combination with other biomaterials or protein chemical modification to develop more robust scaffolds[60]. Besides PL-based hydrogels support cell proliferation[60,61], several studies have reported their application in vascularization[62], wound healing[63], cartilage regeneration[64], periodontal ligament repair[65] and tumor modeling[66].

2.3 Elastin

Elastin is an insoluble protein that constitutes the highly crosslinked elastic fibers of the ECM by a process called elastogenesis. Owing its exceptional physical properties, resilience and structural stability,

elastin provides flexibility to connective tissues such as blood vessels, skin and lung and regulate cell signaling pathways via biomechanical transduction[67]. The suppression of elastin expression in adults and its insolubility limits the isolation process[68]. Therefore, its soluble monomeric precursor tropoelastin and other recombinant elastin-like peptides (ELPs) have contributed with remarkable advances in the engineering of several elastic tissues[69].

The biocompatibility of elastin is recognized, offering adhesion motifs and mechanically robust surfaces that support the adhesion and proliferation of diverse cell types[70,71]. The presence of hydrophobic domains in elastin-derived peptides, which confers them self-assemble ability, has been explored for the production of nanoparticles for drug and/or GF deliver[72,73]. The combination of elastin with other biomaterials, supports the proliferation of human dermal fibroblasts *in vitro* and, *in vivo*, promotes the deposition of *de novo* collagen and neoangiogenesis[72,74,75]. Other applications such as vascular graft[76] and peripheral nerve regeneration[77] have also been reported.

2.4 Keratin

Keratins are insoluble fibrous proteins rich in cysteine groups and important components of epithelial tissues and epidermal structures such as mammalian hair, feathers, nails and hooves[78]. In the biomedical field this protein has been investigated for a variety of applications: wound healing, peripheral nerve regeneration, hemostatic agents, drug delivery vehicles and cell culture systems[79,80]. In fact, the intrinsic mechanical strength of keratins, combined with the protein's biological cues, are key aspects in biomaterials development.

Keratin-derived hydrogels have gained special attention as cell, GF and drug delivery systems[81,82]. Other interesting strategy is the exploitation of keratin negative charge at physiologic pH to selectively deliver GF in a pH-mediated fashion[83]. For decades, keratin hydrogels have been used for wound healing purposes[84]. Moreover, keratin self-assembled hydrogels have demonstrated to be an effective hemostat for liver injury[85].

Peripheral nerve regeneration mediated by nerve conduit filler is another highly explored keratin application owing the neuroinductive and regenerative potential of the hydrogels, improving in vitro Schwann cell migration and in vivo restauration of electrophysiological conduction and myelin thickness[86,87].

2.5 Decellularized ECM tissues and organs

For decades, allogenic tissue grafts have been considered the "gold standard" bioscaffolds for regenerative/reconstructive purposes. However, the shortage of tissue grafts or donated organs alongside the immunogenic response remain a concern. Decellularization of native tissues and organs is recognized as the most promising approach to obtain scaffolds that fully replicate the architecture and physicochemical features of the target tissues[88]. These scaffolds, collected from surgery or cadavers, can be efficiently decellularized to remove the immunogenic cellular material and maintain the ECM components, preserving its structure, mechanical integrity and biological activity[5].

Devoid of immunogenic agents, decellularized tissues/organs can be repopulated by culturing tissue-specific cells through seeding[89,90]. Bone[91], skin[92], cornea[93], tendon[94], cartilage[95], adipose tissue[95,96], heart[95,97], lung[98], pancreas[99], kidney[100] and perinatal tissues[2,101] are some of the tissues explored for these purposes (see Figure X.2). The microenvironmental signals in the decellularized ECMs enhanced cell adhesion, proliferation, migration and differentiation toward a native-like integrated and functional tissue. Relevant preclinical and clinical success over this century has contributed to accelerate the translation of decellularized matrices from bench-to-bedside, emerging several commercially available ECM products (Section 5). Although organ repair, regeneration and augmentation are the main focus of decellularized matrices, recent studies exploring the whole-organ decellularization/recellularization of heart[102], lung[90] and liver[89] have offering new possibilities for regenerative medicine.

3. The chemical modification of proteins

In nature, protein modification represents a powerful post-translational tactic endorsing a powerful chemo diversity[103]. In an attempt to mimic those modifications, synthetic chemists have been exploring a set of reactions that can endow specific features to proteins. As a tool to shape the composition and precisely control their assembly, chemical modifications can afford sophisticated protein-based matrices giving access to advanced materials and platforms for multiple biomedical and biotech applications[104–106].

3.1 Direct and indirect chemical modification

The rich chemical repertoire present in proteins, conferred by the reactive side chains from amino acids, N- and C-terminus, or even non-protein constituents such as carbohydrates, allow their manipulation in a site-specific and chemically defined manner[105,107].

Relying on the natural chemical groups accessible on the side chains residues or N- and C-terminus, direct chemical modification embraces straightforward reactions with a wide-range of active intermediates, and potential for high yielding crosslinking[105,108]. Targeting sulfhydryl groups such as thiols, might not often be the first choice to modify proteins, owing to the inner location on the protein core. Nonetheless, thiols can easily undergo acylation with Michael acceptors, such as maleimides or alkenes, forming thioethers or alkylation forming thioesters (see Fig X.1a-i)[103].

Being faced outward in relation to the surface of proteins, due to its positive character at physiological conditions, amines are probably the most popular targets for this matter, offering well-established protocols and feasible conditions of reaction. Present as primary amines in the side chain of lysine, arginine and histidine, or in the N-terminus, they can be reacted by alkylation or acylation with electrophilic groups such as aldehydes or activated carboxylates (see Fig X.1a-ii)[108,109].

Like primary amines, carboxylate groups are usually on the surface of the protein architecture, as part of glutaric or aspartic acid side chain residues or C-terminus. Carboxylate groups are not typically used in protein modification reactions. The prerequisite to primary activate the carboxylic groups with intermediates like active-ester (like NHS) or reactive carbonyls, to produce an acylating agent, might hinder their exploitation as reactive side chain. Nevertheless, amine-bearing crosslinkers are the top choice to target these groups by using the highly standardized methodology carbodiimide/NHS coupling chemistry.

Just as importantly, tyrosine residues can also expand site-specific modification of proteins (see Fig x.1a-iii)[105]. One of the approaches uses diazodicarboxyamide-bearing crosslinkers**.** In the presence of these, the phenolic ring can undergo an ene-type click reaction, allowing the modification of phenyl side chain groups[110].

Being a valued toolbox for the design of protein bioconjugates, indirect chemical modification or bioorthogonal chemistry allows the insertion of prosthetic functional groups such as carbonyls, alkenes or alkynes, further used for judicious chemoselective couplings[111,112]. Routinely used as oxidative agent, sodium periodate ($NaIO_4$) assists on the creation of carbonyl groups from side-chain structures in serine or threonine, or carbohydrates present in the form of glycoproteins[113]. In the form of aldehydes or ketones, carbonyl groups promptly react with amine-bearing compounds forming imine bonds. The reaction between hydrazide or alkoxyamine compounds are also quite efficient with carbonyls, generating hydrazone or oxime bonds owning greater stability than imine bonds (see Fig X. 1a-iv)[114].

One of the most important advances in bioorthogonal chemistry, for protein bioconjugation, is the ability to use the well-known and highly chemoselective click chemistry. By fashioning the Cu(I)-catalyzed cycloaddition between azides and alkynes it is possible to generate the very stable triazole ring (see Fig X.1a-v)[115]. In an attempt to overcome the limitation of copper ions associated toxicity, the strain-promoted azide-alkyne cycloaddition (SPAAC) with cyclooctyne-bearing crosslinkers, have emerged as important bioorthogonal reactions for protein bioconjugation (see Fig X.1a-v)[115]. Termed as Staudinger ligation,

triphenylphosphine derivatives that contains an electrophilic group next to the phosphorus core, can also be selected to chemically modify proteins bearing azide groups, yielding stable amide bonds[116]. More recently, inverse electron demand Diels-Alder (IEDA) cycloaddition proved to be a promising route to add in the vast portfolio of chemo selective ligation chemistries[112]. With unmatchable kinetics, IEDA is based on the coupling between tetrazine and dienophiles (see Fig X.1a-vi).

