Annals of Biomedical Engineering, Vol. 38, No. 6, June 2010 *(© 2010)* pp. 2142–2154
DOI: 10.1007/s10439-010-0046-y

Tissue Engineering Strategies for the Regeneration of Orthopedic Interfaces

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(Received 27 January 2010; accepted 11 April 2010; published online 27 April 2010)

Associate Editor Michael S. Detamore oversaw the review of this article.

Abstract—A major focus in the field of orthopedic tissue engineering is the development of tissue engineered bone and soft tissue grafts with biomimetic functionality to allow for their translation to the clinical setting. One of the most significant challenges of this endeavor is promoting the *biological fixation* of these grafts with each other as well as the implant site. Such fixation requires strategic biomimicry to be incorporated into the scaffold design in order to re-establish the critical structure–function relationship of the native soft tissue-to-bone interface. The integration of distinct tissue types (e.g. bone and soft tissues such as cartilage, ligaments, or tendons), necessitates a multi-phased or stratified scaffold with distinct yet continuous tissue regions accompanied by a gradient of mechanical properties. This review discusses tissue engineering strategies for regenerating common tissue-to-tissue interfaces (ligament-to-bone, tendon-to-bone, or cartilage-to-bone), and the strategic biomimicry implemented in stratified scaffold design for multi-tissue regeneration. Potential challenges and future directions in this emerging field will also be presented. It is anticipated that interface tissue engineering will enable integrative soft tissue repair, and will be instrumental for the development of complex musculoskeletal tissue systems with biomimetic complexity and functionality.

Keywords—Insertion site, Enthesis, Interface tissue engineering, Strategic biomimicry, Co-culture, Stratified scaffold, Multi-phased scaffold.

INTRODUCTION

Trauma and degeneration of orthopedic tissues are commonly associated with injuries to soft tissues such as cartilage which cover the surface of articulating joints, as well as ligaments and tendons, which connect bone to bone, and muscle to bone, respectively. Tissue-to-tissue interfaces such as those that connect soft tissue (e.g. ligament, tendon, or cartilage) to bone are ubiquitous in the body, and they are essential for facilitating synchronized joint motion and musculoskeletal function. These critical junctions between distinct tissue types are, however, prone to injury and unfortunately not re-established following standard surgical repair methods. Failure to regenerate the intricate tissue-to-tissue interface has been reported to compromise graft stability and long-term clinical outcome,[20,40,61] consequently, the *biological fixation* or *integrative* repair of soft tissues remain a significant clinical challenge.

In the past decade, tissue engineering[32,70] has emerged as a promising approach to orthopedic repair. Utilizing a combination of cells, growth factors and/or biomaterials, the principles of tissue engineering have been readily applied to the formation of a variety of connective tissues such as bone, cartilage, ligament, or tendon *in vitro* and *in vivo*. More recently, the emphasis in the field of orthopedic tissue engineering has shifted from tissue formation to tissue function,[8] with a concentration on imparting biomimetic functionality to orthopedic grafts and enabling their translation to the clinic. Presently, a significant barrier to clinical translation is how to achieve *biological fixation* or functional integration of the tissue-engineered orthopedic grafts,[46] be it bone, ligaments, or cartilage, either with each other and/or with the host environment.

This review focuses on current biological fixation strategies aimed at engineering tissue-to-tissue interfaces, as the elegant design methodologies developed from tissue engineering can be readily applied to regenerate the aforementioned critical junction

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between soft tissue and bone. The nature of this interface tissue engineering challenge is rooted in the complexity of the musculoskeletal system and the structural intricacy of both hard and soft tissues. These tissues, each with a distinct cellular population, must operate in unison to facilitate physiologic function and maintain tissue homeostasis. It is thus not surprising that the transition between various tissue types is characterized by a high level of heterogeneous structural organization that is crucial for joint function. For example, ligaments or tendons with direct insertions into subchondral bone exhibit a multi-tissue transition consisting of three distinct, yet continuous, regions of ligament, fibrocartilage, and bone.[5,13,82] The fibrocartilage interface is further divided into non-calcified and calcified regions. This interface with a gradient of mechanical properties has a number of functions, from mediating load transfer between two distinct types of tissue to sustaining the heterotypic cellular communications required for interface function and homeostasis.[5,40,85] In light of this complexity, functional tissue engineering must incorporate *strategic biomimicry* to facilitate the formation of the tissue-to-tissue interface and enable seamless graft integration.

The detailed mechanisms that drive the development of the tendon-to-bone and ligament-to-bone interface are not fully understood. Published studies have investigated the change of cell type and collagen fiber composition of the interface as a function of age, which have yielded valuable clues to its development. Immunohistologial evaluation of the ligament-to-bone interface of postnatal rats revealed that, at birth, the majority of proliferating cells at the interface were near the ligament region of the insertion site.[52] These cells produced type I and II collagen and slowly developed into fibrochondrocyte-like cells until a month after birth. After that time, rapid longitudinal growth of the ACL took place. These observations suggest that fibrochondrocytes at the ligament-to-bone interface may originate from the ligament. In the case of injury, it has been well established that the native interface is not regenerated between soft tissue and bone. Studies of graft-to-bone healing post ACL reconstruction have provided insights into the neotissue formed when soft tissue is juxtaposed against bone. Liu *et al.* examined the morphology and matrix composition of the interface during the early tendon-to-bone healing process,[37] and found that by 2 weeks after reconstruction, the tendon attached to the bone with scar tissue filling the tendon-to-bone junction. This scar tissue had reorganized into a dense connective tissue matrix by 1 month, with predominantly fibroblasts present. After 6 weeks, contraction of the interface was prominent and significantly less type I collagen was found in the remodeling matrix; however, type II collagen became detectable. As such, no well-defined fibrocartilage interface was observed over time. This study correlates with the biomechanical studies of Rodeo *et al.* and demonstrates that surgically juxtaposing soft tissue and bone does not spontaneously result in the regeneration of the fibrocartilaginous interface.[62] Collectively, these studies suggest that cell source is a significant consideration in interface regeneration, and moreover, the differentiation of these cells into interface-relevant populations is likely driven by both biochemical and mechanical factors during both development and healing.

