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Title: Design and fabrication of biomimetic multiphased scaffolds for ligament-to-bone fixation

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Keywords: Ligament-to-bone, multiphased scaffolds, fixation, PCL anchor, interface

Corresponding Author: Dr. Jiankang He,

Corresponding Author's Institution:

First Author: Jiankang He

Order of Authors: Jiankang He; Wenyou Zhang; Yaxiong Liu; Xiang Li; Dichen Li; Zhongmin Jin

Abstract: Conventional ligament grafts with single material composition can not effectively integrate with host bones due to mismatched properties and eventually affect their long-term function in vivo. Here we presented a multi-material strategy to design and fabricate composite scaffolds including ligament, interface and bone multiphased regions. The interface region consists of triphasic layers with varying material composition and porous structure to mimic native ligament-to-bone interface while the bone region contains polycaprolactone (PCL) anchor and microchanneled ceramic scaffolds to potentially provide combined mechanical and biological implant-bone fixation. Finite element analysis (FEA) demonstrated that the multiphased scaffolds with interference value smaller than 0.5mm could avoid the fracture of ceramic scaffold during the implantation process, which was validated by in-vitro implanting the multiphased scaffolds into porcine joint bones. Pull-out experiment showed that the initial fixation between the multiphased scaffolds with 0.47 mm interference and the host bones could withstand the maximum force of 360.31 ± 97.51 N, which can be improved by reinforcing the ceramic scaffolds with biopolymers. It is envisioned that the multiphased scaffold could potentially induce the regeneration of new bone as well as interfacial tissue with the gradual degradation of the scaffold and subsequently realize long-term biological fixation of the implant with the host bone.

Cover Letter

Jiankang He,
Ph.D. Associate professor
Tel.: 86-029-83395387 Fax: 86-029-83395387
Email: jiankanghe@mail.xjtu.edu.cn

November 13, 2014

Editorial Office, Materials Science & Engineering C

Dear Editor,

We would like to submit the enclosed manuscript entitled "Design and fabrication of biomimetic multiphased scaffolds for ligament-to-bone fixation**"** for publication in *Materials Science & Engineering C*.

In this manuscript, we presented a multi-material strategy to design and fabricate composite scaffolds including ligament, interface and bone regions potentially for effective ligament-to-bone fixation. The interface region consists of triphasic layers with varying material composition and porous structure to mimic native ligament-to-bone interface while the bone region contains polycaprolactone (PCL) anchor and microchanneled ceramic scaffolds to potentially provide combined mechanical and biological implant-bone fixation. Finite element analysis (FEA) demonstrated that the multiphased scaffolds with interference value smaller than 0.5mm could avoid the fracture of ceramic scaffold during the implantation process, which was validated by *in-vitro* implanting the multiphased scaffolds into porcine joint bones. Pull-out experiment showed that the initial fixation between the multiphased scaffolds with 0.47 mm interference and the host bones could withstand the maximum force of 360.31 ± 97.51 N, which can be improved by reinforcing the ceramic scaffolds with biopolymers. It is envisioned that the multiphased scaffold could potentially induce the regeneration of new bone as well as interfacial tissue with the gradual degradation of the scaffold and subsequently realize long-term biological fixation of the implant with the host bone.

We feel that the manuscript will be of relevance and interest to the readership of *Materials Science & Engineering C* for the research scope of biomaterials, tissue engineering and regenerative medicine. We appreciate your careful consideration and should look forward to further correspondence.