3.1.2 The chemistry behind protein-based soft materials

Diverse methods have been explored for the chemical modification of proteins, part of them have found utility in the creation of functional materials. For example, proteins have been frequently conjugated with acrylate groups, a straightforward methodology proved to be valuable on the fabrication of materials with well controlled mechanical properties. The double bonded carbons in acrylates are highly reactive and promote a free radical polymerization when they are exposed to light (see Fig X. 1b-i)[60]. Acrylate groups can also participate in Michael-type addition reactions with thiols (thiol-ene coupling). For example, using a 4-arm thiol-bearing polyethylene glycol (PEG) linker, and triethanolamine as catalyst, the covalent linkage empowered the formation of robust collagen hydrogels (see Fig X. 1b-ii)[117]. By targeting amines-bearing chains, hydrogels made of ELP can be easily fabricated by the straightforward crosslinking with a NHS-bearing 4-arm PEG (see Fig X. 1b-iii)[118]. Keratin, widely recognised by their natural formation of disulfide bonds between cysteine residues, was also explored to fabricate photo-crosslinkable hydrogels in combination with norborene-PEG linkers and a photoinitiator[119].

Bioorthogonal chemistry is also being explored in the engineering of ELP hydrogels. By introducing azides, BCN or triphenylphosphine at lysine residues, protein hydrogels are fabricated via SPAAC or Staudinger ligations (see Fig X. 1b-iv and -v)[116].

Taking advantage from the natural amino acid tyrosine, present in the recombinant mussel foot protein-3 (Mfp-3), it was possible to promote crosslinking via oxidation mediated by tyrosinase (see Fig X. 1b-vi)[120]. Due to its oxidized form and giving the higher amounts of amines in the same protein, hydrogels has been successfully prepared by the formation of amide bonds. In a similar strategy, keratin was subjected to redox reactions to prepare hydrogels with tunable properties[121,122].

Protein conjugation will continue to move towards site-selective and reactions that preserve protein bioactivity. While multiple chemistries and crosslinking agents are available, the target side chain and type of modification must be firstly envisioned and tailored to design hydrogels with a specific biological function[4].

4. Techniques for engineering protein-based 3D soft materials

A major concern in TE and regenerative medicine areas is the development of novel functional approaches to better mimic the natural hierarchical structure of tissues[123]. *In vivo* tissues are very well organized structures that consist of cells and a surrounding ECM that gives support for cells growth and proliferation. In this context, multiple manufacturing techniques that allow for the precisely combination and 3D patterning of cells and biomaterials have been proposed to engineer tissues organized like native tissues[124].

4.1 Electrospinning

Electrospinning is a popular and cost-effective technology to produce nanofiber scaffolds for multiple biomedical applications[125]. This technique allows control over the fiber diameter and orientation of the fibers in a mesh, providing adequate tools to mimic ECM components, thus holding great potential for the fabrication of fibrous scaffolds for biomedical applications[126,127].

Electrospun fibers composed only of ECM are normally fragile, needing another polymer to enhance the mechanical properties. Poly-L-lactic acid (PLLA) or Polycaprolactone (PCL) have been used in combination with ECM proteins to produce hybrid nanofibers, reinforcing their structure[128,129], resulting in suitable structures for TE applications.

Particularly attractive for biomedical applications, the use of electrospinning with solely proteins is also a reality. Many researchers have been developing new techniques to optimize the process and prepare robust fibrillar scaffolds made of proteins such as albumin[26,127], collagen[130], fibrinogen[131,132], elastin[75], gelatin[19] and silk fibroin[133,134]. Dror et al. reported for the first time albumin electrospinning nanofibers with tunable mechanical properties. This protein has limited capacity to be electrospunned, nevertheless by using denatured protein solutions, the authors have afforded nanofibers made entirely of albumin[26]. In an effort to mimic the ECM of native tissues, Dems et al. have proposed an electrospunned membrane of native collagen as a support for cell culture[130]. They were pioneers in the preparation of pure collagen nanofibers by using an innovative aqueous acidic/alcoholic solvent mixture. Bowlin et al. have been used electrospinning to prepare highly porous fibrinogen scaffolds with fiber diameters as small as 80 nm for tissue engineering applications and wound dressing[131,132].

Collagen-based scaffolds currently dominate the dermal TE, but are restricted by their low elasticity and scaffold contraction during tissue repair, the blending of collagen and elastin allows for an extra control over the physical and mechanical properties of collagen based fibrillar soft materials, and have been explored for dermal applications[75].

4.2 Microfluidics

Microfluidics manipulates small amounts of liquids in channeled chips[135]. One of the advantages of microfluidic systems in the production of particles/fibers is the effective control over the shape, size and composition of the resulting structures[61,97].

Proteins like collagen[8,136,137], gelatin[20,138], albumin[135], decellularized ECM[97] and fibrin[139] have been explored to produce soft materials by microfluidics.

Matsunaga et al. developed a microfluidic system to build millimeter scale tissues via molding cell-laden collagen droplets[137]. The platform was used for the rapid preparation of a large number of cell beads that assembly to form millimeter-thick macroscopic tissues. GelMA, synthesized from the methacryloyl modification of gelatin, has widely been used as a soft material for biomedical applications. Sheikhi et al., designed physically crosslinked microgels from GelMA that were subsequently annealed through photo-crosslinking to fabricate bead-based 3D scaffolds with high mechanical resilience[20].

With the principle of ejecting microfibers through capillaries using microfluidics, Takeuchi et al. developed an weaving device with double-coaxial laminar flow to build cell fiber constructs[8]. They were able to produce highly organized 3D macroscopic cellular constructs embedded in the ECM derived proteins collagen and fibrin. Lee et al. reported a strategy for the production of tissue-specific microbeads based on a microfluidic system, they were able to produce microbeads from several decellularized ECM providing tissue-specific environments with optimal biomechanical cues for specific tissue types [97].

4.3 Photolithography

Photolithography has been widely used to create micropatterned protein based soft materials. In this technique the material is exposed to light by using a photomask that enables the production of a precise patterning[140,141]. Besides the disadvantage of the high costs associated with designing and fabricating the

photomasks, photolithography has several advantages as for example the fast production of micro/nanostructures as well as the ease of applying the technique to photocrosslinkable materials[60,141].

Several polymers have been explored in photolithography, both synthetic and natural. In particular, proteins like gelatin[142,21], platelet lysates[60], keratin[143] and silk fibroin[144] have been explored given the ease of combining proteins with photoresponsive moieties as well as their great biological properties. Santos et al.[60] reported for the first time, the formation of 3D microstructures with different shapes and sizes based on human methacrylated platelet lysates[60]. Following the same idea, Ha et al.[142] produced GelMA micropatterned structures. They demonstrated that hydrogel micropatternings guided the self-alignment of stem cells as well as promoted their odontogenic differentiation.

4.4 Bioprinting

3D bioprinting is one of the most recent and promising techniques used to produce 3D scaffolds with high precision and resolution[100,145]. In 3D bioprinting, the bioink is one of the key components of the process and it should have some intrinsic characteristics like printability, structural integrity and biocompatibility. Natural-based materials have been largely explored due to the intrinsic cues that promote cell attachment and growth[50,146–148]. Protein based bioinks made of gelatin[22,149,23,150], fibrin[46,151], collagen[152], silk fibroin[145,153], platelet rich plasma (PRP)[51] and decellularized ECM[100,154] have been proposed. however their printability is harder when compared to synthetic materials due to the lack in mechanical and stability properties. Therefore, alternatives have been proposed such as the combination with other polymers[46], by using sacrificial hydrogels[145] or by using supporting bioinks based on synthetic polymers[150] to improve the printing process.

With increasing importance of personalized medicine, specific biological factors from an autologous source or patient derived are becoming important components of bioinks. Faramarzi et al., reported the

incorporation of human PRP into an alginate bioink to promote a gradual release of proteins and GF that positively affect the function of stem and endothelial cells[51].

Taking advantage of the great features of decellularized ECMs, Ali et al.[100] developed a photocrosslinkable kidney ECM-derived bioink that could better mimic the kidney microenvironment.

As stated here, several techniques have been explored in order to find new approaches for tissue engineering platforms development. Besides all the advantages and disadvantages, the decision about what technique has to be used for a desired end is the most important step when the aim is to produce a platform that can help to understand and recreate what happens *in vivo*.

