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Review

Tissue Engineering in Musculoskeletal Tissue: A Review of the Literature

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Abstract: Tissue engineering refers to the attempt to create functional human tissue from cells in a laboratory. This is a field that uses living cells, biocompatible materials, suitable biochemical and physical factors, and their combinations to create tissue-like structures. To date, no tissue engineered skeletal muscle implants have been developed for clinical use, but they may represent a valid alternative for the treatment of volumetric muscle loss in the near future. Herein, we reviewed the literature and showed different techniques to produce synthetic tissues with the same architectural, structural and functional properties as native tissues.

Keywords: tissue engineering; biocompatible materials; skeletal muscle; biomaterials

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1. Introduction

The musculoskeletal system contains a variety of supporting tissues, including muscle, bone, ligament, cartilage, tendon and meniscus, which support the shape and structure of the body. After severe injuries due to various causes, such as severe diseases or trauma, the lost tissue needs repair or replacement with healthy tissue [1].

Severe muskoloskeletal injuries can lead to volumetric muscle loss, a condition in which massive damage and tissue loss cause a permanent loss of function [2]. Volumetric muscle loss injuries result in permanent disability [3].

In small injuries, muscle is capable of endogenous regeneration and functional recovery. In volumetric muscle loss, self-restoration is impossible due to denervation and the loss of native vasculature, physical and biochemical signaling [4]. In this case, physical therapy alone does not ensure the recovery of muscle strength [5–7]. The standard operative technique consists in replacing the damaged tissue with muscle flaps [8–11]. Muscle flaps are healthy, vascularized, innervated autogenous skeletal muscle tissue from outside of the zone of the injury, which are moved with their neurovascular supply intact [12,13]. Traditional flaps have to be placed in an area depending on the length of their artery and nerve. Free functional muscle transfer (FFMT) differs from a traditional muscle flap because it involves the transplantation of a donor muscle with its artery, vein and nerve transected and sewn in a new location. In this way, skeletal muscle can be moved anywhere on the body, and the surgeon can modify its size and orientation in order to optimize functional outcomes [8].

The limitations of the procedure include the damage of the donor site; extended rehabilitation, which is limited by the reinnervation to the motor endplates in the donor muscle; and long operative times requiring high technical skills, which can limit its widespread

use [14]. Both free functional muscle transfer and traditional muscle flap techniques often lead to infection, graft failure, and donor site morbidity due to tissue necrosis. These complications necessitate surgery or the amputation of the affected limb [15,16].

Vascularized composite allotrasplants (VCAs) are grafts that are composed of multiple different allogenic tissues transplanted together as a single unit. To date, upper extremity, face, abdominal wall, larynx, and genito–uterine tract transplants have been performed [17,18]. This method requires chronic immunosuppression in order to avoid rejection [19,20], which can cause visceral organ toxicity, skin-based malignancies and lymphoproliferative disorders; a suitable donor; and the optimization of the patient's immunosuppressive drug regimen [21].

There is a clinical need for the development of a tissue replacement method to restore function in volumetric muscle loss injuries [22], and tissue engineering may represent a valid option.

Tissue engineering is a component in the field of regenerative medicine, which is intended to produce tissue constructs to repair or replace native tissues [23].

This is a field that uses living cells, biocompatible materials, suitable biochemical and physical factors, and their combinations, to create tissue-like structures [24]. Its ultimate goal is to be a cure, not merely a treatment, by repairing and replacing tissues or organs that fail due to diseases, genetic errors, congenital abnormalities, or traumatic injuries [25].

To date, no tissue-engineered skeletal muscle implants have been developed for clinical use, but it may represent a valid alternative to treat volumetric muscle loss in the near future.

The field of skeletal muscle tissue engineering has taken great strides since Vandenburgh's first work in 1988, using cultured avian myotubes in collagen-coated tissue culture plates [26]. Tissue engineering strategies can be divided into two main categories: scaffold-based and scaffold-free approaches.

1.1. Methods

This systematic review was performed following the guidelines of the Preferred Reporting Items of Systematic Reviews and Meta-analyses (PRISMA).

The PubMed database and Scopus database were queried for the same search string: "tissue engineering for musculoskeletal repair".

1.2. Results

After searching the databases, the total number of records was 4950, divided for PubMed (n = 693) and SCOPUS (n = 4257), respectively. The title and abstract were screened, and duplicates and nonrelevant records were removed, as summarized in Figure 1.

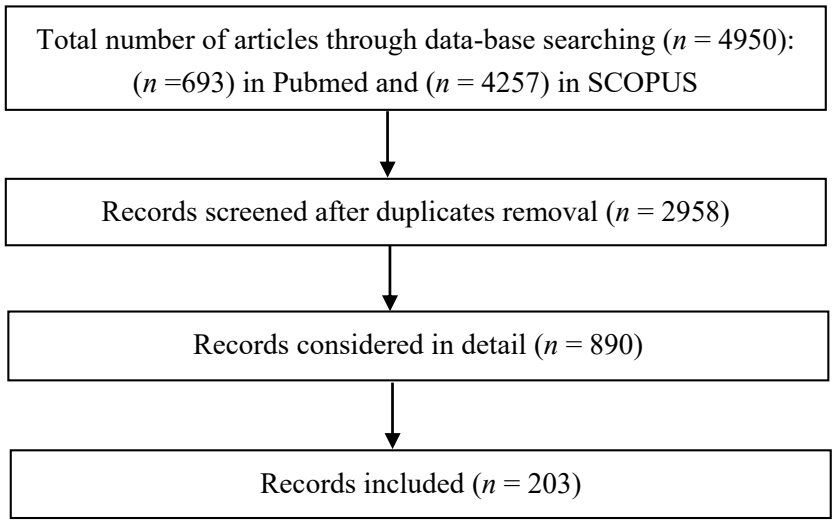

Figure 1. Flow-chart of the study selection.

2. Biomaterials

Robert S. Langer and Joseph Vacanti [27] realized that, in order to build an organ, a framework that guides the cells' growth is needed: a 'scaffold' to define the parts and hold them together. The scaffold's biochemical, topological and geometrical properties and fabrication methods affect its behaviour in terms of differentiation, adhesion, and viability [28,29]. They have a structural framework similar to the extracellular matrix (ECM) in order to provide support for tissue regrowth.