Sincerely,

Jiankang He

State key laboratory for manufacturing systems engineering
Xi'an Jiaotong University
Xi'an 710049, China

***Suggest Reviewers**

Suggested reviewers:

Prof. Chua Chee Kai
Division of Systems and Engineering Management, School of Mechanical and Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798
Tel.: +65-6790-5486
Email: MCKCHUA@ntu.edu.sg

Prof. Yanan Du
Department of Biomedical Engineering, Tsinghua University
Beijing, 100084, China
Email: duyanan@tsinghua.edu.cn
Tel.: +86-10-62781691
Fax: +86-10-62773380

Prof. Jack Zhou
Department of Mechanical Engineering and Mechanics, Drexel University
3141 Chestnut Street， Philadelphia, PA 19104, USA
Email: zhoug@coe.drexel.edu

Design and fabrication of biomimetic multiphased scaffolds for ligament-to-bone fixation

Jiankang He [a*], Wenyou Zhang [a,b], Yaxiong Liu [a], Xiang Li [c], Dichen Li [a] and Zhongmin Jin [a]

[a] State key laboratory for manufacturing systems engineering, Xi'an Jiaotong University, Xi'an 710049, China

[b] Qingdao R&D Institute, Xian Jiaotong University, Qingdao 266300, China

[c] Department of Health Sciences and Technology, ETH Zurich, Switzerland

* Corresponding author: Fax/Tel: 029-83395387, E-mail: jiankanghe@mail.xjtu.edu.cn

Abstract: Conventional ligament grafts with single material composition can not effectively integrate with host bones due to mismatched properties and eventually affect their long-term function *in vivo*. Here we presented a multi-material strategy to design and fabricate composite scaffolds including ligament, interface and bone multiphased regions. The interface region consists of triphasic layers with varying material composition and porous structure to mimic native ligament-to-bone interface while the bone region contains polycaprolactone (PCL) anchor and microchanneled ceramic scaffolds to potentially provide combined mechanical and biological implant-bone fixation. Finite element analysis (FEA) demonstrated that the multiphased scaffolds with interference value smaller than 0.5mm could avoid the fracture of ceramic scaffold during the implantation process, which was validated by *in-vitro* implanting the multiphased scaffolds into porcine joint bones. Pull-out experiment showed that the initial fixation between the multiphased scaffolds with 0.47 mm interference and the host bones could withstand the maximum force of 360.31 ± 97.51 N, which can be improved by reinforcing the ceramic scaffolds with biopolymers. It is envisioned that the multiphased scaffold could potentially induce the regeneration of new bone as well as interfacial tissue with the gradual degradation of the scaffold and subsequently realize long-term biological fixation of the implant with the host bone.

Key Words: Ligament-to-bone, multiphased scaffolds, fixation, PCL anchor, interface

1. Introduction

In vivo, anterior cruciate ligament (ACL) strongly integrates with bone tissue through a transition fibrocartilage interface, which plays an important role in minimizing stress concentrations during load transmission between the dissimilar tissues of ligament and bone [1, 2]. As one of the most frequently injured ligaments, ACL has very limited self-healing potential. Ligament grafts were commonly used to restore the biological functions of injured ACL. For most of the existing ligament grafts, they were clinically inserted into bone tunnels and mechanically fixed with host bones using interference screws. The mismatch between soft ligament grafts and hard bone tissues in mechanical and biological properties has been found to induce the formation of scar tissues and eventually hinder the osteointegration of implanted ligament grafts, which has been considered as one of the important reasons for graft failure [3]. Although several strategies have been developed to promote the osteointegration of ligament grafts by incorporating osteoinductive factors (*e.g.*, bone morphogenetic protein, hydroxyapatite) or altering graft microarchitectures at the graft insertion site [4-7], long-term stability of graft-bone fixation might depend on the regeneration of multiple tissue interface.

Interfacial tissue engineering has shown great promise in regenerating ligament/bone interface by using graded or multiphased scaffolds either with distinct material compositions or seeded with multiple cell types [8-11]. Spalazzi et al [12, 13] developed a triphasic scaffold that contained soft ligament region, intermediate fibrocartilage region and hard bone region to mimic native ligament insertion. The triphasic scaffolds pre-seeded with multiple cell types of osteoblast, chondrocytes and fibroblast were found to support zonal cellular distribution, facilitate phase-specific matrix deposition and enable multitissue regeneration *in vitro* and *in vivo*. Electrospun nanofibrous scaffolds with material or/and structure gradients were also investigated to facilitate the regeneration of multiple tissue interface [14-17]. Although some promising progress has been achieved by using multiphased or gradient scaffolds to regenerate ligament/bone interface, few strategies have been reported so far to effectively integrate

these multiphased scaffolds with ligament grafts as well as consider the initial fixation of ligament grafts with host bones.

