Biodegradable synthetic scaffolds for tendon regeneration

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Summary

Tissue regeneration is aimed at producing biological or synthetic scaffolds to be implanted in the body for regenerate functional tissues. Several techniques and materials have been used to obtain biodegradable synthetic scaffolds, on which adhesion, growth, migration and differentiation of human cells has been attempted. Scaffolds for tendon regeneration have been less frequently proposed, because they have a complex hierarchical structure and it is very difficult to mimic their peculiar mechanical properties. In this review, we critically analyzed the proposed materials and fabrication techniques for tendon tissue engineering and we indicated new preparation processes, based on the use of supercritical fluids, to produce scaffolds with characteristics very similar to the native tendon structure.

Key words: cells cultivation, supercritical fluids, synthetic scaffolds, tendon tissue engineering.

Introduction

Tissue regeneration can be performed using biological scaffolds obtained from mammalian tissues or by synthetic scaffolds. The preparation of biological scaffolds requires a series of processes that include cleaning, lipids and fat removal, disruption of cellular and DNA materials and sterilization. In every case, they are not autologous tissues and their integration with the receiving tissue can be problematic, including inflammatory response in the host. For all these reasons, there is a noticeable interest in developing synthetic extracellular matrices, used to produce the self-regeneration of functional tissue to be implanted in the body. In this review, synthetic biodegradable scaffolds to be used for tendon regeneration will be considered.

The production of biodegradable scaffolds and their colonization/transformation by human cells, is currently investigated for many biomedical applications, including bones, musculoskeletal tissues, nerves, blood vessels, etc.[1]. This strategy has been largely studied in the attempt of inducing bone regeneration[2,3]; but, only in some cases materials and processes aimed at tendon regeneration have been proposed. Indeed, this target is particularly difficult to be obtained, due to the special characteristics of the tendon structure, that can be summarized as:

- presence of nanometric collagen fibrils, that are uniaxially oriented;
- fibrils organization in fiber bundles, according to a hierarchical structure;
- intrinsic elasticity of the tendon structure, that is necessary to exert its functions;
- progressive transition from tendon to bone at the osteo-tendinous junction and from tendon to muscle at the myotendinous junction[4].

Moreover, the proposed artificial structures should be biodegradable at a given rate during tendon regeneration and allow (specialized or stem) cells: adhesion, growth, migration and differentiation. Materials and techniques that can be successful in producing the scaffold should possess all the above described characteristics, thus, mimicking the natural extracellular matrix (ECM).

From the point of view of materials, various biodegradable polymers have been proposed in the literature for tendon tissue engineering (TTE), but only some techniques have been tested among those proposed for other tissues regeneration processes. Electrospinning and freeze drying have been proposed, since these techniques can produce nano/micro structures that can be compatible with the native tendon organization.

The scope of this work is a critical analysis of the materials and of the proposed fabrication techniques for the production of tendon scaffolds and possible alternatives will be proposed that take into a full account the special characteristics of this body structure.

Materials

Materials proposed for TTE can be divided in natural and synthetic polymers, and have also been the subject of some previous reviews[5-7].

Among *natural polymers*, collagen derivatives and some polysaccharides have been proposed for TTE, since they are among major components of the natural tendon ECM;

collagen derivatives are hydrophilic polymers and can support cell adhesion and proliferation. However, they present a relatively poor processability and mechanical properties are far from the ones required in tendon regeneration. Polysaccharides, such as chitin and chitosan, have been traditionally used for hard-tissue regeneration, but have sometimes also been proposed for TTE application.

Among *synthetic polymers,* polyesters, biodegradable polymers like polyglycolic acid (PGA), polylactic acid (PLA) and their copolymer have been proposed for TTE. The major advantage of these polymers is that their degradation products, glycolic and lactic acids, are metabolites present in the human body and their mechanical characteristics are superior with respect to the ones of natural polymers. The main disadvantage is that degradation products are acidic and can produce inflammation reactions; moreover, their hydrophobic structure could give problems for cell adhesion.

Electrospinning

A typical electrospinning apparatus is mainly formed by a syringe pump, a high voltage power supply, a spinneret (typically a syringe capped with a blunt tip needle) and a grounded collector placed at a fixed distance from the needle tip. The polymer solution/suspension is drawn into the spinneret and charged with an electric potential. Once reached a critical value of the electric potential, the electrostatic repulsion of the polymer solution/suspension overcomes the surface tension at the tip of the needle and a jet of polymer is drawn out. When the solution comes into contact with air, evaporation of the solvent produces dry micro or nanofibers, that deposit on the collector surface. Fiber composition and diameter can be modified varying the polymer and its concentration in the liquid solution. Fiber alignment can be increased using a mandrel whose tangential and rotational velocity can be adjusted.