A wide range of materials are used for scaffold processing: natural and synthetic polymers, inorganic biomaterials, and their hybrid combination.

2.1. Natural and Synthetic Polymers

Natural polymers are obtained from plants, animals, algae or microorganisms, and include proteins (silk fibroin, collagen, gelatin, keratin, fibrinogen, elastin, actin), polysaccharides (chitosan, chitin, alginate, gellan gum) and glycosaminoglycans (hyaluronic acid) [30]. They are similar to the macromolecules of the extracellular matrix, reducing the occurrence of immune reactions and inflammation toxicity.

Acellularized tissue scaffolds are natural scaffolds derived from tissues or organs, in which the cellular and nuclear contents are eliminated, but the tridimensional (3D) structure, composition and microenvironment of the extracellular matrix (ECM) are preserved. They have to be similar to the tissue that has to be repaired [31]. Recent works were performed with animal and human skeletal muscle models. Porzionato et al. [32] decellularized human skeletal muscle and used it as a scaffold in a rabbit model with an abdominal wall defect. It gave good results in terms of integration, but recellularization was not completely achieved. Wilson et al. [33] compared decellularized skeletal muscle taken from rectus femoris and supraspinatus, showing that the muscle type influenced its material properties. They demonstrated that these scaffolds biodegrade at a rate that corresponds to the regeneration of the damaged muscle; biological scaffolds cannot be considered as permanent implants, but should rather be used as a temporary support to ECM turnover by resident cells. Davari et al. [34] implanted cryopreserved and decellularized patches, derived from the hemidiaphragm of a deceased, in a canine model. They compared the two methods and demonstrated that the healing process was similar, with fewer inflammatory cells and foreign body granulomas on the decellularized patch. The ECM derived from the decellularization of tissue may also be used for the tissue engineering of another tissue type, as demonstrated by Wolf et al., who compared quadriceps-, hamstring- and intestine derived scaffolds seeded with C2C12 cells implanted in the abdominal wall of a rat. After 35 days, the muscle fiber structure had been restored using each of the scaffolds [35]. A limitation of acellularized tissue scaffolds is the limited donor tissue and the potential for immune rejection. Trials of recellularization have been performed, and have demonstrated that the most promising cell types are mesenchymal stem cells and induced pluripotent stem cells, thanks to their capability of differentiation through different lineages, stimulated by the ECM [36].

Synthetic polymers are also used. The most commonly employed are polyglycolide (or poly glycol acid (PGA)) and polylactide (PLA), poly-lactide-co-glycolide (PLGA), poly-(D, L-lactic acid) (PDLLA), poly-ethylene-glycol (PEG), and polycaprolactone (PCL) [37]. The mechanical properties of these materials changes over time in a physiologic environment according to their molecular weight and degree of crystallinity [38]. They can be hydrolytically and enzymatically degradable, and the hydrolytically degradable examples are the most commonly used [39]. The disadvantage is that these polymers induce an immune reaction [40].

Protein-based scaffolds provide a structural pattern like the fibrous protein components of the ECM. These are available as scaffolds in gel form or suspended into matrices with a pore size determined by cryogelation [41,42]. Synthetic polymers allow control over the scaffold structure and the micro-architecture. The scaffold's structural properties—such as its diameter, alignment and porosity—guide the cell response, proliferation and

differentiation [43], even after implantation [44]. Nigarawa et al. [45] used fibrin-based scaffolds seeded with mesenchymal stem cells to demonstrate their high potential for differentiation into skeletal muscles in vitro. Then, they implanted the construct in vivo, into mouse damaged tibialis anterior muscle, and showed that the transplanted cells can accelerate the functional recovery of injured muscles. Dynamic scaffolds can change their diameter, alignment and porosity through temperature changes or magnetic fields, miming the complexity of environmental changes in vivo. Fraley et al. [46] presented an integrated study of ECM protein parameters in different ECM configurations using self-assembling 3D collagen, and showed how each parameter relates to others and to cell motility; cellular motility was significantly predicted by fiber alignment; the cellular protrusion rate, orientation, speed of migration, and invasion distance showed biphasic responses to increasing collagen density. Wang et al. [47] also demonstrated the relationship between fiber alignment and cells' motility along the direction of the fiber alignment, and the change from an unaligned to an aligned morphology. Although fiber micro-architecture influences cellular behavior, a limitation of a synthetic polymer scaffold is the lack of cell signaling provided by a native ECM. In order to reply to the native ECM environment, proteins and growth factor can be incorporated into the scaffolds [48]. A hydrogel-based scaffold with integrin binding domains improves mesenchymal stem cells' attachment and bone repair upon implantation [49,50].

TGF-b induced the differentiation of MSC toward chondrogenic and osteogenic lineages depending on the fiber alignment [51]. PDGF induced the tenogenic differentiation of adipose-derived stem cells in an aligned collagen nanoparticle composite fiber [52]. Quarta et al. [53] engineered a biomimetic microenvironment that enabled the maintenance of quiescent, potent mouse Muscle Stem Cells (MuSCs) and human Muscle Stem Cells. Both mouse and human Muscle Stem Cells showed an enhanced engraftment and self-renewal potential. The possibility of culturing MuSCs for a long time period without the loss of potency gives the chance to correct genetic mutations, in order to transplant only the corrected cells and replace the pathological tissue in muscle disorders [54,55].

2.2. Inorganic Biomaterials

Metallic and ceramic biomaterials have been used for musculoskeletal and periodontal repair.

Metallic biomaterials (titanium) are characterized by high strength, low elasticity and low density; ceramic biomaterials (aluminia, zirconia, CaPs, calico phosphate cements (CPCs), silicates) are biocompatible, osteoconductive, and osteogenic [56].

Natural inorganic biomaterials are derived from marine shells, corals, sponges, nacres, and animal bones, as they contain a great amount of calcium compounds.

Synthetic inorganic biomaterials are obtained by different methods (wet precipitation, hydrolysys, mechanochemical syntehesis, microwave processing, spray drying), resulting in materials with an increased crystal size and morphology.

They can be classified into: bioinert, bioactive, and bioresorbable.

Bioinert materials have no interactions with native living tissue, and are used essentially to give structural support for bones. Examples of bioinert materials are aluminia, zirconia, and titanium.

Bioactive materials tie with tissue, and are used to repair small bone or periodontal defects. They are bioglasses and glass-ceramics. They have been treated with ionic elements in order to accelerate natural tissue formation after implantation [57,58].