Here we sought to fabricate a multiphased ligament-bone composite scaffold mainly including biodegradable polycaprolactone (PCL) anchor, microfluidic bone tissue-engineered scaffold, microfibrous ligament graft and biomimetic interface, which could provide initial mechanical implant-bone fixation and potentially facilitate new bone ingrowths and interface regeneration for long-term biological fixation. A multiple-step molding process was proposed to fabricate the multiphased scaffold. The structure of PCL anchor was theoretically and experimentally optimized to enhance initial implant-bone fixation and simultaneously avoid the crack of ceramic scaffold during implantation process. The microstructure as well as the initial mechanical fixation of the multiphased scaffolds to host bone was investigated.

2. Materials and methods

2.1 Materials

β-tricalcium phosphate (β-TCP) was purchased from Shanghai Bio-lu Biomaterials Company (China). Poly (L-lactide-co-glycolide) (PLGA) and PCL were purchased from Jinan Daigang Biomaterial Company (China). PLGA solution with different concentration was prepared in a mixed solvent of 1, 4 dioxane and deionized water with a volume ratio of 9:1. Poly-lactide acid (PLA) microfibers with an average diameter of 50 μm were purchased from Haining Fibers & Non-wovens Company (China). All other chemicals were local products with analytical grade unless specifically mentioned.

2.2 Design of the multiphased scaffold with biomimetic ligament-to-bone interface

Natural ligaments were indirectly connected with bony tissues through non-calcified and calcified fibrocartilage layers (**Figure 1a**). To mimic such characteristic features, the scaffold (**Figure 1b**) was designed to mainly contain three regions: ligament region, bone region and ligament-to-bone interface region. The ligament

region consisted of parallel PLA microfibers. The bone region was comprised of microchanneled β-TCP ceramic scaffolds and PCL anchor. The PCL anchor has a specific sawtooth structure that facilitates the implantation of the constructs and simultaneously prevents pull-out. The ligament region was mechanically bonded with the bone region through a triphasic structure. Phase A and B were designed to mimic the non-calcified and calcified fibrocartilage layer respectively. Phase C was directly inserted into the microchannels of bone region to fix the interface region with the bone region.