5. The market of protein-based soft materials

The global market of biomaterials has expanded rapidly in recent years[155]. Although over the last decades biomedical companies have produced and marketed mainly ceramic and metallic devices intended for the replacement of hard tissues like bone and teeth, this industry is now more focused on the development of soft materials that stimulate the self-healing ability of patient's tissues[156]. Natural biopolymers have attracted considerable attention as they are inherently biocompatible, biodegradable, and bioactivity[3]. This is especially true in the case of scaffolds produced from animal or human proteins, two classes of materials that have gained clinical importance and consequently market space in the last years.

One popular approach of companies using proteins as raw materials is the development of 3D substrates to support the culture of cells *in vitro* (see Table 1). Currently, the time and investment required to develop new drugs are a major concern and one of the main causes is the lack of physiological relevant platforms to perform the pre-clinical tests. It is therefore paramount that innovative solutions reach the market as soon as possible. One of these solutions may be the use of tissue-engineered products which aim to replicate most of the functions of real tissues. Matrigel, for example, is a hydrogel produced from basement membrane extracted

from murine Engelbreth-Holm-Swarm (EHS) tumors that has been used to grow a large variety of cells with some degree of predictability[157]. Although this is a quite more realistic environment when compared to other commonly used substrates, such as the single protein-based solutions made of collagen or laminin, it continues to lack critical features of the human physiology. In alternative, two main approaches have been commercially explored in order to increase the accuracy of *in vitro* models: the first one is the coupling of protein-based scaffolds with sophisticated technologies such as 3D printing[152] or microfluidic chips[158], and the second one the use of tissue-specific extracellular matrices[98].

3D printing leveraged the market of bioinks that gained great popularity and potential for further growth[147]. CELLINK is a key company acting in the field of 3D bioprinting, and the first to offer a universal bioink designed to optimize bioprinting of human tissues made of non-animal proteins. Today, it offers a wide variety of bioinks including collagen and gelatin-based materials, and also methacrylated versions of these products. Although this was a pioneering company, competition has increased rapidly with the emergence of companies such as Allevi and ROKIT Healthcare. Simultaneously, a novel market has been arising from the commercialization of ready-to-use bioprinted skin models. For example, BioDan and Poietis developed human skin models by printing different skin layers composed of distinct cell types and protein matrices (collagen, fibrin)[159,160].

Whereas 3D bioprinting has been mainly used to produce stratified skin models, microchip technologies have been idealized mostly for disease modelling and drug toxicity assays of specific target tissues. For instance, Mimetas developed and sells a microfluidic 3D tissue culture plate (OrganoPlate®) composed of different compartments that allow to create biomimetic environments and produce models of specific tissues (liver, gut, blood vessel) and conditions (angiogenesis, pancreatic and breast cancer)[158,161]. In 2014, TARA Biosystems, Inc. was founded offering "heart-on-a-chip" tissue models derived from the patients' own cells for drug discovery and risk assessment applications[162].

Regarding the use of tissue-specific ECMs and decellularized matrices, companies such as Tissuelabs and Xylyx bio have been on the leading edge of the technology [163,164]. Of note, Xylyx bio has recently announced a strategic partnership with Allevi to create liver-specific bioinks for 3D printing of more reliable cell culture substrates towards a better understanding of disease and development of more effective drugs and treatments. Furthermore, it is also believed that this kind of models can be used in future to decrease the number of animals used during *in vivo* testing.

Table 1 List of commercially available protein-based cell culture substrates.

Product	Description	Company
GrowDex	Cellulose (birch)	UPM Biomedicals
JellaGel	Collagen (jellyfish)	Jellagen
Silk Fibroin	Silk fibroin (bombyx mori silkworm)	Advanced BioMatrix
Collagen	Collagen	Corning
ECM Gels	EHS mouse sarcoma BME	Sigma-Aldrich
Matrigel	EHS mouse sarcoma BME	Corning
Cultrex BME	EHS mouse sarcoma BME	Travigen
Geltrex	EHS mouse sarcoma BME	ThermoFisher Gibco
RAFT	Type I Collagen	Lonza
PhotoCol	Methacrylated collagen	Advanced BioMatrix
PhotoGel	Methacrylated gelatin	
PhotoHA	Methacrylated HA	
LunaGel	Photocrosslinkable gelatin	Gelomics Pty
AlphaBioGel	Cell-derived ECM	Alphabioregen

MaxGel	Cell-derived BME	Sigma-Aldrich
MatriXpec	Tissue-specific ECM	TissueLabs
MatriXpec	Photocrosslinkable ECM	
TissueSpec	Human Tissue-derived ECM	Xylyx Bio
Engitix platform	Human Tissue-derived ECM	Engitix
MucilAir	Airway Epithelia model	Epithelix
SmallAir	Small Airway Epithelia model	

Creating solid organs at the laboratory that fulfil all the requirements for clinical use is a quite challenging and complex process, breakthrough solutions have been released onto the market. In fact, since Organogenesis and Integra LifeSciences launched the first FDA approved bio-engineered skin grafts, several companies committed to this type of products[165]. For example, MedSkin Solutions and Symatese are two companies commercializing collagen-based scaffolds for dermal TE. Another successful example is that of the spin-off company CUTISS, which develops personalized, permanent skin grafts to treat skin defects, such as burn injuries, using its own proprietary technology[166,167]. Although skin regeneration has been the focus of most of the companies working on the field, other tissues have also benefit from these technologies. CelGro™, for example, is a collagen-based scaffold marketed by ORTHOCELL LTD which has been used to repair a variety of tissues including nerve, tendon and bone[168].

Collagen-based biomaterials have definitely been a big bet on the TE market. This approach is aligned with the basic premise that complex bioengineered tissues can be produced by combining different elements of the natural ECM. However, the reality is that, to date, no product has been designed using this methodology that incorporates all the mechanical and biochemical cues required by the biomedical industry. The difficulties in creating tissue constructs from scratch hence redirected research towards top-down approaches as a way to

harness the native properties of tissues. As a result, decellularized matrices became a popular source of protein-based scaffolds[88].The two main sources of decellularized matrices continue to be the small intestinal submucosa (SIS) and the urinary bladder matrix (UBM) from porcine tissues. For instance, whereas Cook Biotech commercializes SIS sheets (Biodesign®) as a plastic surgery matrix for soft tissue reinforcement[169], Aziyo biologics engineers' vascular grafts (VasCure™) for the repair or reconstruction of the peripheral vasculature[170]. ACell, inc commercializes products based on UBM for wound management and surgical soft tissue repair[171].

Although most of the currently available solutions are derived from animal sources, human tissues and proteins have also been used as starting materials in the production of scaffolds foreseen to have superior biological performance and clinical value[5,172]. However, it has not always been easy for researchers to find the adequate tissue sources and processing techniques. Fortunately, recent progress in biomaterials science and ECM biology have accelerated the development of human-based scaffolds bringing exciting technologies to the field.

Up-to-date a major focus of companies working on the development of human protein-based soft materials has been the design of functional scaffolds for repair of the skin, ocular surface and musculoskeletal tissue. Placental tissues have been considered "gold-standards" owing their natural regenerative potential and immune privilege character, but also due to their availability, cost-effectiveness and ethical acceptance[2]. Several companies are therefore dedicated to the research and marketing of these products, including MiMedx, Bio-Tissue and Surgenex. Even though placental tissues are definitely the most actively explored, tissues like dermis (Alloderm®[173], Graftjacket™[174]) are also under use.

In alternative to decellularized tissues, ECM derived from the patient's own cells have also been found to be a commercially viable and potent source of humanized and personalized scaffolds[175]. For instance, Organogenesis Inc. developed and sells a clinical approved fibroblast-sourced 3D human dermal substitute

for the treatment of diabetic ulcers[176]. Other companies, such as Lineage Cell Therapeutics, Inc. and Cellf BIO, are exploring the implementation of this technique in the production of bioengineered retinas[177] and sphincters[178], respectively.

Many companies have focused their interest on protein based materials taking into account the promising pre-clinical and clinical results. However, moving basic findings out of the lab and into the clinic has not been an easy task. While numerous articles are published and patents filed every year, few commercial products are effectively launched to the marketplace, mostly due to the, commercial and regulation hurdles. Nevertheless, in the coming years, it is very likely that the number of people treated with these materials will increase significantly, also boosting the development of more sophisticated, efficient and accessible products.