In addition to developmental cues, characterization studies[5,7,45,49,55,60,71,79,85] of the structure–function relationship inherent at the soft tissue-to-bone insertion have revealed remarkable organizational similarities between many tissue-to-tissue interfaces, as they often consist of a multi-tissue, multi-cell transition as described above for ligaments or tendons, as well as being associated with a controlled distribution of non-mineralized and mineralized interface regions which, along with other structural parameters such as collagen fiber organization, are reported to be responsible for engineering a gradient of mechanical properties progressing from soft tissue to bone. These observations have provided invaluable clues for the design of biomimetic scaffolds for engineering the tissue-to-tissue interface. Specifically, a stratified or multi-phased scaffold will be essential for recapturing the multi-tissue organization observed at the soft tissue-to-bone interface. In order to minimize the formation of stress concentrations, the scaffold should exhibit phase-specific structural and material properties, with a gradual increase in mechanical properties across the scaffold phases, similar to that of the native tissue. To this end, introducing spatial control over mineral distribution on a stratified scaffold can impart controlled mechanical heterogeneity similar to that of the native interface. Compared to a homogenous structure, a scaffold with pre-designed, tissue-specific matrix inhomogeneity can better sustain and transmit the distribution of complex loads inherent at the multi-tissue interface. It is emphasized that while the scaffold is stratified or consists of different phases, a key criteria is that these phases must be interconnected and pre-integrated with each other, thereby supporting the formation of distinct yet continuous multi-tissue regions. Furthermore, interactions between interface-relevant cells serve important functions in the formation, maintenance, and repair of interfacial tissue.[83] Therefore, precise control over the spatial distribution of these cell populations is also critical for multi-tissue formation and interface regeneration. Consideration of these biomimetic parameters will collectively enable the design of stratified scaffolds optimized for

promoting the formation and maintenance of controlled matrix heterogeneity and tissue-to-tissue integration.

This review will highlight current tissue engineering efforts in the regeneration of three characteristic connective tissue interfaces, namely, the ligament-to-bone, tendon-to-bone, and the cartilage-to-bone interface, focusing on biomimetic scaffold design and biomaterial- as well as cell-based strategies to engineer a functional gradient of mechanical properties that approximates that of the native interface. Each section will begin with a brief description of the current understanding of the requirements for biomimetic and functional interface scaffold design, which have been distilled through characterization and structure–function understandings of the native interface. This is followed by a brief review of stratified scaffold and gradient-based scaffold design currently researched in soft tissue-to-bone interface tissue engineering. Lastly, potential challenges and future direction in this rapidly expanding area of functional tissue engineering will be discussed.

STRATIFIED SCAFFOLD DESIGN FOR LIGAMENT-TO-BONE INTERFACE TISSUE ENGINEERING

The site of anterior cruciate ligament (ACL) insertion into bone (Fig. 1a) is a classic example of a complex soft tissue-to-bone interface consisting of spatial variations in cell type and matrix composition resulting in three distinct tissue regions of ligament, fibrocartilage, and bone,[5,13,82] whereby the fibrocartilage region is further divided into mineralized and non-mineralized regions. From a structure–function perspective, the complex organization of this interface is likely related to the nature and distribution of mechanical stress experienced at the region. It has been reported that matrix organization at this site is optimized to sustain both tensile and compressive stresses.[45,85] For example, fibrocartilage is often localized in anatomical regions subjected to compressive loading.[45] These region-specific mechanical properties facilitate a gradual transition in strain across the insertion and provide valuable cues for ligament-to-bone interface scaffold design.

The multi-tissue transition from ligament to bone at the ACL-to-bone interface represents a significant challenge for functional interface tissue engineering. Initial attempts to improve ligament graft to bone fixation focused on augmenting the surgical graft with a material that would encourage bone tissue growth. For example, Tien *et al.* used calcium phosphate cements to fill the tendon-to-bone junction in a rabbit ACL reconstruction study and found that the addition of this ceramic helped to improve bone growth and organization.[81] In a similar study, the addition of injectable tricalcium phosphate (TCP) cement to the interface region in a canine ACL reconstruction model