Bioresorbable materials are absorbed and gradually replaced by the adjacent tissue. Examples include CaPs, CPCs, calcium carbonates, and calcium silicates.

2.3. Hybrid Biomaterials

Hybrid biomaterials are composed of a combination of organic polymers and inorganic materials with good compatibility between the phases, maintaining the porosity and the mechanical strengths of the scaffolds.

These combinations have been used to produce tissues with enhanced osteoconductivity and mechanical properties. Nanostructured hybrids also show an enhanced bonding capacity of the tissue to the organic matrices [59–62].

3. Biomaterials Modifications

The interaction between biomaterials and a biological environment depends on the physico-chemical and morphological properties of their surface [63]. Polymers are promising materials to be used for tissue engineering. In their original form, they are biologically inert, and the modification of the surface properties is necessary to allow the adhesion of cells to the substrate, and cell proliferation [64]. Different methods of surface modification have been studied. The following are the most used.

3.1. Laser Treatment

The laser irradiation of a solid substrate can induce the formation of a periodic pattern on the surface of the substrate. This leads to laser-induced periodic surface structures (LIPSS).

According to the relationship between the wavelength of the laser radiation and the period of the structure, these structures can be classified into: low spatial frequency LIPSS, when the period is similar to the wavelength of the incident laser beam; high spatial frequency LIPSS, when the period is smaller than the wavelength of the laser beam [65].

The periodic surface structures have different shapes; the most common are ripples. The formation of the ripples depends on the interference between the incident laser beam and the perpendicularly-reflected beam, which causes the formation and accumulation of energy. This energy causes the heating of the non-crystalline phase of the polymer and the melting of the crystalline phase, resulting in a periodic pattern on the polymer's surface [66].

The dimensions of the ripples vary according to the characteristics of the polymer substrate and the conditions of the laser treatment: the height of the ripples remains constant and the period increases with the increasing of the angle of incidence of the laser beam.

Surface modifications induce variation in the chemical modification of the surface itself, which promotes cell adhesion and proliferation. They also induce the cellular orientation on the surface morphology, depending on the ripple period.

Human myoblasts proliferate in a systematic orientation parallel to the orientation of the ripples only on ripples of large periods [67].

3.2. Ion Implantation

High energy ions are separated by a magnetic field and accelerated by an electric field, and then implanted on the surface of the substrate. Their energy is thus dissipated, inducing the breaking of macromolecular chains and the consequent change in the structure of the polymer, inducing cell adhesion and proliferation. The depth of penetration depends on the weight and energy of the ions. The ions of noble gasses and of oxygen or nitrogen are the most commonly used for biomedical applications. Low energies are usually used, because the interactions happen between the upper layers of the material [68].

3.3. Plasma Treatment

Plasma is defined as an ionized gas consisting of positive and negative charges in equal density. There are two categories of plasma: thermal and non-thermal plasma, depending on its thermal equilibrium state.

In thermal (or equilibrium) plasma, the electron and ion temperatures are in equilibrium. In non-equilibrium (or cold) plasma, the temperatures of the electrons and ions are not in equilibrium. Equilibrium plasmas are applied for the surface modification of materials that can stand high temperatures, while non-equilibrium plasmas are ideal for the modification of thermosensitive materials, such as polymeric nanofibrous scaffolds.

The most commonly used plasma type for the treatment of nanofibrous scaffolds is dielectric barrier discharge (DBD). It consists of two parallel electrodes separated by a gas gap of a few millimeters to a few centimeters. At least one electrode is covered with a dielectric layer, such as glass, quarz or aluminia. When a high voltage with a frequency in the range of kHz is applied, it creates an electric field. Collisions between the accelerating electrons and the gas determines the ionization of the gas, generating and sustaining the plasma. When it is exposed to plasma discharges, plasma–surface interactions are possible. The modified surfaces allow biomolecule immobilization, which is necessary for the realization of functional substrates [69,70]. Bourkoula et al. cultured fibroblasts on plasma-treated surfaces of increasing roughness, with effects on their adhesion proliferation rate and morphology [71].

Kitsara et al. used plasma treatment, combined with electrospinning, to develop a scaffold capable of activating osteoblasts [72].

Table 1 summarizes the in vitro cells studies performed on plasma-activated nanofibrous scaffolds.

Micropatterned substrates are synthetic polymers designed to control many aspects of cellular behavior, including spatial organization, migration, proliferation, and differentiation [73–75]. Skeletal muscle is made of multiple bundles of fiber formed by the fusion and alignment of myoblasts into myotubes, which is necessary to produce appropriate contraction. This is the reason why the alignment and fusion of myotubes are crucial aspects for muscle tissue engineering. Micropatterned scaffolds have been used to guide these processes.

Soft lithography uses an elastomeric master that is easy to mold or emboss, which can be used directly as a substrate for biological applications or as a mold. It is widely used for the patterning of cells and proteins through various techniques, such as microcontact printing, microfluidic patterning, and stencil micropatterning [76–78]. It can be used for the patterning of ECM proteins such as collagen, fibronectin or laminin; and the printing of self-assembled monolayers with cell-repellant molecules, and a combination of cell-repellant and cell-adhesive molecules [79–81]. Shimizu et al. [82] used a stencil membrane seeded with C2C12 myoblasts that proliferated and differentiated, forming a pattern of single myotubes. A direct inkjet printing technique has been used to obtain myoblasts' patterning, improving the cell alignment and tissue formation [83]. Contractile C2C12 myotube line patterns embedded in a fibrin gel have been developed in order to afford a physiologically relevant and stable bioassay system by Nagamine et al. [84]. Huang et al. [75] used micropatterned polydimethylsiloxane (PDMS) or poly2-hydroxyethyl methacrylate (pHEMA) as a scaffold, and transferred the aligned myotubes to biodegradable collagen gel. Functional nanomembranes, ultrathin polymeric films of fibronectin and fibril carbon nanotubes, can be micropatterned in order to promote myoblasts' alignment, elongation and differentation [85]. In five-layer 3D tissue made of human umbilical vein endothelial cells sandwiched between myoblasts sheets, endothelial cell connections and capillary-like structures were found throughout the layers. After trasplantation into the subcutaneous tissue of nude rats, the endothelial networks connected the host vessels, allowing the graft's survival [86]. Takahashi et al. [87] discovered that an anisotropic cell sheet placed on top of other cell sheets induced myoblast alignment. Guillarme-Gentil et al. [88] used an electrochemical strategy for the micropatterning and detachment of heterotypic cell sheets. These methods present the possible creation of co-cultured cell sheets, and the creation of cellular constructs which mimic the cellular complexity of native tissue.