6. Conclusions

Among natural polymers, proteins are probably the most promising candidates in multiple biomedical applications due to their excellent biodegradability, biocompatibility and immunocompatibility. As evidenced, herein, protein-based soft materials have the potential to regenerate a wide range of tissues by promoting cell proliferation and tissue regrowth, to be applied in drug delivery systems or in the engineering of disease models. Noteworthy, the biochemical composition of proteins provides them with versatile physical, chemical, and biological properties and recent advances in the chemical modification of biomolecules offer tools to produce materials with unique mechanical and biological properties, overcoming the limited processability and crosslinking mechanisms of native proteins. The scientific community is particularly interested in exploring human proteins derived from blood and ECM and multiple technologies have been applied for the production of customized or patient-specific materials. Exciting advancements are therefore expected for the biomedical and pharmaceutical industries where humanized and personalized technologies can significantly impact the time to the market of novel therapies.

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1 D. F. Williams, *Front. Bioeng. Biotechnol.*, 2019, **7**, 255.
2 I. A. Deus, J. F. Mano and C. A. Custódio, *Acta Biomater.*, 2020, **110**, 1.
3 J. F. Mano, G. A. Silva, H. S. Azevedo, P. B. Malafaya, R. A. Sousa, S. S. Silva, L. F. Boesel, J. M. Oliveira, T. C. Santos, A. P. Marques, N. M. Neves and R. L. Reis, *J. R. Soc. Interface*, 2007, **4**, 999.
4 S. A. Fisher, A. E. G. Baker and M. S. Shoichet, *J. Am. Chem. Soc.*, 2017, **139**, 7416.
5 F.-M. Chen and X. Liu, *Prog. Polym. Sci.*, 2016, **53**, 86.
6 A. Sorushanova, L. M. Delgado, Z. Wu, N. Shologu, A. Kshirsagar, R. Raghunath, A. M. Mullen, Y. Bayon, A. Pandit, M. Raghunath and D. I. Zeugolis, *Adv. Mater.*, 2019, **31**, 1801651.
7 E. E. Antoine, P. P. Vlachos and M. N. Rylander, *Tissue Eng. Part B Rev.*, 2014, **20**, 683.
8 H. Onoe, T. Okitsu, A. Itou, M. Kato-negishi, R. Gojo, D. Kiriya, K. Sato, S. Miura, S. Iwanaga, K. Kuribayashi-shigetomi, Y. T. Matsunaga, Y. Shimoyama and S. Takeuchi, *Nat. Mater.*, 2013, **12**, 584.
9 B. Patel, Z. Xu, C. B. Pinnock, L. S. Kabbani and M. T. Lam, *Sci. Rep.*, 2018, **8**, 3294.
10 E. Davison-Kotler, W. S. Marshall and E. García-Gareta, *Bioengineering*, 2019, **6**, 56.
11 A. Yahyouche, X. Zhidao, J. T. Czernuszka and A. J. P. Clover, *Acta Biomater.*, 2011, **7**, 278.
12 J. L. Chen, Z. Yin, W. L. Shen, X. Chen, B. C. Heng, X. H. Zou and H. W. Ouyang, *Biomaterials*, 2010, **31**, 9438.
13 Z. Xia, M. M. Villa and M. Wei, *J. Mater. Chem. B*, 2014, **2**, 1998.
14 P. A. Parmar, J.-P. St-Pierre, L. W. Chow, C. D. Spicer, V. Stoichevska, Y. Y. Peng, J. A. Werkmeister, J. A. M. Ramshaw and M. M. Stevens, *Acta Biomater.*, 2017, **51**, 75.
15 C. F. Monteiro, C. A. Custódio and J. F. Mano, *Adv. Ther.*, 2018, **2**, 1800108.
16 L. Zheng, S. Liu, X. Cheng, Z. Qin, Z. Lu, K. Zhang and J. Zhao, *Adv. Sci.*, 2019, **6**, 1900099.
17 C. M. Murphy, A. Schindeler, J. P. Gleeson, N. Y. C. Yu, L. C. Cantrill, K. Mikulec, L. Peacock, F. J. O'Brien and D. G. Little, *Acta Biomater.*, 2014, **10**, 2250.
18 C. Mölzer, S. P. Shankar, M. Griffith, M. M. Islam, J. V. Forrester and L. Kuffová, *J. Tissue Eng. Regen. Med.*, 2019, **13**, 1528.
19 H. A. Alam, A. D. Dalgic, A. Tezcaner, C. Ozen and D. Keskin, *Int. J. Polym. Mater. Polym. Biomater.*, 2019, **69**, 339.
20 A. Sheikhi, J. de Rutte, R. Haghniaz, O. Akouissi, A. Sohrabi, D. Di Carlo and A. Khademhosseini, *Biomaterials*, 2018, **192**, 560.
21 N. Peela, F. S. Sam, W. Christenson, D. Truong, A. W. Watson, G. Mouneimne, R. Ros and M. Nikkhah, *Biomaterials*, 2016, **81**, 72.