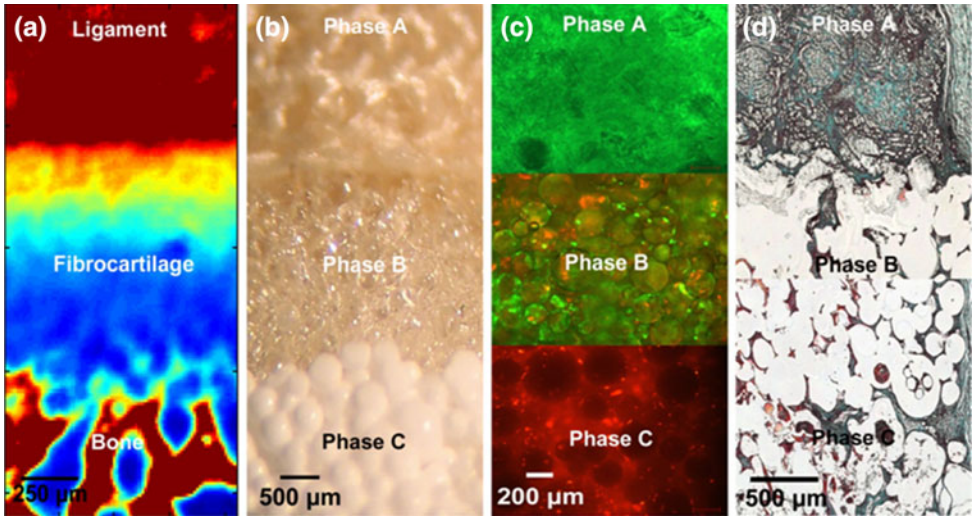

FIGURE 1. Biomimetic stratified scaffold design for ligament-to-bone interface tissue engineering. (a) Fourier transform infrared spectroscopic imaging (FTIR-I) revealed that relative collagen content is the highest in the ligament and bone regions, with a decrease in collagen across the fibrocartilage interface from ligament to bone (neonatal bovine, bar 250 μm, with blue to red representing low to high collagen content, respectively). (b) A tri-phasic stratified scaffold designed to mimic the three interface regions (ligament, fibrocartilage and bone, bar 200 μm). (c) *In vitro* co-culture of fibroblasts and osteoblasts on the tri-phasic scaffold resulted in region-specific cell distribution and cell-specific matrix deposition. Fibroblasts (Calcein AM, green) were localized in Phase A and osteoblasts (CM-DiI, red) in Phase C, and both osteoblasts and fibroblasts migrated into Phase B over time (bar 200 μm). (d) *In vivo* evaluation of the tri-phasic scaffold tri-cultured with fibroblasts (Phase A), chondrocytes (Phase B), and osteoblasts (Phase C) showed abundant host tissue infiltration and matrix production (wk 4, Modified Goldner Masson Trichrome Stain, bar 500 μm).

resulted in more organized bone tissue formation than the uncemented control.[25] Using a different approach, Mutsuzaki *et al.* soaked tendon grafts in a series of solutions which coated the tendons with a calcium phosphate layer prior to implantation.[51] The modified graft was tested using a rabbit ACL reconstruction model, and it was found that the precoated tendons enhanced healing and promoted integration. Other approaches to improve bone tunnel osteointegration have included the addition of periosteum grafts to the region of the graft that interacts with the bone[9,31,56,87] and growth factors such as rhBMP-2.[63] Although these methods have improved osteointegration between the ACL graft and the bone tunnel, these efforts do not result in the regeneration of the fibrocartilage interface. Moreover, single-factor systems do not fully mimic the complexity of events at the healing interface, thus a systematic and controlled approach which uses a biomimetic stratified scaffold to direct the growth of the multi-tissue interface may overcome these shortcomings as it can be designed to recapitulate the inherent complexity of this multi-layered ligament-to-bone interface (Fig. 1).

The ideal scaffold has several functions including supporting the growth and differentiation of the relevant cell populations, directing cellular interactions, and promoting the formation and maintenance of controlled matrix heterogeneity. The scaffold must also exhibit a gradation in mechanical properties, mimicking the native insertion site, with magnitudes comparable to those of the ligament-to-bone interface. In addition, the scaffold must be biodegradable to be gradually replaced by living tissue. Lastly, for *in vivo* integration, the engineered graft must be easily adaptable within current ACL reconstruction grafts, or pre-incorporated into the design of the ligament replacement grafts.

Traditional efforts for developing tissue-engineered grafts for ACL reconstruction have centered on regenerating the ligament proper,[3,16,17] with more recent studies focusing on the restoration of the anatomic ACL–bone interface.[12,14,27,38] Cooper *et al.* reported on a multi-phased design of a synthetic ACL graft fabricated from 3D braiding of polylactide-*co*-glycolide fibers, with a ligament proper as well as two bony regions.[12] *In vitro*[38] and *in vivo*[14] evaluation demonstrated scaffold biocompatibility, healing, and mechanical strength in a rabbit model. Recently, Altman *et al.* developed a multi-region, porous knitted silk ACL graft which was evaluated in a goat model with promising results.[4] Using a cell-based approach, Ma *et al.* reported that it is possible to form bone–ligament–bone constructs by introducing engineered bone segments to ligament monolayers.[43] Specifically, the monolayer rolled up around the bone pieces and self-assembled into a ligament–bone–ligament construct. Paxton *et al.* utilized a similar methodology with promising results when evaluating the use of a poly(ethylene glycol) hydrogel incorporating HA and the RGD cell-adhesion peptide to engineer functional ligament-to-bone attachments.[57] These novel ACL graft designs represent significant improvement over single-phased ACL grafts, and the next step is to address the challenge of graft integration with bone, by considering the fibrocartilage interface in the ACL scaffold design.

To this end, Spalazzi *et al.* pioneered the design of a tri-phasic scaffold (Fig. 1) for the regeneration of the ACL-to-bone interface.[72,73] Modeled after the native insertion, the scaffold consists of three distinct yet continuous phases, each engineered for a specific tissue region found at the interface: Phase A is designed with PLGA (10:90) mesh for fibroblast culture and soft tissue formation, Phase B consists of PLGA (85:15) microspheres and is the interface region intended for fibrochondrocyte culture, and Phase C is composed of sintered PLGA (85:15) and 45S5 bioactive glass composite microspheres for bone formation.[39] The scaffold is innovative in that it is essentially a ''single'' scaffold system with three distinct yet continuous phases, intended to support the formation of the multi-tissue regions observed across the ACL–bone junction.