Many polymers and their blends have been electrospun into nanofibrous matrices[8-10]. Cross-linking procedures are required in the case of hydrophilic polymers to obtain a more stable-non hydrophilic structure. Newton et al.[11] studied the importance of cross-linking on the structure of electrospun collagen cross-linked with glutaraldehyde at different degrees, using an ethanol-based solvent system. Many electrospinning works for tissue engineering applications have been published[12-15]; but, only a limited number of them is dedicated to soft tissues related materials[8]. The most studied electrospun biopolymers used to obtain nanofibrous scaffolds, considered potential constructs for tendon tissue engineering have been collagen[9,10,16-21], gelatin[10,18,19], elastin[10,16], chitosan, PLGA[19,22-24], PLLA[25,26] and combinations of couples of them[1,17,25,27]. Matthews et al.[16] electrospun type I and type III collagen dissolved at various concentrations in 1,1,1,3,3,3 hexafluoro-2-propanol (HFP). A matrix composed of 100 nm collagen fibers was obtained. They proposed that, by controlling fiber orientation, it is possible to tailor mechanical properties of the matrix. Similar papers, in which electrospinning of collagen, elastin or gelatin was obtained using HFP or trifluoroethanol, were published by other authors[9,18,19]. The mechanical properties of a single electrospun fiber have been measured, in a dedicated paper written by Carlisle et al.[21].

Liu et al.[27] electrospun PLGA plus collagen in HFP, producing 250 nm average nanofibers. They found that the produced membranes were functionally active in response to human fibroblasts and were effective as wound-healing accelerators in early-stage wound healing. Chen et al.[1] electrospun collagen and chitosan blends in HFP/trifluoroacetic acid mixtures, producing an average fiber diameter of about 400-700 nm. The results of cell behavior on scaffolds, showed that both endothelial cells and smooth muscle cells proliferated on or within the nanofibers.

A critical paper on the electrospinning of collagen was published by Zeugolis et al.[19]; they demonstrated that electrospinning of collagen using fluoroalcohols denatures the polymer producing gelatin, that is the degradation product of collagen. To demonstrate this denaturation, the authors analyzed the electrospun polymers observing that they were not crystalline, not triple-helical, not quarter-staggered arranged and had denaturation temperature similar to gelatin. They concluded that the use of the organic solvents based electrospinning fails in maintaining the relevant properties of collagen!

To avoid the use of organic solvents, Buttafoco et al.[17] electrospun meshes of collagen and/or elastin (diameter from about 200 to 600 nm) from aqueous solutions. To spin homogeneous and continuous fibers, poly (ethylene oxide) (PEO) and sodium chloride (NaCl) were added to the solutions. To avoid the use of glutaraldehyde (that is also cytotoxic), the cross-linking of the fibers was obtained using a mixture of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Preliminary *in-vitro* studies performed using smooth muscle cells seeded on the crosslinked scaffolds were performed and a layer of cells was observed after 14 days, on the surface of different meshes.

Bashur et al.[22] studied how diameter and orientation of electrospun PLGA fibers can affect morphology, orientation and proliferation of NIH 3T3 fibroblasts. Fiber diameter was varied from 140 nm to 3.6 μm and fiber orientation was introduced by electrospinning onto a drum rotating at surface velocities in the range 2-4.7 m/s. Cells were analyzed 2 days after seeding to characterize cell morphology, and 3, 7 and 14 days after seeding to determine cell number. In this study, fibroblast cell density was not sensitive to fiber diameter and grew well on all fiber topographies.

Moffat et al.[24] electrospun PLGA fibers using a mixture of N,N-dimethylformamide and ethyl alcohol. To fabricate unaligned nanofiber scaffolds, the collecting surface consisted of a stationary plate, whereas a custom rotating mandrel was used to produce aligned scaffolds. These authors evaluated the attachment, alignment, gene expression, and matrix elaboration of human rotator cuff fibroblasts on the aligned and the unaligned PLGA nanofiber scaffolds. Tendon fibroblasts proliferated on the nanofiber scaffolds over the 2-week culturing period, both on the aligned and unaligned structures.