Table 1. In vitro cell studies on plasma-activated nanofibrous scaffolds.

Application	Cell Type	Plasma Source	Gas	Substrate	Results	Reference
Bone	Human-induced pluripotent stem cells (iPSCs)	MW	O_2	Polyethersulfone (PES)	Enhanced proliferation and osteogenesis	[89]
	Human primary osteosarcoma cells (Saos-2)	RF	O_2 and Ar	PCL	Improved cell viability and proliferation	[90]
	Mouse osteoblast cells (MC3T3-E1)	RF	Ar/O_2, NH_3/O_2 and N_2/H_2	PCL	Improved cell attachment and proliferation	[91]
	MC3T3	RF	O_2	PCL	Improved cell adhesion and ALP activity	[92]
	Human mesenchymal stem cells (hMSCs)	Not specified	Ar and N_2	PCL	Improved cell attachment. Accelerated differentiation towards osteoblasts	[93]
	hMSCs	Not specified	He	PCL/CMC	Enhanced osteoinductivity without external osteogenic differential agent, did not support the proliferation	[94]
	hMSCs	RF	O_2	PolyActive	Significant upregulation of bone sialoprotein and osteonectin expression	[95]
	hMSCs	Not specified	Air	PLGA	Greatly enhances peptide immobilization which increases the ALP activity, calcium content and expression osteogenic markers of collagen type-I, osteocalcin (OC) and osteopontin (OP)	[96]
	hMSCs	RF	O_2	PLLA	Improved expression of genes associated with osteoblast linkage	[97]
	hMSCs	Not specified	Air	PLLA	Improved cell proliferation, ALP activity and mineralization	[98]
	hMSCs	Not specified	Air	PLLA/PVA	Increases the ALP activity level, protein content and calcium deposition	[99]

Table 1. *Cont.*

Application	Cell Type	Plasma Source	Gas	Substrate	Results	Reference
Cartilage	Mouse chondrocyte teratocarcinoma-derived cells (ATDC5)	RF	O_2 and Ar	PCL	Improved cell viability and proliferation	[90]
	Neonatal human knee articular chondrocytes (nHAC-kn)	MW	Ar	Silk fibroin	Improved cell attachment, proliferation and glycosaminoglycan synthesis	[100]
	Schwann cells (RT4-D6P2T)	RF	Air	PCL	Improved cell proliferation	[101]
	MSCs	Not specified	Air	PCL	Improved cell attachment and proliferation, chondro-differentiation in a non-differential medium	[102]
	Mouse lung fibroblasts (L929)	RF	O_2 and Ar	PCL	Improved cell viability and proliferation	[90]
	Human foreskin fibroblasts (HFFs)	DBD	Ar, N_2 and He/NH_3	PCL	Improved cell adhesion and proliferation	[103]
	HFFs	DBD	Ar, N_2 and He/NH_3	Chitosan/PEO	Improved cell adhesion and proliferation	[104]
	Normal human epidermal keratinocytes and fibroblasts (NHEKs and NHEFs)	RF	O_2	Silk fibroin	Improved cell attachment	[105]
Epithelial	3T3 fibroblasts	DBD	O_2 and NH_3	PLGA	Improved cell adhesion and proliferation	[101]
	Mouse embryonic fibroblasts (MEFs)	Corona	N_2	PLLA	More elongated and dendritic cell morphology. Improved cell vitality	[106]
	Bovine aorta endothelial cells (BAECs)	RF	Ar and $Ar\text{-}NH_3/H_2$	-	Improved cell adhesion, spreading and infiltration	[107]
Stem cells	Porcine mesenchymal stem cells (pMSCs)	RF	O_2	PLLA	Improved cell adhesion	[100]
	Adipose-derived stem cells (ADSCs)	DBD	Ar and Air	PCL	Improved cell adhesion, proliferation, spreading and viability	[108]
Muscle	Primary porcine smooth muscle cells (SMCs)	RF	Air	PCL	Improved spread-out cell morphology	[100]
	Bovine smooth muscle cells (BSMCs)	RF	Ar and $Ar\text{-}NH_3/H_2$	-	Improved adhesion, spreading and infiltration	[107]
Immune System	Human monocyte	RF	Air	PLLA	Disruption of macrophage polarization balance towards an anti-inflammatory profileImproved cell morphology with filopodia-like and podosome-like structures on plasma-treated samples	[109]

4. Scaffolding Strategies

Tissue engineering mainly uses 3D porous scaffolds and hydrogels that are fabricated with multiple technologies. Here, we focus on phase separation, 3D printing, and electrospinning.

Table 2 compares the advantages and disadvantages of these techniques.

Table 2. Advantages and disadvantages of three different scaffold techniques.

Technique	Advantages	Disadvantages
Phase separation	• 3D pore arrangement • Pore size and shape controllable • Tailorable mechanical properties	• Complex procedures • Lack of control of fiber arrangement • Low yield • Limited range of polymers can be used
3D printing	• Multiple extrusions with different materials • Simplicity • Cost effectiveness • High speed • Solvent free • Tailorable internal and external architecture	• Limited range of biocompatible materials can be used
Electrospinning	• Simple • Continous fibers • Wide range of materials • Control on fiber diametres and orientations • Tailorable mechanical properties	• Use of toxic solvents • Difficult to control pore size and shape

4.1. Phase Separation

Phase separation can be non-solvent–induced or thermally-induced.

The non-solvent–induced phase separation process produces a heterogeneous pore structure [110], so it is not suitable for scaffold production, since it generally requires a uniform structure.

Thermally-induced phase separation (TIPS) allows us to obtain homogeneous porous scaffolds [111]. During this process, a row polymer is dissolved in an appropriate solvent; then, there is phase separation between the polymer-rich phase (which becomes a gel) and the polymer-poor phase (the solvent); the solvent is extracted from the gel, and the gel is frozen and dried. In this way, the polymer-rich phase forms a porous 3D matrix [112] (Figure 2).