22 D. Chimene, L. Miller, L. M. Cross, M. K. Jaiswal, I. Singh and A. K. Gaharwar, *ACS Appl. Mater. Interfaces*, 2020, **12**, 15976.
23 C. K. Bektas and V. Hasirci, *Biomater. Sci.*, 2020, **8**, 438.
24 D. Sleep, J. Cameron and L. R. Evans, *Biochim. Biophys. Acta BBA - Gen. Subj.*, 2013, **1830**, 5526.
25 J. Ong, J. Zhao, A. W. Justin and A. E. Markaki, *Biotechnol. Bioeng.*, 2019, **116**, 3457.
26 Y. Dror, T. Ziv, V. Makarov, H. Wolf, A. Admon and E. Zussman, *Biomacromolecules*, 2008, **9**, 2749.
27 N. Amdursky, M. M. Mazo, M. R. Thomas, E. J. Humphrey, J. L. Puetzer, J.-P. St-Pierre, S. C. Skaalure, R. M. Richardson, C. M. Terracciano and M. M. Stevens, *J. Mater. Chem. B*, 2018, **6**, 5604.
28 L. Gallego, L. Junquera, Á. Meana, M. Álvarez-Viejo and M. Fresno, *J. Biomater. Appl.*, 2010, **25**, 367.
29 B. Scholz, C. Kinzelmann, K. Benz, J. Mollenhauer, H. Wurst and B. Schlosshauer, *Eur. Cell. Mater.*, 2010, **20**, 24.
30 Y. Zhou, L. Gao, J. Peng, M. Xing, Y. Han, X. Wang, Y. Xu and J. Chang, *Adv. Healthc. Mater.*, 2018, **7**, 1800144.
31 S. Fleischer, A. Shapira, O. Regev, N. Nseir, E. Zussman and T. Dvir, *Biotechnol. Bioeng.*, 2014, **111**, 1246.
32 J. W. Weisel and R. I. Litvinov, *Blood*, 2013, **121**, 1712.
33 A. C. Brown and T. H. Barker, *Acta Biomater.*, 2014, **10**, 1502.
34 T. Fattahi, M. Mohan and G. T. Caldwell, *J. Oral Maxillofac. Surg.*, 2004, **62**, 218.
35 W. Li, J. Sigley, M. Pieters, C. C. Helms, C. Nagaswami, J. W. Weisel and M. Guthold, *Biophys. J.*, 2016, **110**, 1400.
36 R. I. Litvinov and J. W. Weisel, *Matrix Biol.*, 2017, **60**–**61**, 110.
37 T. N. Snyder, K. Madhavan, M. Intrator, R. C. Dregalla and D. Park, *J. Biol. Eng.*, 2014, **8**, 10.
38 G. K. Levy, J. Ong, M. A. Birch, A. W. Justin and A. E. Markaki, *Polymers*, 2019, **11**, 1743.
39 D. G. Harkin, R. A. Dawson and Z. Upton, *Wound Repair Regen.*, 2006, **14**, 354.
40 L. M. Ricles, P.-L. Hsieh, N. Dana, V. Rybalko, C. Kraynak, R. P. Farrar and L. J. Suggs, *Biomaterials*, 2016, **102**, 9.
41 A. A. Ucuzian, L. P. Brewster, A. T. East, Y. Pang, A. A. Gassman and H. P. Greisler, *J. Biomed. Mater. Res. A*, 2010, **94**, 988.
42 I. Muhamed, E. P. Sproul, F. S. Ligler and A. C. Brown, *ACS Appl. Mater. Interfaces*, 2019, **11**, 3771.
43 X. Yuan, R. J. Smith, H. Guan, C. N. Ionita, P. Khobragade, R. Dziak, Z. Liu, M. Pang, C. Wang, G. Guan, S. Andreadis and S. Yang, *Tissue Eng. Part A*, 2016, **22**, 928.
44 J. S. Park, H. N. Yang, D. G. Woo, S. Y. Jeon and K.-H. Park, *Biomaterials*, 2011, **32**, 1495.
45 M. Marcinczyk, H. Elmashhady, M. Talovic, A. Dunn, F. Bugis and K. Garg, *Biomaterials*, 2017, **141**, 233.
46 S. A. Kumar, M. Alonzo, S. C. Allen, L. Abelseth, V. Thakur, J. Akimoto, Y. Ito, S. M. Willerth, L. Suggs, M. Chattopadhyay and B. Joddar, *ACS Biomater. Sci. Eng.*, 2019, **5**, 4551.
47 T. Aper, M. Wilhelmi, C. Gebhardt, K. Hoeffler, N. Benecke, A. Hilfiker and A. Haverich, *Acta Biomater.*, 2016, **29**, 21.
48 C. B. Pinnock, E. M. Meier, N. N. Joshi, B. Wu and M. T. Lam, *Methods*, 2016, **99**, 20.
49 E. Anitua, I. Andia, B. Ardanza, P. Nurden and A. Nurden, *Thromb. Haemost.*, 2004, **91**, 4.
50 S. C. Santos, Ó. E. Sigurjonsson, C. A. Custódio and J. F. Mano, *Tissue Eng. Part B Rev.*, 2018, **24**, 454.
51 N. Faramarzi, I. K. Yazdi, M. Nabavinia, A. Gemma, A. Fanelli, A. Caizzone, L. M. Ptaszek, I. Sinha, A. Khademhosseini, J. N. Ruskin and A. Tamayol, *Adv. Healthc. Mater.*, 2018, **7**, 1701347.
52 X. Liu, Y. Yang, X. Niu, Q. Lin, B. Zhao, Y. Wang and L. Zhu, *Acta Biomater.*, 2017, **62**, 179.
53 S. C. Bir, J. Esaki, A. Marui, K. Yamahara, H. Tsubota, T. Ikeda and R. Sakata, *J. Vasc. Surg.*, 2009, **50**, 870.
54 G. Cheng, X. Ma, J. Li, Y. Cheng, Y. Cao, Z. Wang, X. Shi, Y. Du, H. Deng and Z. Li, *Int. J. Pharm.*, 2018, **547**, 656.
55 G. Astori, E. Amati, F. Bambi, M. Bernardi, K. Chieregato, R. Schäfer, S. Sella and F. Rodeghiero, *Stem Cell Res. Ther.*, 2016, **7**, 93.
56 D. Thieme, L. Reuland, T. Lindl, F. Kruse and T. Fuchsluger, *J. Tissue Eng. Regen. Med.*, 2018, **12**, 557.
57 C. A. Custódio, V. E. Santo, M. B. Oliveira, M. E. Gomes, R. L. Reis and J. F. Mano, *Adv. Funct. Mater.*, 2014, **24**, 1391.
58 S. M. Oliveira, V. E. Santo, M. E. Gomes, R. L. Reis and J. F. Mano, *Biomaterials*, 2015, **48**, 56.
59 G. Walenda, H. Hemeda, R. K. Schneider, R. Merkel, B. Hoffmann and W. Wagner, *Tissue Eng. Part C Methods*, 2012, **18**, 924.

60 S. C. Santos, C. A. Custódio and J. F. Mano, *Adv. Healthc. Mater.*, 2018, **7**, 1800849.
61 C. R. Martins, C. A. Custódio and J. F. Mano, *Carbohydr. Polym.*, 2018, **202**, 91.
62 T. M. Fortunato, C. Beltrami, C. Emanueli, P. A. De Bank and G. Pula, *Sci. Rep.*, 2016, **6**, 25326.
63 R. Ito, N. Morimoto, L. H. Pham, T. Taira, K. Kawai and S. Suzuki, *Tissue Eng. Part A*, 2013, **19**, 1398.
64 V. E. Santo, E. G. Popa, J. F. Mano, M. E. Gomes and R. L. Reis, *Acta Biomater.*, 2015, **19**, 56.
65 P. S. Babo, A. Klymov, J. teRiet, R. L. Reis, J. A. Jansen, M. E. Gomes and X. F. Walboomers, *Tissue Eng. Part C Methods*, 2016, **22**, 914.
66 C. F. Monteiro, S. C. Santos, C. A. Custódio and J. F. Mano, *Adv. Sci.*, 2020, **7**, 1902398.
67 G. Faury, *Pathol. Biol. (Paris)*, 2001, **49**, 310.
68 W. Daamen, J. Veerkamp, J. Vanhest and T. Vankuppevelt, *Biomaterials*, 2007, **28**, 4378.
69 S. M. Mithieux, S. G. Wise and A. S. Weiss, *Adv. Drug Deliv. Rev.*, 2013, **65**, 421.
70 B. Kinikoglu, J. C. Rodríguez-Cabello, O. Damour and V. Hasirci, *Biomaterials*, 2011, **32**, 5756.
71 S.-K. Choi, J.-K. Park, K.-M. Lee, S.-K. Lee and W. B. Jeon, *J. Biomed. Mater. Res. B Appl. Biomater.*, 2013, **101**, 1329.
72 J. Devalliere, K. Dooley, Y. Hu, S. S. Kelangi, B. E. Uygun and M. L. Yarmush, *Biomaterials*, 2017, **141**, 149.
73 P. Pal, Q. C. Nguyen, A. H. Benton, M. E. Marquart and A. V. Janorkar, *Macromol. Biosci.*, 2019, **19**, 1900142.
74 P. Koria, H. Yagi, Y. Kitagawa, Z. Megeed, Y. Nahmias, R. Sheridan and M. L. Yarmush, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 1034.
75 J. Rnjak-Kovacina, S. G. Wise, Z. Li, P. K. M. Maitz, C. J. Young, Y. Wang and A. S. Weiss, *Acta Biomater.*, 2012, **8**, 3714.
76 J. M. Caves, W. Cui, J. Wen, V. A. Kumar, C. A. Haller and E. L. Chaikof, *Biomaterials*, 2011, **32**, 5371.
77 J. R. Soucy, E. Shirzaei Sani, R. Portillo Lara, D. Diaz, F. Dias, A. S. Weiss, A. N. Koppes, R. A. Koppes and N. Annabi, *Tissue Eng. Part A*, 2018, **24**, 1393.
78 H. H. Bragulla and D. G. Homberger, *J. Anat.*, 2009, **214**, 516.
79 A. Shavandi, T. H. Silva, A. A. Bekhit and A. E.-D. A. Bekhit, *Biomater. Sci.*, 2017, **5**, 1699.
80 M. Rajabi, A. Ali, M. McConnell and J. Cabral, *Mater. Sci. Eng. C*, 2020, **110**, 110612.
81 S. Han, T. R. Ham, S. Haque, J. L. Sparks and J. M. Saul, *Acta Biomater.*, 2015, **23**, 201.
82 T. R. Ham, R. T. Lee, S. Han, S. Haque, Y. Vodovotz, J. Gu, L. R. Burnett, S. Tomblyn and J. M. Saul, *Biomacromolecules*, 2016, **17**, 225.
83 R. C. de Guzman and S. Y. Rabbany, *J. Drug Deliv.*, 2016, **2016**, 1.
84 X. Chen, D. Zhai, B. Wang, S. Hao, J. Song and Z. Peng, *J. Mater. Sci. Mater. Med.*, 2020, **31**, 28.
85 L. R. Burnett, M. B. Rahmany, J. R. Richter, T. A. Aboushwareb, D. Eberli, C. L. Ward, G. Orlando, R. R. Hantgan and M. E. Van Dyke, *Biomaterials*, 2013, **34**, 2632.
86 P. Sierpinski, J. Garrett, J. Ma, P. Apel, D. Klorig, T. Smith, L. A. Koman, A. Atala and M. Van Dyke, *Biomaterials*, 2008, **29**, 118.
87 P. S. Hill, P. J. Apel, J. Barnwell, T. Smith, L. A. Koman, A. Atala and M. Van Dyke, *Tissue Eng. Part A*, 2011, **17**, 1499.
88 L. F. Tapias and H. C. Ott, *Curr. Opin. Organ Transplant.*, 2014, **19**, 145.
89 H. C. Ott, B. Clippinger, C. Conrad, C. Schuetz, I. Pomerantseva, L. Ikonomou, D. Kotton and J. P. Vacanti, *Nat. Med.*, 2010, **16**, 927.
90 T. H. Petersen, E. A. Calle, L. Zhao, E. J. Lee, L. Gui, M. B. Raredon, K. Gavrilov, T. Yi, Z. W. Zhuang, C. Breuer, E. Herzog and L. E. Niklason, *Science*, 2010, **329**, 538.
91 M. Sladkova, J. Cheng, M. Palmer, S. Chen, C. Lin, W. Xia, Y. E. Yu, B. Zhou, H. Engqvist and G. M. de Peppo, *Tissue Eng. Part A*, 2019, **25**, 288.
92 I. Belviso, V. Romano, A. M. Sacco, G. Ricci, D. Massai, M. Cammarota, A. Catizone, C. Schiraldi, D. Nurzynska, M. Terzini, A. Aldieri, G. Serino, F. Schonauer, F. Sirico, F. D'Andrea, S. Montagnani, F. Di Meglio and C. Castaldo, *Front. Bioeng. Biotechnol.*, 2020, **8**, 229.
93 H.-J. Lin, T.-J. Wang, T.-W. Li, Y.-Y. Chang, M.-T. Sheu, Y.-Y. Huang and D.-Z. Liu, *Tissue Eng. Part A*, 2019, **25**, 652.