Interactions between interface relevant cell types (e.g. fibroblasts, chondrocytes, osteoblasts) on the tri-phasic scaffold have been evaluated both *in vitro*[73] and *in vivo*.[72] In order to form the ligament and bone regions, fibroblasts and osteoblasts were seeded onto Phase A and Phase C, respectively. This controlled cell distribution resulted in the elaboration of cell type-specific matrix on each phase of the scaffold, with a mineralized matrix detected only on Phase C, and an extensive type I collagen matrix found on both Phases A and B. *In vivo* evaluation[72] of co-culture of fibroblasts and osteoblasts on the tri-phasic scaffold revealed extensive tissue infiltration and abundant matrix deposition on Phase A and Phase C. Cell migration and increased matrix production and vascularization were observed on Phase B, the interface region. Moreover, tissue continuity was maintained across all the three scaffold phases. Interestingly, extracellular matrix production compensated for the decrease in mechanical properties accompanying scaffold degradation, and the phase-specific controlled matrix heterogeneity was maintained *in vivo*.[72]

In order to form a fibrocartilage interface-like tissue at the interface phase, Spalazzi *et al.* extended the *in vivo* evaluation of the above scaffold system to tri-culture,[73,74] including chondrocytes along with fibroblasts and osteoblasts.[72] Specifically, articular

chondrocytes were encapsulated in a hydrogel matrix and loaded into Phase B of the scaffold, while ligament fibroblasts and osteoblasts were pre-seeded onto Phase A and Phase C, respectively. At 2 months post-implantation, an extensive collagen-rich matrix was prevalent in all the three phases of the tri-cultured scaffolds (Fig. 1d). Moreover, a fibrocartilaginous region of chondrocyte-like cells embedded within a matrix containing types I and II collagen as well as glycosaminoglycans was observed. Interestingly, both cell shape and matrix morphology of the neo-fibrocartilage resembled that of the neonatal ACL–bone interface.[82] Moreover, the neo-fibrocartilage formed was continuous with the ligament-like tissue observed in Phase A as well as the bone-like tissue found in Phase C.[74]

These promising results demonstrate that biomimetic stratified scaffold design coupled with spatial control over the distribution of interface relevant cell populations can lead to the formation of cell type- and phase-specific matrix heterogeneity *in vitro* and *in vivo*, with a fibrocartilage-like interface formed in tri-culture. These observations not only validate the feasibility of the stratified scaffold for promoting biological fixation of ACL grafts to bone, but also highlight the potential for continuous multi-tissue regeneration on a single scaffold system. In terms of clinical application, the tri-phasic scaffold can be used to guide the re-establishment of an anatomic fibrocartilage interfacial region directly on soft tissue grafts. Specifically, the scaffold can be used as a graft collar during ACL reconstruction surgery, and the feasibility of such an approach was recently demonstrated in a study by Spalazzi *et al.*, where a mechanoactive scaffold system was formed based on a composite of poly-α-hydroxyester nanofibers and sintered microspheres.[74,75] It was observed that scaffold-induced compression of tendon grafts resulted in significant matrix remodeling and the expression of fibrocartilage interface-related markers such as type II collagen, aggrecan, and transforming growth factor-β3 (TGF-β3). These results suggest that the stratified scaffold can be used to induce the formation of an anatomic fibrocartilage interface directly on biologically derived ACL reconstruction grafts.

In summary, current strategies in ligament-to-bone interface tissue engineering first tackles the difficult problem of soft tissue-to-bone integration *ex vivo* by pre-engineering the soft tissue-to-bone interface through stratified scaffold design for mutli-tissue regeneration, and then focuses on the relatively less challenging task of bone-to-bone integration *in vivo*. Moreover, functional and integrative ligament repair may be achieved by coupling both cell-based and scaffold-based approaches.

STRATIFIED SCAFFOLD DESIGN FOR TENDON-TO-BONE INTERFACE TISSUE ENGINEERING

Similar to the ligament-to-bone interface, the tendon-to-bone interface displays a zonal distribution of extracellular matrix components.[5,6] Thus, the biomimetic scaffold design and multi-lineage cell culture methods previously discussed for the ligament-to-bone interface are also applicable for the regeneration of tendon-to-bone insertions, such as that of the rotator cuff tendons and bone. However, while tendon-to-bone and ligament-to-bone insertions are physiologically and biochemically similar, the tissue engineering strategy applied is expected to differ as the two interfaces do vary in terms of loading environment, mineral distribution, and surgical repair methods which would also influence the subsequent healing response.

The debilitating effect of rotator cuff tears coupled with the high incidence of failure associated with existing repair techniques[11,15,26] underscore the clinical need for functional solutions for integrative tendon-to-bone repair. In order to address this challenge, several groups have evaluated the feasibility of integrating tendon grafts with bone or biomaterials through the formation of an anatomic insertion site. By surgically reattaching the Achilles tendon to bone, Fujioka *et al.* reported that cellular reorganization occurred at the reattachment site, along with the formation of non-mineralized and mineralized fibrocartilage-like regions.[21] In addition, Inoue *et al.* were able to successfully promote supraspinatus tendon integration with a metallic implant using a bone marrow-infused bone graft.[27] These promising results indicate that the tendon-to-bone interface may be regenerated and underscore the need for functional grafting solutions that can promote biological fixation.