Kumbar et al.[8] reviewed nanofibrous matrices in the re-

generation of various soft tissues including skin, blood vessels, tendon/ligament. They stated that electrospun matrices were, from a nanometric point of view, morphologically very similar to the human extra-cellular matrix. The papers explicitly dedicated to the electrospinning of polymers to obtain scaffolds for tendon tissue engineering (TTE) are a small subset of the previously cited manuscripts, since the most part cites only the generic possibility to regenerate soft tissues.

More complex structures based on electrospinning were in some cases produced. Barber et al.[26] produced aligned PLLA nanofibers by electrospinning and then fabricated braided nanofibrous scaffolds formed by 3, 4 or 5 aligned bundles of PLLA nanofibers. The fibers were electrospun from a solution of PLLA in HFP (15% wt polymer solution). To assure the alignment of the fibers the mandrel rotated at a linear velocity of 9.0 m/s. To standardize the amount of nanofibers in each of the braided nanofibrous scaffolds (BNFSs), the process was carried out for a total of 350 min per scaffold. Three groups of fibrous bundles were braided into BNFSs with 3, 4, or 5 bundles; once braided, the scaffolds were cut to a length of 27 mm and melted at each end to generate a cohesive construct. Scaffolds seeded with human mesenchymal stem cells (hMSCs) were cultured up to 21 days. When cultured on the BNFSs, hMSCs adhered, aligned parallel to the length of the nanofibers, proliferated and a tenogenic differentiation was obtained, when a mechanically stimulation in a multi-chamber bioreactor was performed.

Sahoo et al.[28-31] produced a hybrid fibrous "nano-microscaffold" system, combining knitted silk microfibers covered by electrospun PLGA nanofibers. They produced a biohybrid scaffold by coating bioactive basic fibroblast growth factor (bFGF)-releasing electrospun PLGA fibers over the surfaces of a knitted degummed microfibrous silk scaffold. The slowly-degrading silk microfibres ensured mechanical integrity, and the scaffold provided sufficient mechanical support, whereas the ultrafine fibrous PLGA coatings provided a temporary template for cell attachment, proliferation and new ECM deposition. Moreover, they demonstrated the feasibility of bFGF, releasing blend-electrospun nanofibers for TTE, evaluating bFGF bioactivity on BMSCs and proliferation and differentiation of seeded BMSCs into tendon fibroblasts. After seven days of culture, the scaffolds were rolled up into cylindrical ligament/tendon analogues. They observed that the released bFGF stimulated proliferation and tenogenic differentiation of the bone marrow stromal cells (BMSC).

Freeze drying

In some, limited, cases, freeze-drying has been proposed for tendon related tissue engineering. This process and related techniques is based on thermally induced phase variation of the solute[32]. Phase separation occurs due to solid-liquid demixing. Lowering of temperature below freezing point of the solvent induces phase separation of the solution into a solvent phase and a polymer rich phase[32]. The scope of freeze drying in this application is to try to preserve the micro-nanostructure of hydrogels formed by collagen and other polymeric materials that form a relevant part of the native ECM of many tissues. Lv et al.[33] prepared porous fibroin-based scaffolds from fibroin and collagen blends aqueous solutions using a freeze-drying method. Scaffolds were made water-stable with a methanol treatment, that induces the transition from random coil and -helix conformation to -sheet. Morphological analysis showed highly homogeneous and interconnected pores with pore sizes ranging from about 130 to 830 μm, depending on the fibroin concentration. Adhesion, spreading and proliferation of HepG2 cells on fibroin and fibroin/collagen blend scaffolds was observed to investigate the biocompatibility of the obtained structure. Results showed that fibroin/collagen scaffolds had a better biocompatibility than fibroin alone scaffolds.

Sloviková et al.[34] developed hybrid porous scaffold via lyophilisation of frozen hydrogels to be used for cartilage tissue engineering. The polymers used were collagens modified with various polymers: chitosan nanofibres, hyaluronic acid, copolymers based on poly (ethylene glycol), poly (lactic-co-glycolic acid), itaconic acid and hydroxyapatite nanoparticles. According to their results, scaffolds containing hydrophilic chitosan nanofibres had the highest swelling ratio; the faster degradation rate was observed for scaffolds containing synthetic copolymers. The addition of hydroxyapatite or hyaluronic acid to the collagen matrix increased the rigidity in comparison with the collagen-chitosan scaffold.