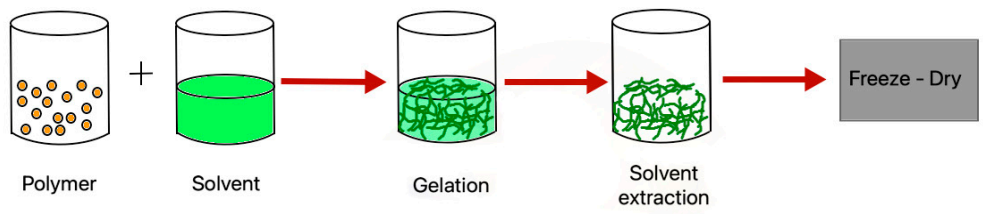

Figure 2. Thermally-induced phase separation steps.

Ma et al. [113] prepared, for the first time, 3D nanofibrous scaffolds from poly-L-lactic acid (PLLA) dissolved in tetrahydrofuran (THF).

The porosity and fiber size of the scaffolds can be modified by controlling each step of the process: the polymer/solvent system, polymer concentration, gelation temperature, and gelation duration [114,115].

Da et al. [116] used a biphasic scaffold composed of a bony phase, a chondral phase, and a compact layer. The bony phase was seeded with autogenic osteoblast-induced bone marrow stromal cells, and the chondral phase was seeded with chondrocyte-induced bone marrow stromal cells. The biphasic scaffold-cell constructs were implanted into osteochondral defects of rabbits' knees, and showed an enhanced regeneration of the osteochondral tissue.

4.2. 3D Printing

Three-dimensional (3D) printing plays an important role in the production of scaffolds for tissue engineering. The main 3D printing categories are presented here.

4.2.1. Fused Deposition Modeling

A thermoplastic polymer is introduced into the heating chamber and melted. This is extruded through a nozzle onto a platform, onto which it is deposited layer-by-layer, creating a 3D form. The position of the nozzle and its movement are controlled by a computer program, and they continue until the desired form is created [117]. The diameter of the nozzle, the print speed, the angle and the distance between fibers, and the number of layers define the resolution of the details [118].

The advantages of this technique are the possibility of multiple extrusions with different materials, and its simplicity, cost-effectiveness, high speed, and solvent-free nature [119,120].

The disadvantages are the limited number of biocompatible thermoplastic materials [121].

4.2.2. Selective Laser Sintering

A laser beam heats a layer of powder materials (ceramic, plastic, thermoplastic polymers) and fuses them together, with the shape being defined by a computer program. The process is repeated layer-by-layer until the final structure is obtained [122].

This method allows us to fabricate large and complex scaffolds, and does not need support structures and solvents [123]. The main disadvantage is that the product obtained is rough and needs polishing [124].

4.2.3. Stereolithography

Stereolithography uses a narrow beam of light to induce the polymerization of a liquid resin, thus obtaining the desired pattern according to a computer model. The process is repeated layer-by-layer until the desired product is obtained [125].

Each layer is printed at the same time when multiple objects are being printed, decreasing the printing time [126]. Since the width of the light source is small and highly-controlled, the external and internal architecture of the scaffold can be controlled [127]. The main disadvantage is the limited number of biocompatible materials available for this method [128].

4.2.4. Bioprinting

Bioprinting prints scaffolds using hydrogels seeded with cells. There are three main technologies: inkjet, extrusion, and laser bioprinting (Figure 3).

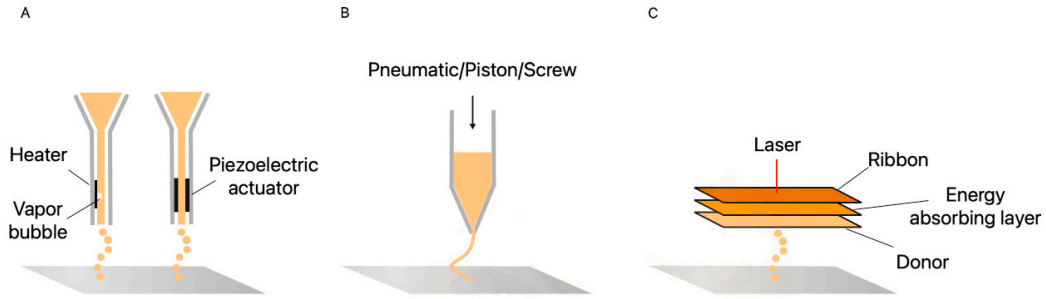

Figure 3. Three main bioprinting technologies: (**A**) inkjet bioprinting; (**B**) extrusion; (**C**) laser-assisted bioprinting.

4.2.5. Inkjet Bioprinting

In thermal inkjet bioprinting, a bioink—that is, a prepolymer solution containing cells—is loaded into an ink cartridge. The cartridge is placed in the printer, and small droplets of ink are created thanks to the air bubbles produced by heat. The size of the droplets depends on ink's viscosity and the temperature applied [129].

In piezoelectric inkjet bioprinting, different potentials are applied to the piezoelectric crystal in the bioprinting, generating a pressure that ejects the bioink droplets [130].

4.2.6. Extrusion

The extrusion method extrudes bioink using a pneumatic or a mechanical system. In a pneumatic system, air pressure allows the extrusion of bioink from the nozzle as an uninterrupted filament [131].

The mechanical system dispenses bioink using a screw or a piston [132].

Cells are subjected to high mechanical stresses that can decrease cell viability [133]. The main problem of this technique is the clogging of the nozzle due to cell aggregation or the drying of the material in the nozzle.

4.2.7. Laser-Assisted Bioprinting

Laser-Assisted Bioprinting consists of a pulsed laser source, a donor layer, and a receiving substrate. Bioink is placed below a ribbon that contains an energy-absorbing layer. The pulsed laser source is focused on the laser-absorbing layer, generating a vapor bubble, which creates a pressure that deforms the bioink and forms droplets. These cell-loaded hydrogel droplets are collected and crosslinked on the receiver [134]. Laser assisted bioprinting prevents clogging due to the absence of a nozzle and the lack of mechanical stress on cells, increasing cell viability [133].