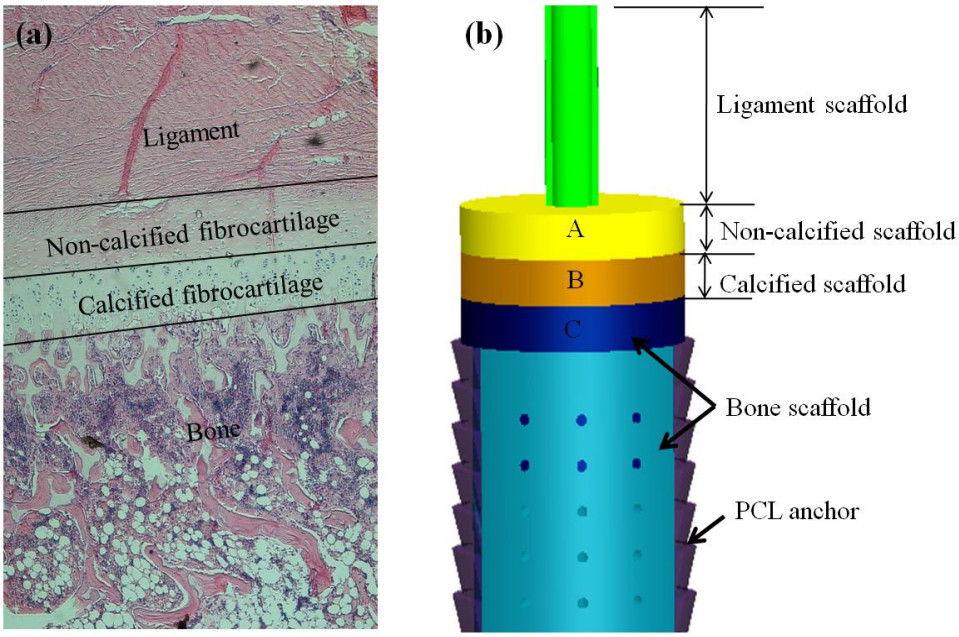

Figure 1 Design of the multiphased scaffolds with biomimetic ligament-to-bone interface. (a) Native ligament-to-bone interface, (b) specifically-designed multiphased scaffolds.

2.3 Finite element analysis (FEA) for the structure optimization of PCL anchor

When the multiphased scaffold is implanted into host bone, it depends on the interference fit between PCL anchor and host bone to provide the scaffold initial fixation (Supplementary Figure 1a). Since the bone ceramic scaffold is fragile and likely to fracture under relatively large compressive force, it is important to optimize the interference value, δ, between PCL anchor and host bone. In this study, FEA was employed to simulate the implantation process and predict the effect of different δ value (0.1, 0.3, 0.5 and 0.7mm) on the maximum

compressive stress of the ceramic scaffold. To simplify the simulation process, the scaffold was simplified to only include ceramic scaffold and PCL anchor and 1/4 symmetric model was used (Supplementary Figure 1b). Tetrahedron element was used for the implant and the mesh size was set at 0.6mm. For host bone, hexahedron element was used and the mesh size was set at 0.4mm. The friction coefficient between PCL anchor and host bone was set at 0.2. The elastic modulus for ceramic scaffold and PCL anchor was experimentally measured as 1228.9 ± 148.6 MPa and 237.4 ± 13.7 MPa respectively. The Poisson's ratio for ceramic scaffold and PCL was set at 0.28 and 0.47[18]. The elastic modulus and Poisson's ratio for host bone were set at 500 MPa and 0.25 [19].

2.4 Fabrication of the multiphased scaffold

A multi-step molding process was proposed to fabricate the multiphased scaffolds as shown in Figure 2. Briefly, microfluidic bone ceramic scaffolds were fabricated according to our previously developed indirect rapid prototyping methods [20, 21]. Ligament grafts were obtained by weaving PLA microfibers, the number of which determines the final tensile strength. The ligament graft was initially bonded with the bone ceramic scaffold as shown in Figure 2b. Stereolithography, a 3D printing technique, was employed to build the resin prototype of PCL anchor, based on which a PDMS mould with negative pattern was replicated. PCL material was melt in the PDMS mould at 190 ^{o}C and the ligament-bone bonded scaffold was pressed into the molten PCL as shown in Figure 2c. Since the diameter of the PDMS chamber is close to that of the bone scaffold, the molten PCL could only fill the predefined area as shown in Figure 2d. Ligament-bone scaffold with PCL anchor was obtained after solidification at room temperature (Figure 2e). To fabricated ligament-to-bone interface, another PDMS mould with cylindrical chamber was prepared and assembled with the ligament-bone scaffold as shown in Figure 2f. 20wt% PLGA solution with higher β-TCP mass content (PLGA: β-TCP =7:3) was slowly injected into the chamber to form zone C and part of the solution was allowed to flow into the predefined microchannels of bone ceramic scaffolds. 15wt% of PLGA solution with lower β-TCP mass content (PLGA: β-TCP =8.5:1.5) was gently added onto the top

layer of zone C to form zone B. Zone A was generated by adding 10wt% PLGA pure solution on the top of zone B as shown in Figure 2f-h. The whole construct (Figure2i) was subsequently placed in a -20 ^{o}C refrigerator to freeze the triphasic solutions together and then freeze-dried for 3 days to obtain the multiphased scaffolds (Figure 2j).