Based on the aforementioned characteristics of the interface, nanofiber scaffolds have been explored for tendon and tendon-to-bone interface tissue engineering applications.[44] The biomimetic potential and physiological relevance of nanofibers makes them advantageous for orthopedic tissue engineering. These scaffolds can be tailored to resemble the native tendon extracellular matrix as they exhibit a high surface area to volume ratio, permeability, and porosity.[34,35,44,58] In addition, nanofiber organization and alignment can be modulated during fabrication and used to guide cell response.[50] Recently, the potential of a degradable PLGA nanofiber-based scaffold system for rotator cuff repair was evaluated *in vitro*.[47] Moffat *et al.* examined the effects of nanofiber organization on fibroblast attachment and alignment as well as gene expression and matrix deposition.[47] It was reported that nanofiber orientation (aligned vs. unaligned) was the primary

factor guiding tendon fibroblast morphology, alignment, and integrin expression. Types I and III collagen, the dominant collagen types of the supraspinatus tendon, were synthesized on the nanofiber scaffolds, and it was shown that their deposition was also controlled by the underlying fiber orientation. Furthermore, scaffold mechanical properties, directly related to fiber alignment, decreased as the polymer degraded but remained within range of those reported for the native supraspinatus tendon.[28] Building upon these promising results, Moffat *et al.* also designed a composite nanofiber system of PLGA and hydroxyapatite (HA) nanoparticles in an effort to regenerate both the non-mineralized and mineralized fibrocartilage regions of the supraspinatus insertion site.[48] The responses of interface-relevant cell populations such as osteoblasts, chondrocytes, and fibroblasts have been examined on the polymer–ceramic composite nanofibers with promising results (Fig. 2).

In addition to engineering the tendon-to-bone interface, the muscle–tendon interface is another critical research area for integrative tendon repair, although to date, it has been relatively underexplored. As the tendon joins the muscle to bone, thus the myotendinous junction (MTJ), which connects muscle to tendon, acts as a bridge to distribute mechanical loads.[86] This interface consists of a band of fibroblast-laden, interdigitating tissue that connects the dense collagen fibers of the tendon to the more elastic muscle fibers while displaying a gradient of structural properties.[80] Current tissue engineering approaches, as demonstrated by Swasdison and Mayne, include the culturing of myoblasts in collagen gels *in vitro* to form contractile muscle constructs with fibrils that terminate in a manner similar to the native MTJ.[76,77] Adopting a cell-based approach, Larkin *et al.* evaluated a novel self-organizing system for *in vitro* myotendinous junction formation by co-culturing skeletal muscle constructs with engineered tendon constructs. Interestingly, upregulation of paxillin was observed at the neo-interface, and the MTJ formed was able to sustain tensile loading beyond the physiological strain range.[33]

The aforementioned studies demonstrate the promise of the biodegradable scaffold system for tendon-to-bone interface tissue engineering as well as the potential of harnessing cellular interaction for engineering both tendon-to-bone and muscle-to-tendon interface and ultimately, functional and integrative tendon repair.

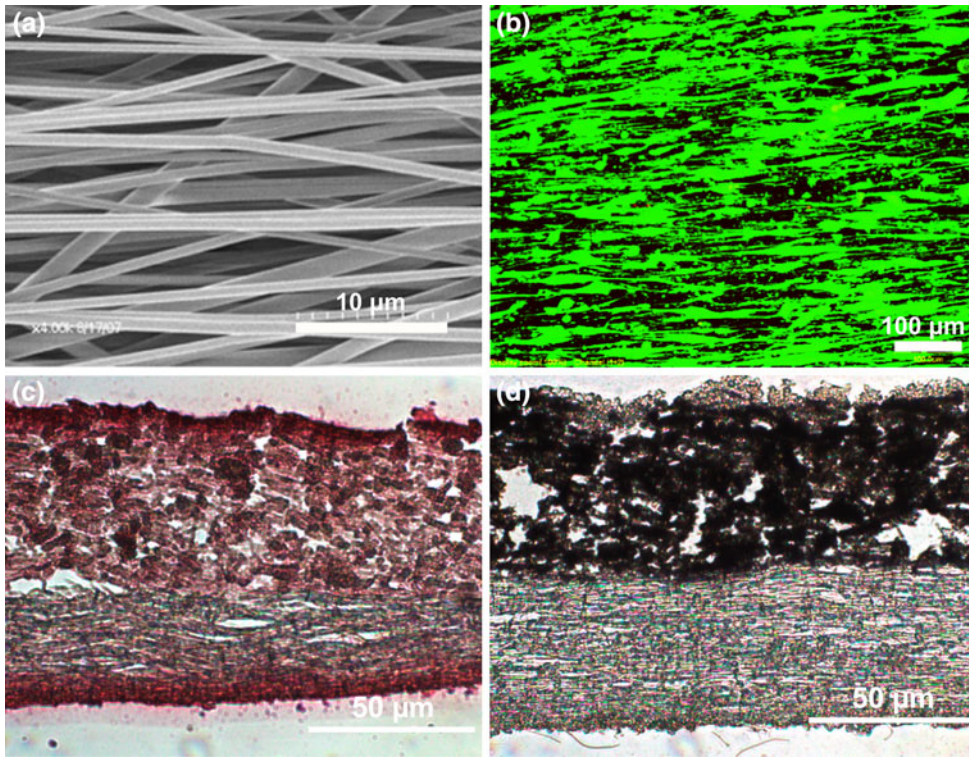

FIGURE 2. Nanofiber-based scaffold for tendon-to-bone integration. (a) SEM micrograph depicting aligned fiber organization (4000×, bar 10 μm). (b) Fluorescence microscopy of human rotator cuff fibroblasts cultured on aligned nanofibers, cellular attachment and alignment is directed by the underlying substrate morphology. Histological analysis of a bi-phasic scaffold after 3 weeks of subcutaneous implantation, exhibiting (c) collagen matrix deposition and in-growth (picrosirius red, 20×, bar 50 μm) and (d) region-dependent distribution of mineral (von Kossa, 20×, bar 50 μm).