4.3. Electrospinning

Electrospinning is based on the use of electrical forces to produce fibers with sizes from micro- to nanometers. The electrospinning process needs three components: a nozzle tip attached to a high voltage direct current (HVDC) source, a flow rate controller, and a grounded collector [135]. When an electric field is applied between the nozzle tip and the grounded collector, a microsphere is formed at the end of the nozzle. The microsphere elongates itself as the strength of the electric field increases, assuming a conical shape called the Taylor cone. The electrostatic force in the cone becomes greater than the surface tension, generating a liquid jet from the cone. The jet causes the whipping of fibers, generating a randomly-orientated fibrous mat (Figure 4). These microsized or nanosized fibers provide a large surface-area-to-volume ratio which can enhance cellular activities such as attachment, proliferation and differentiation. They also simulate the structure of the native extracellular matrix (ECM), which has a fundamental role in cell survival, polarity, proliferation, migration, and differentiation [136–139].

Electrospun scaffolds are used in biomedical applications to regenerate various tissues. Since some tissues—like muscles, nerves and tendons—are made of aligned structures, studies have been conducted to align fibers. Aviss et al. [140] achieved the alignment of poly(lactide-co-glycolide) fibers for skeletal muscle regeneration, using a rotating mandrel as the grounded collector.

The limitations of the procedure are the use of toxic solvents, poor cell infiltration, and non-homogeneous cell-distribution.

Cell electrospinning was introduced in order to overcome the limitations of electrospinning. It differs from conventional electrospinning in its use of viable cells. Townsend-Nicholson et al. [141] introduced this method using astrocytoma cells (1321N1) embedded in a cell suspension, which was supplied to the needle of the nozzle. Then, various cell types and biocompatible materials were used [142–144].

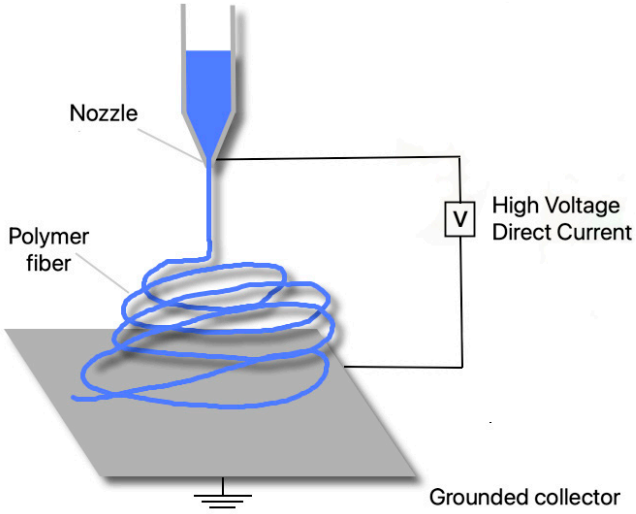

Figure 4. The basic components of electrospinning.

Cell electrospinning shows compatibility with different types of muscle cells, such as cardiac, skeletal, and smooth muscle cells [145–148].

For muscle regeneration, a uniaxially-arranged micropattern is important in order to mimic the structure of the native extracellular matrix. Yeo et al. [149] used a cell-electrospun scaffold seeded with C2C12 myoblasts and, as a control, a 3D cell-printed scaffold in order to compare the efficiency of the cell alignment and differentiation on myoblasts. A highly-arranged, multinucleated cell morphology was confirmed in the cell-electrospun scaffold, which facilitates myogenic differentiation.

Table 3 summarize advantages and disadvantages of conventional electrospinning and cell-electrospinning [150].

Table 3. Advantages and disadvantages of conventional electrospinning and cell-electrospinning.

Advantages and Disadvantages	Electrospinning	Cell-Electrospinning
Advantages	• Simple process • Provide controllable micro/nano-sized fibers • Mimic the native ECM structure	• All the same advantages of electrospinning • High resolution (nanoscale) • Efficient and fast nutrients/oxygen exchange • Excellent cell-to-cell interaction • Homogeneous cell distribution
Disadvantages	• Use of toxic solvents • Insufficient cell infiltration • Inhomogeneous cell distribution	• Low mechanical properties • Restrict to develop into 3D structure • Low cell density controllability • Low precision in fiber deposition

Electrospun scaffolds, fabricated using biodegradabl polymers such as ECM proteins [140,151–155], have been used for muscle tissue engineering [156]. Elastin-like recombinamer fibers are cytocompatible, and allow for the incorporation of different functionalities, such as cell adhesion domains for fibroblasts and keratinocytes [157]. Collagen electrospun scaffolds seeded with myoblasts facilitate the regeneration of muscle fibers with low biocompatibility [153]. This method gives control over the scaffold's parameters, which is important for cell adhesion and myotube formation, and is quick and cost effective [158,159].

Recent studies using different scaffold strategies for tissue engineering are summarized in Table 4.

Table 4. Recent studies using different scaffold strategies.

Technology	Materials	Cells/Growth Factors	Outcomes	Application	References
3D bioprinting	Polycaprolactone (PCL)	Saos-2 cell seeding	positive influence on the biological performance of the cells; higher values for the mineralization, activity of osteogenic-related genes, and deposition of the mineralized matrix.	Bone tissue engineering	[160]
	Alginate/alginate-sulfate	MC3T3-E1 cells/BMP-2	Alginate/alginate sulfate bioinks allowed good 3D cell printing. Improvement of the release of BMP-2 was achieved using alginate sulfate. Proliferation and differentiation of the printed osteoblasts were enhanced	Bone tissue engineering	[161]
	GelMA and methacrylated hyaluronic acid (HA) modified with HAp	hASCs	Positive effects on bone matrix production and remodelling	Bone tissue engineering	[162]
	Collagen/dECM/silk fibroin (SF)	MC3T3-E1 cells	High compressive modulus mainly due to the methanol-treated SF; high cellular activities in in vitro tests using MC3T3-E1 cells, induced by Collagen and dECM.	Bone tissue engineering	[163]
	α-TCP/collagen	MC3T3-E1 cells	The scaffold showed good mechanical properties and cellular activities	Bone tissue engineering	[164]
	collagen type I/agarose with sodium alginate	Primary chondrocytes	Addition of collagen or agarose had an impact on gelling behavior and improving mechanical strength. The collagen facilitated cell adhesion, accelerated cell proliferation, and enhanced the expression of cartilage-specific genes, (*Acan*, *Sox9*, and *Col2a1*)	Bone tissue engineering	[165]
	Fibrin and wollastonite	Loaded with rabbit BMSCs	Possible extensive regeneration of both cartilage and subchondral bone induced by in vivo transplantation of the scaffolds	Osteochondral tissue	[166]
	CS/PCL	dECM coating/WJMSCs seeding	Improved osteogenic differentiation in vitro and bone regenerative potential in vivo	Bone	[167]
	PCL/β-TCP	dECM coating/MC3T3-E1 seeding	Improved osteogenic differentiation in vitro and bone regenerative potential in vivo	Bone	[168]