94 S. Farnebo, C. Y. L. Woon, T. Schmitt, L.-M. Joubert, M. Kim, H. Pham and J. Chang, *Tissue Eng. Part A*, 2014, **20**, 1550.
95 F. Pati, J. Jang, D.-H. Ha, S. Won Kim, J.-W. Rhie, J.-H. Shim, D.-H. Kim and D.-W. Cho, *Nat. Commun.*, 2014, **5**, 3935.
96 A. Kayabolen, D. Keskin, A. Aykan, Y. Karslıoglu, F. Zor and A. Tezcaner, *Biomed. Mater.*, 2017, **12**, 35007.
97 J. S. Lee, Y. H. Roh, Y. S. Choi, Y. Jin, E. J. Jeon, K. W. Bong and S. W. Cho, *Adv. Funct. Mater.*, 2019, **29**, 1807803.
98 X. Tian, M. E. Werner, K. C. Roche, A. D. Hanson, H. P. Foote, S. K. Yu, S. B. Warner, J. A. Copp, H. Lara, E. L. Wauthier, J. M. Caster, L. E. Herring, L. Zhang, J. E. Tepper, D. S. Hsu, T. Zhang, L. M. Reid and A. Z. Wang, *Nat. Biomed. Eng.*, 2018, **2**, 443.
99 S. D. Sackett, D. M. Tremmel, F. Ma, A. K. Feeney, R. M. Maguire, M. E. Brown, Y. Zhou, X. Li, C. O'Brien, L. Li, W. J. Burlingham and J. S. Odorico, *Sci. Rep.*, 2018, **8**, 10452.
100 M. Ali, P. R. A. Kumar, J. J. Yoo, F. Zahran, A. Atala and S. J. Lee, *Adv. Healthc. Mater.*, 2019, **8**, 1.
101 M. Song, W. Wang, Q. Ye, S. Bu, Z. Shen and Y. Zhu, *Mater. Sci. Eng. C*, 2017, **77**, 739.
102 H. C. Ott, T. S. Matthiesen, S.-K. Goh, L. D. Black, S. M. Kren, T. I. Netoff and D. A. Taylor, *Nat. Med.*, 2008, **14**, 213.
103 J. A. Shadish and C. A. DeForest, *Matter*, 2020, **2**, 50.
104 C. A. Custódio and J. F. Mano, *ChemNanoMat*, 2016, **2**, 376.
105 O. Boutureira and G. J. Bernardes, *Chem Rev*, 2015, **115**, 2174.
106 E. A. Hoyt, P. M. S. D. Cal, B. L. Oliveira and G. J. L. Bernardes, *Nat. Rev. Chem.*, 2019, **3**, 147.
107 C. D. Spicer and B. G. Davis, *Nat Commun*, 2014, **5**, 4740.
108 O. Koniev and A. Wagner, *Chem. Soc. Rev.*, 2015, **44**, 5495.
109 C. M. Madl and S. C. Heilshorn, *Bioconjug Chem*, 2017, **28**, 724.
110 S. Sato, M. Matsumura, T. Kadonosono, S. Abe, T. Ueno, H. Ueda and H. Nakamura, *Bioconjug Chem*, 2020, **31**, 1417.
111 K. Lang and J. W. Chin, *ACS Chem Biol*, 2014, **9**, 16.
112 B. L. Oliveira, Z. Guo and G. J. L. Bernardes, *Chem Soc Rev*, 2017, **46**, 4895.
113 R. J. Spears and M. A. Fascione, *Org Biomol Chem*, 2016, **14**, 7622.
114 S. M. Agten, P. E. Dawson and T. M. Hackeng, *J Pept Sci*, 2016, **22**, 271.
115 E. Kim and H. Koo, *Chem Sci*, 2019, **10**, 7835.
116 C. M. Madl, L. M. Katz and S. C. Heilshorn, *Adv Funct Mater*, 2016, **26**, 3612.
117 R. Ravichandran, M. M. Islam, E. I. Alarcon, A. Samanta, S. Wang, P. Lundstrom, J. Hilborn, M. Griffith and J. Phopase, *J Mater Chem B*, 2016, **4**, 318.
118 M. S. Desai, E. Wang, K. Joyner, T. W. Chung, H. E. Jin and S. W. Lee, *Biomacromolecules*, 2016, **17**, 2409.
119 A. Yosef, O. Kossover, I. Mironi-Harpaz, A. Mauretti, S. Melino, J. Mizrahi and D. Seliktar, *Adv Heal. Mater*, 2019, **8**, e1801436.
120 J. Luo, X. Liu, Z. Yang and F. Sun, *ACS Appl. Bio Mater.*, 2018, **1**, 1735.
121 V. P. Ribeiro, J. Silva-Correia, C. Goncalves, S. Pina, H. Radhouani, T. Montonen, J. Hyttinen, A. Roy, A. L. Oliveira, R. L. Reis and J. M. Oliveira, *PLoS One*, 2018, **13**, e0194441.
122 Y. Cao, Y. Yao, Y. Li, X. Yang, Z. Cao and G. Yang, *J Colloid Interface Sci*, 2019, **544**, 121.
123 B. C. Isenberg and J. Y. Wong, *Mater. Today*, 2006, **9**, 54.
124 A. G. Mikos, S. W. Herring, P. Ochareon, J. Elisseeff, H. H. Lu, R. Kandel, F. J. Schoen, M. Toner, D. Mooney, A. Atala and M. E. V. A. N. Dyke, *Tissue Eng.*, 2006, **12**, 3307.
125 J. Xue, T. Wu, Y. Dai and Y. Xia, *Chem. Rev.*, 2019, **119**, 5298.
126 M. He, M. Chen, Y. Dou, J. Ding, H. Yue, G. Yin, X. Chen and Y. Cui, *Polymers*, 2020, **12**, 305.
127 B. H. Noszczyk, T. Kowalczyk, M. Łyzniak, K. Zembrzycki, G. Mikułowski, J. Wysocki, J. Kawiak and Z. Pojda, *Biofabrication*, 2015, **7**, 15011.
128 J. M. Patel, A. R. Merriam, J. Kohn, C. J. Gatt and M. G. Dunn, *Tissue Eng. Part A*, 2016, **22**, 1116.