STRATIFIED SCAFFOLD DESIGN FOR CARTILAGE-TO-BONE INTERFACE TISSUE ENGINEERING

Another common tissue-to-tissue interface of the musculoskeletal system is the osteochondral interface which acts as a barrier between articular cartilage and subchondral bone. The ultrastructure of articular cartilage can be divided into three regions: the tangential (surface) zone, the transitional (middle) zone, and the radical (deep) zone. Directly below the deep zone is the calcified cartilage region containing hypertrophic chondrocytes embedded in a densely mineralized matrix, which constitutes the osteochondral interface.[7,19,42,54] As with the ligament-to-bone and tendon-to-bone interfaces, the heterogeneity at the cartilage-to-bone interface is important for load bearing and force distribution[55] (Fig. 3). Thus, the regeneration of this controlled heterogeneity is a critical component of integrative and functional cartilage tissue engineering strategies.

Stratified scaffold design has been researched for osteochondral tissue engineering,[22,24,53,64,88] with the first generation of scaffolds consisting of two distinct cartilage or bone regions joined together using either sutures or sealants.[22,64] Schaefer *et al.* seeded bovine articular chondrocytes on polyglycolic acid (PGA) meshes and periosteal cells on poly(lactic-*co*-glycolic acid) (PLGA)/polyethylene glycol foams, and subsequently sutured the separate constructs together at 1 or 4 weeks after seeding.[64] Integration between the two scaffolds was observed to be superior when brought together at week one instead of four, suggesting the importance of *immediate* osteoblast–chondrocyte interactions for phase-to-phase integration. Similarly, Gao *et al.* seeded mesenchymal stem cells (MSCs) stimulated with TGF-β1 for chondrogenic differentiation on a hyaluronan sponge, and MSCs stimulated with osteogenic media on a porous calcium phosphate scaffold.[22] These scaffolds were then joined by a fibrin sealant and implanted subcutaneously in syngeneic rats, with continuous collagen fibers observed between the two scaffolds at 6 weeks following implantation. Shortly after, Sherwood *et al.* designed a *continuous* biphasic scaffold and evaluated chondrocyte response

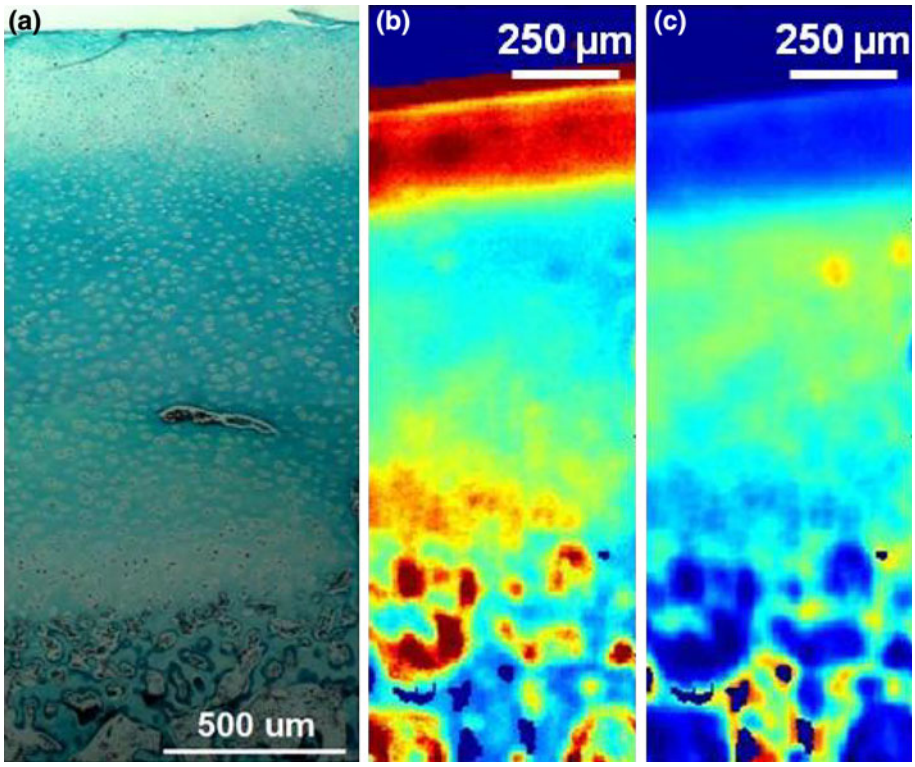

FIGURE 3. Structure of the osteochondral interface. (a) Histological evaluation of the osteochondral interface region revealed a collagen-rich tissue interface region (neonatal bovine, bar 500 μm, Goldner's Masson Trichrome Stain). (b) Fourier transform infrared spectroscopic imaging (FTIR-I) revealed that relative proteoglycan content is the highest on the articular surface, with a decrease in proteoglycan content across the interface region from cartilage to bone (neonatal bovine, bar 250 μm, with blue to red representing low to high proteoglycan content, respectively). (c) Fourier transform infrared spectroscopic imaging (FTIR-I) revealed that relative collagen content is the highest in the interface region with a decrease in proteoglycan content on the articular surface and the subcondral bone (neonatal bovine, bar 250 μm, with blue to yellow representing low to high proteoglycan content, respectively).[30]

on the scaffold.[66] Utilizing a sequential photo-polymerization technique, Alhadlaq and Mao formed a bi-layered human mandibular condyle-shaped osteochondral construct based on polyethylene glycol-diacrylate hydrogel. The top hydrogel layer contained MSC-derived chondrocytes while the bottom layer contained MSC-derived osteoblasts.[1] After 12 weeks *in vivo*, distinct cartilaginous and osseous regions were observed in a subcutaneous SCID mouse model, with histological integration between the two layers. Swieszkowski *et al.* also reported similar results from hyaluronan/ceramic and PCL/tricalcium phosphate composite scaffolds seeded with MSCs,[22,78] and these observations have been confirmed by other studies with MSC culture on biphasic scaffolds.[10,65]