Table 4. *Cont.*

Technology	Materials	Cells/Growth Factors	Outcomes	Application	References
Electrospinning	Graphene-incorporated electrospun PCL/gelatin	PC12 cells	Good cell attachment and proliferation	Nerve tissue engineering	[169]
	PCL/collagen	Human endometrial stem cells seeding	Good cell attachment and proliferation Higher wettability, attachment, and proliferation rates of hEnSCs on the PCL/collagen scaffold	Skin	[170]
	Polyhydroxybutyrate-co-hydroxyvaletare (PHBV) containing bredigite	-	Bredigite nanoparticles increased the mechanical properties, biodegradability, and bioactivity of the scaffolds	Bone tissue	[171]
	PLLA/β-TCP	hMSCs seeding	Enhanced water uptake ability, in vitro bio-mineralization, and bioactivity promoted by the incorporation of β-TCP	Bone	[172]
Electrospinning combined with 3D bioprinting	PCL	Laden with L929 mouse fibroblasts	Multi-layered structures—3D scaffolds—with loosely packed nanofibers, with better surface wettability (when compared to the 2D scaffolds)	Not defined	[173]
Electrospinning electro-spraying	PCL/HAp	Murine embryonic cell seeding	High capacity to guide the migration of differentiated bone cells throughout the cavities and the ridge of the scaffolds	Bone regeneration	[174]
	PCL/gelatin and multi-walled carbon nanotubes (MWNTs)	Adult rabbit chondrocytes seeding	Increased hydrophilicity and tensile strength, and higher bioactivity and slower degradation rate due to presence of MWNTs; 99% antibacterial properties against gram-positive and gram-negative bacteria.	Cartilage tissue	[175]
Phase separation process	Cartilage ECM-derived/PLGA-β-TCP-collagen type I	BMSCs seeding	Enhanced OC regeneration. Chondro and osteogenic-induced BMSCs with independent environments	Osteochondral tissue	[116]

BMSCs: bone marrow stem cells; ECM: extracellular matrix; HA: hyaluronic acid; hMSCs: human mesenchymal stem cells; hASCs: human adipose stem cells; PCL polycaprolactone; PLLA: poly-L-lactic acid; SF: silk fibroin; TCP: tricalcium phosphate.

5. Scaffold-Free Approaches

Although classical tissue engineering is based on a combination of cells, scaffolds and signals, scaffold-free techniques have emerged. They exploit the cells' capability of synthetizing tissues and responding to signals, offering advantages over traditional scaffold-based techniques. Cell viability is increased because they do not use electric fields, elevated temperatures or toxic chemicals [176]. Scaffold-free approaches provide a biomimetic microenvironment allowing cell communication and the maintenance of cell phenotypes in order to increase ECM production [177–180]. Tissue regeneration can happen rapidly, because there is no need for scaffold degradation. They also do not cause immune rejection in the host [181]. The advantages of scaffold-free technology over classical scaffold-based tissue engineering make them promising solutions for use in clinical practice in the near future.

5.1. Self-Organization Process

The self-organization process produces organized tissues with the use of external forces, such as physical manipulation or thermal input [182,183]. Cell-sheet engineering, pellet culture aggregates or spheroids are examples of self-organization.

5.1.1. Cell-Sheet Engineering

Cells are cultured in monolayers either on functionalized substrates or on thermo-responsive polymers. In the first case, they are removed via the mechanical or enzymatic cleavage of their cell-matrix attachments to the surface; in the second case, they are removed through the change in conformation induced by the variation in temperature [184]. The last method preserves the cell-matrix–binding interactions. After that, monolayers go through the process of tissue fusion. Through tissue fusion, isolated cell populations make contact and adhere [185]. The fusion of multiple cell-sheet layers can be used to generate tissues of greater thickness in order to replicate the architecture of a target tissue [186].

5.1.2. Pellet Culture

Pellet culture consists in the centrifugation of cells inside a conical-shaped tube, and then cell pellets are cultured in a medium in order to cause cell differentiation and ECM deposition [187,188]. This method fails in creating tissues with mechanical properties suitable for clinical use.

5.1.3. Aggregate Culture

Aggregate cultures are based on various methods that induce aggregate formation. The aggregate cells lie in an environment that can induce cell differentiation, ECM synthesis, and the formation of neotissue. These methods, like pellet culture, are not suitable alone for in vitro biomimetic tissue engineering, because they produce small cell aggregates. In any case, these approaches can be used in tissue engineering with other technologies for the maintenance of many cell types [189,190].

Self-organizing musculoskeletal tissues show structural features and mechanical functions similar to those of the native tissues [191,192]. Donnelly et al. [193] showed that the physiology and function of muscle can be improved in vitro using an electrical stimulation bioreactor. Electrical stimulation applied to monolayers in a culture caused an increase in protein synthesis, while the stimulation of three-dimensional engineered muscle improved force production and excitability.

5.2. Self-Assembling Process

The self-assembling process produces tissues with spontaneous organization without the influence of external energy. These tissues are characterized by the use of a non-adherent substrate to minimize the tissue's free energy, sequential phases that reassume native tissue formation, tissue constructs with clinically relevant size and morphology, and functional properties like those of native tissue [194].

This process has been used especially in articular cartilage tissue engineering [195]. The differential adhesion of surface-bound molecules and differential interfacial tension are mechanisms that contribute to the explanation of self-assembly.

Williams et al. [196] produced scaffold-free constructs and implanted them into the hind limb of a rat along the sciatic nerve. After one week in vivo, the engineered constructs showed phonotypical modification: a developing capillary system, an epimysium-like outer layer of connective tissue, and an increase in myosin-heavy chain content. In addition, they increased in contractile strength, and no signs of immune rejection were observed.

Carosio et al. [197] engineered three-dimensional vascularized skeletal muscle tissue and implanted it in place of the extensor *digitorum longus* muscle in mice, restoring the functionality of the damaged muscle.