129 A. P. Rameshbabu, S. Datta, K. Bankoti, E. Subramani, K. Chaudhury, V. Lalzawmliana, S. K. Nandi and S. Dhara, *J. Mater. Chem. B*, 2018, **6**, 6767.
130 D. Dems, J. Rodrigues, C. He, F. Wien, M. Marchand, N. Debons, L. Muller, Y. Chen, C. Laberty-robert, N. Krins and C. Aime, *ACS Appl. Biomater.*, 2020, **3**, 2948.
131 M. C. McManus, E. D. Boland, H. P. Koo, C. P. Barnes, K. J. Pawlowski, G. E. Wnek, D. G. Simpson and G. L. Bowlin, *Acta Biomater.*, 2006, **2**, 19.
132 M. Mcmanus, E. Boland, S. Sell, W. Bowen, H. Koo, D. Simpson and G. Bowlin, *Biomed. Mater.*, 2007, **2**, 257.
133 Q. Dong, J. Cai, H. Wang, S. Chen, Y. Liu, J. Yao, Z. Shao and X. Chen, *Acta Biomater.*, 2020, **106**, 102.
134 M. Sun, Y. Zhang, Y. Zhao, H. Shao and X. Hu, *J. Mater. Chem.*, 2012, **22**, 18372.
135 M. K. Kim, M. A. Kim, R. Jenjob, D. H. Lee and S. G. Yang, *Mater. Sci. Eng. C*, 2016, **62**, 391.
136 M. Jang, I. Koh, S. J. Lee, J. H. Cheong and P. Kim, *Sci. Rep.*, 2017, **7**, 41541.
137 Y. T. Matsunaga, Y. Morimoto and S. Takeuchi, *Adv. Healthc. Mater.*, 2011, **23**, 90.
138 B. Kim, S. W. Han, S. E. Choi, D. Yim, J. H. Kim, H. M. Wyss and J. W. Kim, *Biomacromolecules*, 2018, **19**, 386.
139 E. Vardar, H. M. Larsson, S. Allazetta, E. M. Engelhardt, K. Pinnagoda, G. Vythilingam, J. A. Hubbell, M. P. Lutolf and P. Frey, *Acta Biomater.*, 2018, **67**, 156.
140 C. A. Custódio, R. L. Reis and J. F. Mano, *Adv. Healthc. Mater.*, 2014, **3**, 797.
141 C. G. Garcia and K. L. Kiick, *Acta Biomater.*, 2019, **84**, 34.
142 M. Ha, A. Athirasala, A. Tahayeri, P. P. Menezes and L. E. Bertassoni, *Dent. Mater.*, 2020, **36**, 88.
143 S. Zhu, W. Zeng, Z. Meng, W. Luo, L. Ma, Y. Li, C. Lin, Q. Huang, Y. Lin and X. Y. Liu, *Adv. Mater.*, 2019, **31**, 1900870.
144 W. Liu, Z. Zhou, S. Zhang, Z. Shi, J. Tabarini, W. Lee, Y. Zhang, S. N. G. Corder, X. Li, F. Dong, L. Cheng, M. Liu, D. L. Kaplan, F. G. Omenetto, G. Zhang, Y. Mao and T. H. Tao, *Adv. Sci.*, 2017, **4**, 1700191.
145 A. M. Compaan, K. Christensen and Y. Huang, *ACS Biomater. Sci. Eng.*, 2017, **3**, 1519.
146 B. Zhang, L. Gao, L. Ma, Y. Luo, H. Yang and Z. Cui, *Engineering*, 2019, **5**, 777.
147 N. Ashammakhi, S. Ahadian, C. Xu, H. Montazerian, H. Ko, R. Nasiri, N. Barros and A. Khademhosseini, *Mater Today Bio*, 2019, **1**, 100008.
148 M. P. Nikolova and M. S. Chavali, *Bioact. Mater.*, 2019, **4**, 271.
149 M. Du, B. Chen, Q. Meng, S. Liu, X. Zheng, C. Zhang, H. Wang, H. Li, N. Wang and J. Dai, *Biofabrication*, 2015, **7**, 44104.
150 J. H. Kim, I. Kim, Y. J. Seol, I. K. Ko, J. J. Yoo, A. Atala and S. J. Lee, *Nat. Commun.*, 2020, **11**, 1025.
151 X. Cui and T. Boland, *Biomaterials*, 2009, **30**, 6221.
152 J. P. Jiang, X. Y. Liu, F. Zhao, X. Zhu, X. Y. Li, X. G. Niu, Z. T. Yao, C. Dai, H. Y. Xu, K. Ma, X. Y. Chen and S. Zhang, *Neural Regen. Res.*, 2020, **15**, 959.
153 L. Wei, S. Wu, M. Kuss, X. Jiang, R. Sun, P. Reid, X. Qin and B. Duan, *Bioact. Mater.*, 2019, **4**, 256.
154 S. Das, S. W. Kim, Y. J. Choi, S. Lee, S. H. Lee, J. S. Kong, H. J. Park, D. W. Cho and J. Jang, *Acta Biomater.*, 2019, **95**, 188.
155 MarketsandMarkets Research Private, *Biomaterials Market by Type of Materials (Metallic, Ceramic, Polymers, Natural) & By Application (Cardiovascular, Orthopedic, Dental, Plastic Surgery, Wound Healing, Neurological disorders, Tissue Engineering, Ophthalmology) - Global Forecast to 2024*, 2019.
156 F. J. O'Brien, *Mater. Today*, 2011, **14**, 88.
157 G. Benton, I. Arnaoutova, J. George, H. K. Kleinman and J. Koblinski, *Adv Drug Deliv Rev*, 2014, **79**–**80**, 3.
158 C. Beaurivage, E. Naumovska, Y. X. Chang, E. D. Elstak, A. Nicolas, H. Wouters, G. van Moolenbroek, H. L. Lanz, S. J. Trietsch, J. Joore, P. Vulto, R. A. J. Janssen, K. S. Erdmann, J. Stallen and D. Kurek, *Int J Mol Sci*, 2019, **20**, 5661.
159 European Union, EP2165678A1, 2010.
160 B. Guillotin, A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, R. Bareille, M. Remy, L. Bordenave, J. Amedee and F. Guillemot, *Biomaterials*, 2010, **31**, 7250.
161 H. L. Lanz, A. Saleh, B. Kramer, J. Cairns, C. P. Ng, J. Yu, S. J. Trietsch, T. Hankemeier, J. Joore, P. Vulto, R. Weinshilboum and L. Wang, *BMC Cancer*, 2017, **17**, 709.

162 N. T. Feric, I. Pallotta, R. Singh, D. R. Bogdanowicz, M. Gustilo, K. Chaudhary, R. N. Willette, T. Chendrimada, X. Xu, M. P. Graziano and R. Aschar-Sobbi, *Toxicol Sci*, 2019, **172**, 89.
163 I. Germanguz, E. Aranda, J. C. Xiong, N. Kissel, A. Nichols, E. Gadee and J. D. O'Neill, *bioRxiv*, 2019, 833913.
164 A. Marturano-Kruik, A. Villasante and G. Vunjak-Novakovic, *Methods Mol Biol*, 2016, **1502**, 203.
165 L. Zaulyanov and R. S. Kirsner, *Clin Interv Aging*, 2007, **2**, 93.
166 D. Marino, J. Luginbuhl, S. Scola, M. Meuli and E. Reichmann, *Sci Transl Med*, 2014, **6**, 221ra14.
167 M. Meuli, F. Hartmann-Fritsch, M. Huging, D. Marino, M. Saglini, S. Hynes, K. Neuhaus, E. Manuel, E. Middelkoop, E. Reichmann and C. Schiestl, *Plast Reconstr Surg*, 2019, **144**, 188.
168 United States, US9096688B2, 2015.
169 G. K. Bejjani, J. Zabramski and G. Durasis Study, *J Neurosurg*, 2007, **106**, 1028.
170 F. G. Scholl, M. M. Boucek, K. C. Chan, L. Valdes-Cruz and R. Perryman, *World J Pediatr Congenit Heart Surg*, 2010, **1**, 132.
171 B. N. Brown, R. Londono, S. Tottey, L. Zhang, K. A. Kukla, M. T. Wolf, K. A. Daly, J. E. Reing and S. F. Badylak, *Acta Biomater*, 2012, **8**, 978.
172 R. Edri, I. Gal, N. Noor, T. Harel, S. Fleischer, N. Adadi, O. Green, D. Shabat, L. Heller, A. Shapira, I. Gat-Viks, D. Peer and T. Dvir, *Adv Mater*, 2019, **31**, e1803895.
173 H. Xu, H. Wan, M. Sandor, S. Qi, F. Ervin, J. R. Harper, R. P. Silverman and D. J. McQuillan, *Tissue Eng Part A*, 2008, **14**, 2009.
174 S. J. Snyder, S. P. Arnoczky, J. L. Bond and R. Dopirak, *Arthrosc.- J. Arthrosc. Relat. Surg.*, 2009, **25**, 329.
175 H. Lu, T. Hoshiba, N. Kawazoe and G. Chen, *Biomaterials*, 2011, **32**, 2489.
176 C. E. Hart, A. Loewen-Rodriguez and J. Lessem, *Adv Wound Care New Rochelle*, 2012, **1**, 138.
177 R. K. Singh, R. K. Mallela, P. K. Cornuet, A. N. Reifler, A. P. Chervenak, M. D. West, K. Y. Wong and I. O. Nasonkin, *Stem Cells Dev*, 2015, **24**, 2778.
178 P. Dadhich, J. L. Bohl, R. Tamburrini, E. Zakhem, C. Scott, N. Kock, E. Mitchell, J. Gilliam and K. N. Bitar, *Sci Rep*, 2019, **9**, 18096.