Verma et al.[32] synthesized polyelectrolyte complex (PEC) fibrous scaffolds. Scaffolds were synthesized from polygalacturonic acid and chitosan, using freeze drying. Scaffold pore sizes ranged between 5 and 20 μm and the thickness of the fibers was around 1-2 μm. Biocompatibility studies showed that scaffolds containing chitosan, polygalacturonic acid and hydroxyapatite, promoted cell adhesion and proliferation.

Wu et al.[35] prepared gelatin scaffolds crosslinked with glutaraldehyde, with a microtubular orientation by unidirectional freeze-drying technology. In this case, the gelatin solution was unidirectionally frozen in liquid nitrogen from the bottom to the top, and then, the solidified gelatin/water system was freeze-dried. Width and length of microtubules ranged from 50 to 100 μm and 100 to 500 μm, respectively. Morphological analysis showed that pores were not round in shape and were randomly distribuited; the porous structure looked less regular when the amount of crosslinking agent was high. Since gelatin scaffolds prepared by unidirectional freeze-drying had an anisotropic structure, they showed different compressive strength along the transverse and longitudinal directions, and the difference became more obvious as the gelatin concentration or the amount of crosslinking agent was increased. Rabbit cartilage cells were used as model cells for seeding and culturing these scaffolds. Results showed that gelatin scaffolds had good cell affinity and were beneficial for cell attachment and growth.

Zhang et al.[36] produced a 3-D macroporous gelatin/hyaluronic acid (HA) hybrid scaffolds by freeze-drying. Scaffolds were crosslinked to increase their structural stability and mechanical integrity. Morphological studies revealed a uniaxial orientation in the porous structure in the vertical section, and, depending on the ratio of gelatin to HA, scaffolds possessed different porous struc-

tures. To evaluate *in vitro* cytocompatibility, mouse L929 fibroblasts were seeded onto scaffolds; the results showed that the hybrid scaffolds did not induce cytotoxic effects and were suitable for cell growth. Moreover, authors found that cell adhesion and proliferation could be enhanced by increasing the gelatin content in HA-based scaffolds.
Caliari et al.[37] developed scaffold-membrane composites based on Collagen-Glycosaminoglycan (CG). CG membranes were produced by a solvent evaporative process, whereas aligned CG scaffolds were produced via freeze-drying. Scaffold-membrane composites were crosslinked with carbodiimide. The goal of this research was to create core-shell CG composites with high bioactivity and improved mechanical properties; indeed, the CG scaffold core maintained an open-pore structure (pore size: 243 ± 29 μm), conductive for cell penetration and efficient metabolite transport, whereas, the addition of the CG membrane shell, covering about 75% of the scaffold surface, significantly increased the core-shell composite tensile modulus. Moreover, these composites demonstrated the capability to support tendon cell attachment, out to 14 days at comparable levels to CG scaffolds alone.

Other techniques

Some authors used biological derived scaffolds for tissue engineering applications. For example, Whitlock et al.[38] employed a process combining decellularization and chemical oxidation to decellularize and modify the dense micro-architecture of the chicken tendons to increase the porosity and pore size of the scaffolds in comparison to native tendons.
Other authors studied how cellular response is influenced varying the morphological parameters of the scaffolds, using purchased scaffolds (gel and sponge). They evaluated the individual and combined effects of scaffold type, construct length and mechanical stimulation on *in vitro* implant stiffness. Results showed that changes in all three treatment factors improved the construct *in vitro* linear stiffness after 14 days in culture[39].
Inui et al.[40] textured poly(L-lactic acid) (PLLA) fibers to obtain two types of scaffolds to be used in tendon regeneration: a plain-woven PLLA scaffold with a smooth surface and a double layered PLLA scaffold with a smooth surface on one side and a rough surface on the other side. These scaffolds were implanted into the back muscles of rabbits and evaluated at three and six weeks after implantation. Both scaffolds showed linear cell attachment on their smooth surface.