Collectively, these pioneering studies demonstrate the feasibility of engineering multi-tissue formation (cartilage and bone) on a multi-phased scaffold; the next step is to incorporate the osteochondral interface into this scaffold design. To this end, several groups have reported on stratified scaffold designs that mimic the structural organization of the native osteochondral interface. Lu *et al.* and later Jiang *et al.* evaluated 3D osteoblast–chondrocyte co-culture on a biomimetic, continuous multi-phased osteochondral construct consisting of a hydrogel-based cartilage region, a polymer–ceramic composite microsphere bone region, and an interfacial region consisting of a hybrid of the hydrogel and polymer–ceramic composite.[29,41] It was found that osteoblast and chondrocyte co-culture on this scaffold system supported the formation of distinct yet continuous cartilaginous and osseous matrices, with pre-designed integration between these regions achieving a mineralized interfacial region within which direct osteoblast–chondrocyte interactions are encouraged. In this tri-phasic scaffold system, the calcified interface region was pre-incorporated into scaffold design by the inclusion of a mineralized scaffold phase consisting of osteoblasts seeded on the polymer–ceramic microsphere-based scaffold infused with chondrocyte-laden agarose hydrogel. The formation of a calcified cartilage-like zone has also been investigated by directly seeding deep zone articular chondrocytes on a calcium polyphosphate scaffold.[2] As the cartilaginous tissue forms, the chondrocytes infiltrated into the superficial region of the calcium polyphosphate scaffold, resulting in three distinct regions—cartilage, mineralized cartilage, and a proteoglycan-rich layer directly above the bone scaffold. More recently, Harley *et al.* reported on design strategies associated with the formation of an osteochondral scaffold with cartilage and bone regions as well as a continuous osteochondral interface-like region in between these two phases.[23] While it remains to be validated *in vitro* and *in vivo*, this multi-phased design is intended to promote scaffold-mediated integration within an osteochondral defect site.

In summary, stratified scaffold design in conjunction with the use of interface relevant cell populations as well as mesenchymal stem cells is a promising approach to engineer the osteochondral interface. Results from the studies highlighted above demonstrate the importance of the cartilage-to-bone interface, thus advanced scaffold design aimed at the formation of integrated osteochondral grafts must take into consideration the regeneration of a functional and stable interface region between these distinct tissue types.

GRADIENT SCAFFOLD DESIGN FOR INTERFACE TISSUE ENGINEERING

The stratified scaffolds described here have been designed to mimic the multi-tissue transition inherent at many tissue-to-tissue interfaces. As such, given the well-documented changes in tissue type in the interface structural organization and the need to support the related tissue-specific cell phenotypes, there is emerging interest in designing scaffolds with a gradient of properties.[67] These novel scaffolds with either a compositional[18,69] or chemical factor[36,59] gradient have the potential to address the need to recapitulate the complex transition of mechanical and chemical properties that occurs in interface regions, offering direct regional control and allow for scaffold heterogeneity that can mimic the complex native interface. In contrast to the previously discussed stratified designs, these scaffolds consist of relatively gradual and continuous transition in either compositional or mechanical properties.

Utilizing an ethanol-based solvent evaporation technique, Singh *et al.* developed 3D multi-phased PLGA microsphere scaffolds with continuous macroscopic gradients in stiffness. Structural gradients were produced by incorporating a high stiffness nano-phase material ($CaCO_3$ or TiO_2) into portions of the microspheres during the scaffold fabrication process.[69] Preliminary *in vitro* studies showed that structurally homogenous scaffolds fabricated using this method can support the attachment of human umbilical cord-derived MSCs.[69] Utilizing a novel extrusion method, Erisken *et al.* fabricated nanofiber scaffolds containing a mineral gradient or a region-dependent calcium phosphate concentration. *In vitro* studies showed that culturing MC3T3 cells on these scaffolds led to the formation a gradient of calcified matrix within 4 weeks.[18] More recently, Li *et al.* formed a variable calcium phosphate coating on a nonwoven mat of gelatin-coated PCL nanofibers by incubating the scaffolds in a simulated body fluid (SBF) with physiologic or supra-physiologic calcium and/or

phosphate ion concentrations. The results showed that this gradient in mineral content resulted in spatial variations in scaffold stiffness and affected the number of MC3T3 cells that adhered to the scaffold.[36] Exploring a novel alternative approach, Phillips *et al.* achieved a gradient of mineralized matrix deposition by seeding fibroblasts onto collagen scaffolds with a composition gradient of retrovirus coating for the osteogenic transcription factor RUNX2. It was found that by controlling the spatial patterning of transcription factor expression, fibroblasts were stimulated to deposit a gradient of mineralized matrix on the collagen scaffold and this matrix heterogeneity was maintained *in vivo*.[59]

In addition to scaffolds with compositional gradient, Singh *et al.* have also designed microsphere scaffolds with spatially controlled growth factor release profiles.[68] This method offers a unique advantage by avoiding the relative high temperatures required in classic sintering techniques which can compromise growth factors bioactivity as they are incorporated. In addition, Singh *et al.* also formed cell-encapsulating microsphere scaffolds using a subcritical CO_2 fabrication technique. Both human umbilical cord MSCs and porcine chondrocytes were used to demonstrate potential of this fabrication method in cartilage tissue engineering applications.[69] It is promising that this technique resulted in high initial cell viability, although long-term cell response still remains to be evaluated.