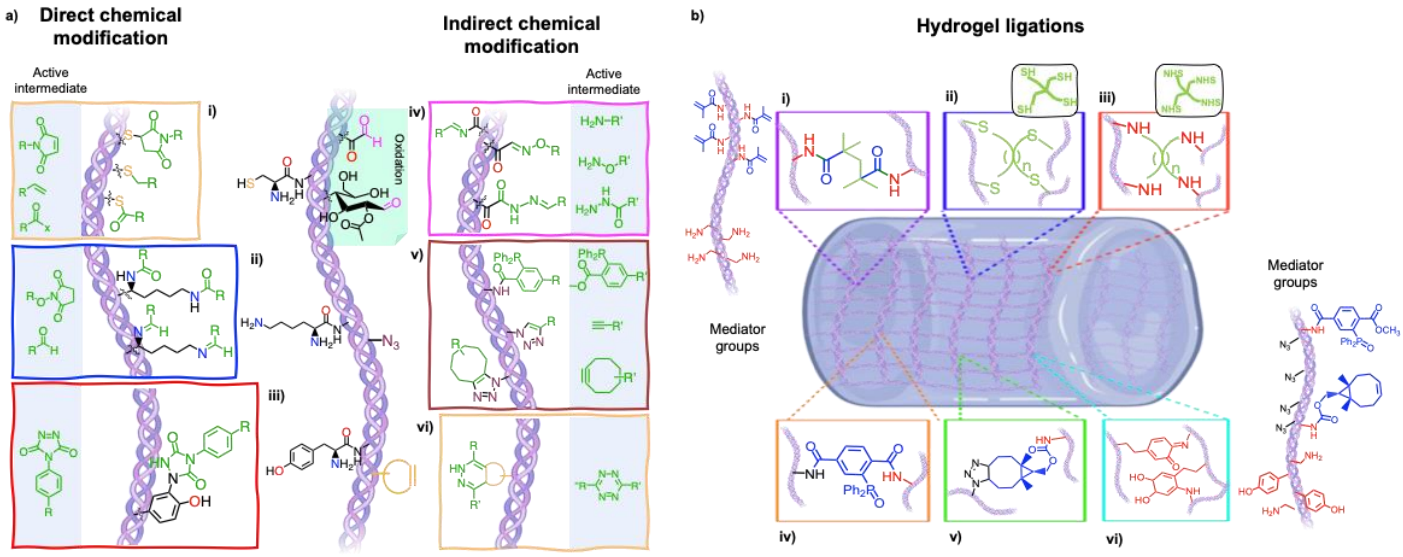

Figure X.1 a) Direct and indirect chemical modification of proteins. The most widespread reactions focused

on the modification of the side chains naturally present on proteins like i) cysteine, ii) lysine, and iii) tyrosine, or focused on prosthetic groups like iv) aldehydes, v) alkynes and vi) alkenes. b) Outline of chemical ligations with contribute to the fabrication of protein-based soft materials. i) radical, ii) Michael-type addition, iii) NHS coupling, iv) Staudinger, v) SPAAC and vi) enzymatic reactions.

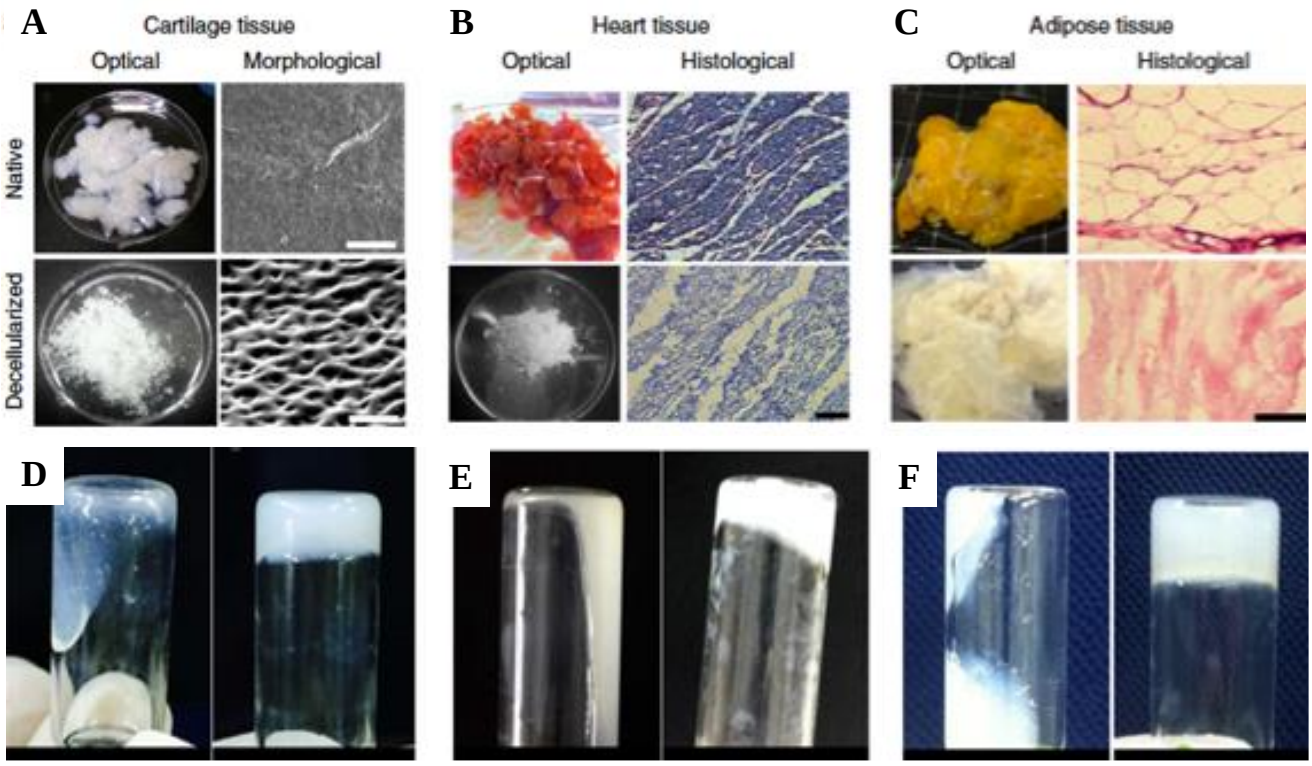

Figure X.2. Decellularization of the native tissues and their biochemical analysis. Optical and microscopic images of native and decellularized (A) cartilage tissue (scale bar, 50 mm), (B) heart tissue (scale bar, 100 mm), and (C) adipose tissue (scale bar, 100 mm). Sol to gel transition of the pre-gels prepared from (D) cartilage, (E) heart and (F) adipose decellularized matrices. (Adapted from 95, Copyright © 2014, Springer Nature).