The advantages of scaffold-free techniques consist in the native tissue integration, enhanced ECM deposition and direct mechanic transduction, and the avoidance of the release of harmful by-products. They also avoid issues of cytotoxicity caused by the processing conditions required for the production of some biomaterials. By avoiding the use of synthetic materials, biocompatibility issues are mitigated.

These technologies present some limitations. The engineered tissues have to present the same mechanical characteristics as the tissue that needs to be repaired. Dennis and Kosnik [197] produced a self-assembling skeletal muscle tissue in which the myotubes remained arrested in an early developmental state due to the absence of signals to promote the expression of adult myosin isoforms. This limitation can be resolved by the development of a vascular network in vitro [198].

The needs of the cell source are also an important issue. Stem cells are an attractive cell source, since they can differentiate into different cells and tissues, so co-cultures of primary cells and stem cells should be explored [199]. The problem is that, in many cases, they don't totally differentiate into the target tissue, compromising the properties of the neo-tissue. Another limitation for the creation of functional muscle tissue is the time required for myoblasts and fibroblasts to assemble a robust tissue through ECM. Even if the process is faster than the scaffold-based approaches, it takes almost a month to assemble an implantable muscle–tendon construct in vitro. For this reason, scaffold-free constructs could be more appropriate for chronic reconstruction, rather than the acute repair of traumatic injuries.

6. Clinical Use of Tissue Engineered Products

The progress in the field of tissue engineering led to the development of tissue-based products that could be a valid alternative to conventional approaches in clinical practice. The development of a tissue engineered product is complex and time consuming, because it requires the application of legal and regulatory frameworks established by health care agencies. A marketing authorization (MA) by the competent authority is necessary for commercialization. These depend on the nation in which they are intended to be marketed [200]. Currently, only 12 tissue-based products have been authorized worldwide [201] (Table 5).

Table 5. Tissue-based products authorized worldwide.

Name (MA Holder)	Therapeutic Indication	Jurisdiction
Spherox (CO. DON AG)	Articular cartilage defects of the femoral condyle and the patella of the knee up to 10 cm^2	European Union
MACI (Vericel Denmark ApS.)	Full-thickness cartilage defects of the knee of 3–20 cm^2	European Union
CHONDROCELECT (TiGenix N.V.)	Cartilage defects of the femoral condyle of the knee of 1–5 cm^2.	European Union
Holoclar (Holostem Terapie Avanzate S.R.L)	Moderate to severe limbal stem cell deficiency due to physical or chemical ocular burns.	European Union
MACI (Vericel Denmark ApS)	Full-thickness cartilage defects of the knee with or without bone involvement in adults.	Unied States of America
GINTUIT (Organogenesis, Inc.)	Topical treatment for vascular wound bed postsurgery with mucogingival conditions in adults	United States of America
Carticel (Vericel Denmark ApS)	Cartilage defects of the femoral condyle, in patients who have had an inadequate response to a prior surgical repair procedure	United States of America
HeartSheet (TerumoCorporation, Ltd.)	Severe heart failure	Japan
JACC (Japan Tissue Engineering Co., Ltd.)	Osteochondritis and traumatic cartilage defects	Japan
JACE (Japan Tissue Engineering Co., Ltd.)	Treatment for severe burns Giant congenital melanocytic nevus Dystrophic and junctional epidermolysis bullosa	Japan
Kaloderm (Tego Science, Inc.)	Second-degree burn Diabetic foot ulcer	South Korea
Holoderm (Tego Science, Inc.)	Second- and third-degree burns	South Korea

7. Conclusions and Future Directions

Tissue engineering studies different techniques to produce synthetic tissues with the same architectural, structural and functional properties as native tissues. In the last few decades, it has made significant progress in creating functional tissues that are able to replace the ones damaged by age, disease or trauma. Scaffold-based and scaffold-free approaches have been developed and studied over the years.

The main goal is to produce a material that is able not only to replicate the structural components of the extracellular matrix of the native tissue, but also the interactions with cells, regulating adhesion, proliferation and differentiation. This presents a challenge because the production of an engineered tissue is a multistep process which consists of multiple components, and acting on one of its properties can negatively affect the others. Different types of natural and synthetic biomaterials, used alone or in combination, have been developed for musculoskeletal tissue engineering.

Even if the development and maturation of the musculoskeletal system are difficult to reproduce in laboratory, deepening the knowledge of the processes regulating endogenous regeneration will be critical for the development of engineered materials. This could help us to understand the interactions between materials and cell environments in the tissue, in order to improve their effectiveness after implantation in vivo.

For example, a scaffold that provides mechanical stability, fiber alignment and controlled bioactive molecules is able to cause functional skeletal muscle regeneration. These scaffolds should be engineered in order to promote vascularization and innervation, which are necessary for normal muscle function and regeneration.

Engineered muscle tissue has to be biocompatible in order to permit muscle regrowth without immune reactions. It also has to be scaled up in order to replace clinically-relevant volumes of tissue. Studies in vivo could be more effective than in vitro to obtain biocompatible and large-enough tissue. The development and standardization of the appropriate

animal models are needed in order to create valuable long-term results and allow clinical application.

The simultaneous use of scaffold and scaffold-free approaches is a promising method, depending on the characteristics of the tissue that needs to be repaired. The choice of the method should be based on the knowledge of the development of the tissue in vivo, in order to use an approach that is as close as possible to the biological process. For example, the phases of the self-assembling process are similar to the development of articular cartilage in vivo.

Further research into the biological mechanisms at the basis of muscular endogenous regeneration could guide new approaches that can be used in skeletal muscle tissue engineering. The ultimate aim of tissue engineering is to synthetize neo tissues with a high level of complexity that present the exact features of native tissue, in order to regenerate it.

To date, the commercialization and clinical use of tissue-engineered devices is still poor. This is due to scientific and technical challenges, but also regulatory ones. Although the current research shows promising results, long-term studies are necessary in order to evaluate the implant–tissue interactions and turn them into a clinical strategy. The clinical translation of these products has led to the creation of regulatory schemes by regulatory bodies. Tissue-engineered products follow different regulatory approaches depending on the jurisdiction in which they have to be marketed. The creation of common regulatory schemes and market authorization requirements could accelerate their commercialization and use in clinical practice.

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