When comparing stratified and gradient scaffold systems, the primary advantage of the aforementioned compositional gradient-based scaffolds resides in their ability to more closely mimic the native transition in composition, and potentially result in a gradation of functional properties which facilitates load transfer between distinct tissue types. As such, given the structural complexity and relatively small scale of the interface, which averages from 50 μm to 1 mm in length depending on species and age,[13,82,84] it remains a significant design challenge for the gradient scaffolds to recapitulate the micro- to nano-scale gradients that have been reported at the interface. In other words, design parameters for interface regeneration must be prioritized and *strategic biomimicry* be adopted in functional interface scaffold design. To this end, the multi-phased scaffold represents a simpler approach, whereby a gradation of composition and functional properties is established by engineering the specific tissue region of interest and pre-integrating these tissue regions through stratified design. For these scaffolds, it is anticipated that cellular contributions will play a pivotal role in mediating the regeneration and homeostasis of the gradation of compositional and mechanical properties inherent at the interface. Consequently, controlling cellular response via co-culture, tri-culture, or growth factor distribution on the multi-phased scaffolds is a critical strategy to enable the development of cell-mediated local gradients on a physiologically relevant scale.

SUMMARY AND FUTURE DIRECTIONS

This review has provided an overview of current concepts in interface tissue engineering, focusing on strategies for the design of scaffolds with a gradation of mechanical and structural properties aimed at the regeneration of the complex tissue-to-tissue interface. Specifically, these multi-phased scaffolds have been designed to mimic the structure and function of the native soft tissue-to-bone interface while employing spatial control over heterotypic cell interactions and supporting the formation of integrated multi-tissue systems. The vast potential of stratified scaffold systems is evident from the *in vitro* and *in vivo* evaluations described here for the integrative repair of cartilage, ligament, and tendon injuries. Moreover, these novel scaffolds are capable of multi-tissue regeneration by mediating heterotypic cellular interactions, and can be further refined by incorporating well-controlled compositional and growth factor gradients, as well as the use of biochemical and biomechanical stimulation to encourage tissue growth and maturation. Furthermore, functional and integrative soft tissue repair may be achieved by coupling both cell-based and scaffold-based approaches.

Clinically, it is anticipated that stratified scaffolds would significantly improve current soft tissue repair strategies by attaching to existing tissues and stimulating the formation of native insertion tissue in a controlled manner. While integration of osteochondral grafts with host tissue could be accomplished via simple layering techniques or press-fit implants to repair focal defects, the incorporation of stratified scaffolds with tendon and ligament tissue would require more complex methods. Specifically, in terms of ACL–bone integration, multi-phased scaffolds could be fabricated as a cylinder, so that it is inserted into the bone tunnels while encasing the ACL graft and promoting interface formation directly on the graft. In addition, fully integrative ACL grafts with distinct yet continuous ligament, interface, and bone regions can be developed. In terms of rotator cuff repair; scaffold patches could be fabricated to bridge the gap between torn tendon tissue and bone through interface generation, while surgically sutured to the tendon and potentially augment tendon repair as well.

It is emphasized that interface tissue engineering will be instrumental for the *ex vivo* development and *in vivo* regeneration of integrated musculoskeletal

tissue systems with biomimetic functionality; however, there remains a number of challenges in this exciting area. These include the need for a greater understanding of the structure–function relationship existing at the native tissue-to-tissue interface as well as the mechanisms governing interface development and regeneration. Furthermore, the *in vivo* host environment plus the precise effects of biological, chemical, and physical stimulation on interface regeneration must be thoroughly evaluated to enable the formation and homeostasis of the neo-interface. Physiologically relevant *in vivo* models are also needed to determine the clinical potential of the designed scaffolds.

Additional challenges remain to be addressed for successful clinical translation of stratified scaffolds including the integration of these scaffolds with the native tissue post-surgical reconstruction. This will require determining how existing tissues will attach to stratified scaffolds to direct tissue growth and re-establishing the native interfacial organization. In addition, as is evident in many of the reported studies, selection of multiple cell sources is typically necessary to ensure or enhance heterogeneous tissue formation. Clinical implementation of these scaffolds will require identifying an optimal cell source which is readily available, such as an adult stem cell source which can be quickly isolated and expanded. Translation of these findings may also require reassessing measurable outcomes to ensure adequate interface tissue formation following surgical intervention. Finally, to extend clinical translation of stratified scaffolds, methods for sterilization and potentially long-term shelf storage are needed.

In summary, regeneration of tissue-to-tissue interfaces through interface tissue engineering represents a promising strategy for achieving biological fixation and integrative soft tissue repair, using either biological or tissue engineering grafts. It is anticipated that these efforts will lead to the development of a new generation of functional fixation devices for orthopedic repairs as well as augmenting the clinical translation potential of tissue engineered orthopedic grafts. Moreover, by bridging distinct types of tissue, interface tissue engineering will be instrumental for the development of integrated musculoskeletal tissue systems with biomimetic complexity and functionality.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding support from the National Institutes of Health (AR056459 and AR055280), the Wallace H. Coulter Foundation, the National Science Foundation Graduate Fellowship (SDS), and the National Sciences and Engineering Research Council of Canada (XZ).

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