Tendon-to-bone interface regeneration

The insertion of the tendon in the bone consists of three continuous regions in which the soft tissue transforms in fibro cartilage and, then, in bone. The fibro-cartilaginous region is further divided in calcified and non calcified regions. These complex transitions are essential for the biological fixation of the tendon and exhibit a gradient of structural and mechanical properties to transmit the complex loads between tendon and the bone insertion site. To mime these structures, complex scaffolds with engineered matrix inhomogeneities and structures are required[41-43].
An example of graded scaffolds with the production of composition gradients (instead of homogeneous scaffolds) was proposed by Erisken et al.[41], that used a twin-screw extruder coupled to an electrospinning apparatus to produce polycaprolactone (PCL) incorporated with tricalcium phosphate nanoparticles. Another attempt of mimicking the tendon-to-bone insertion was performed by Li et al.[42]; they used electrospinning with a linear gradient in calcium phosphate content to increase scaffold stiffness and influence cells activity.
Also the muscle-tendon junction is a critical part of tendon regeneration in which dense collagen fibers are progressively substituted by the more elastic muscle fibers, producing also in this case a gradient of structural properties. For example, Saxena et al.[43] incorporated myoblasts on a scaffold constituted by fibronectin hydrogel and polyglycolic acid.

Limits of the proposed techniques

Most of the works on TTE have been performed using electrospinning, trying to take advantage of the possibility to produce nanofibers resembling the natural ECM of tendons and using in many cases biopolymers that participate to ECM formation.
However, the fiber deposition by electrospinning is intrinsically random and rotating or translating mandrels can only mitigate this situation. Moreover, there is also a limit on the thickness of the constructs obtained: above several microns thickness, the ground (receiving) plate can get substantially insulated. Some authors[20] also analyzed electrospun collagen and found that it was denatured, due the use of aggressive organic solvents. It is also very important to note that this technique produces scaffolds that have poor mechanical properties that are far from the ones required to mime tendon properties. At last, the hierarchical structure, typical of tendons, is also missing.
To try to overcome some of the described limitations, hybrid/complex processes have been in some cases proposed, using multibundles of electrospun materials[26] or proposing a substrate on which electrospun fibers were deposited[28-31], as in the case of knitted silk proteins as support for electrospun polymer.
In the case of freeze dried structures, starting from hydrogels of natural ECM proteic materials, the nanostructure of the hydrogel is partly or totally lost and also the obtained microstructure is disordered. Again the hierarchical tendon structure is absent and the mechanical properties are poor.

Proposals and perspectives

Considering the limitations and the open problems in TTE, it should be necessary to develop new preparation processes to produce scaffolds possessing with more (or all) the characteristics of the native tendon structure. From this point of view, a new approach consists of the

supercritical carbon dioxide (SC-CO_2) drying of natural (chitosan) or synthetic (Poly-l-lactide acid - PLLA) gels. These gels, when appropriately prepared, present a nanofibrous architecture[44,45]; but, they have been rarely considered for TTE applications as it is difficult to maintain this structure during the elimination of the solvents to produce the corresponding aerogels: the nanostructure collapses because of the surface tension exerted by the solvent during its elimination.

A supercritical drying procedure that has been tested on some polymers (chitosan, PLLA), has been successfully used to produce PLLA scaffolds, mimicking the bone-like structure with adequate mechanical resistance[46,47]. Human mesenchymal stem cells (hMSC) adhered, grew, migrated and differentiated during the cultivation in a perfusion reactor. They uniformly colonized all the structure, differentiated to osteoblasts, and some early bone formation proteins were consistently expressed[48]. In the overall, gels formation plus supercritical drying is a very flexible process and the formed material possesses the intrinsic nanofibrous structure that is the prime requisite for a tendon scaffold. However, the nanofibers are randomly organized within the gel. Therefore, for TTE application some modifications of the bone-like structures, previously proposed, are required.

To obtain bundles of nanofilaments of selected length/diameter, mimicking primary and secondary tendon fiber bundles (chitosan or PLLA), gels can be produced in thin macrofibers (around 500 □m diameter), that will confine the nanofibers in a limited transverse diameter. Collagen and/or elastin will be added to the synthetic structure to improve its elastic properties. The various bundles will be coated with collagen/elastin, so as to mimic tendon hierarchical organization and will be fused altogether.

Using this technique it is also possible the formation of hybrid scaffolds with the ends characterized by a gradient structure, to attempt the regeneration of the osteotendinous junction, using different cell populations and different scaffold substructures. This result can be obtained because of the high flexibility of the supercritical gel drying process that allows to freely modulate nano, micro and macro structure of the polymeric scaffold.

The scaffolds produced can be processed in a perfusion reactor, cultivating tenocytes or tendon precursor stem cells, to verify and to optimize the interaction between the tissue engineered structure and the cells.

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