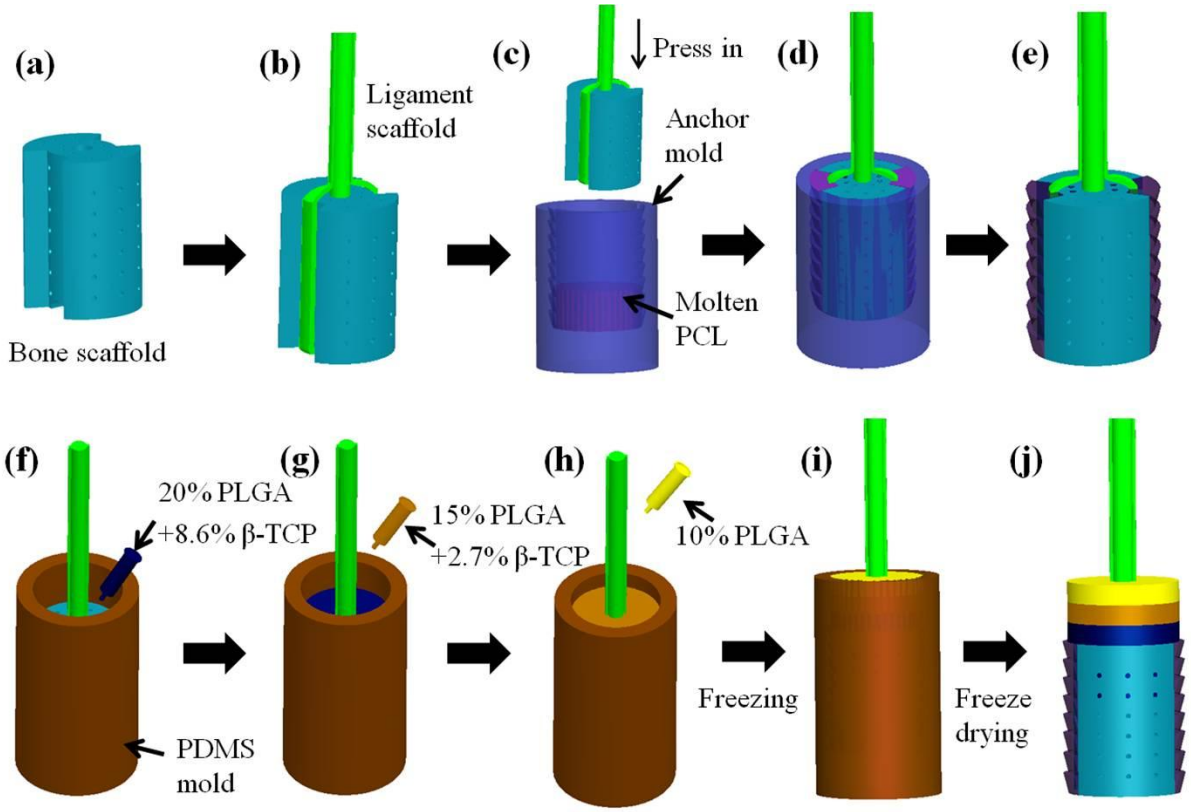

Figure 2 Schematic of the multi-step molding process to fabricate multiphased ligament-bone scaffolds with biomimetic interface.

2.5 Microstructural characterization of the multiphased scaffolds with biomimetic interface

To characterize the microstructure of the multiphased scaffold, the scaffold was sectioned, coated with platinum in a metal sputter (Explorer 14, Denton, America) and viewed with scanning electron microscope (SEM, SU8010, Hitachi). Micro-CT (Y. Cheetah, YXLON) was also used to observe the internal microchannels of bone ceramic scaffold.

2.6 *In-vitro* implantation of the multiphased scaffolds into host bone

To validate the FEA results for PCL anchor with different δ as well as the mechanical property of initial

implant-bone fixation, the multiphased scaffolds were implanted into host bones *in vitro*. A bone tunnel with diameter of 10 mm was drilled in a fresh porcine joint bone using an electric driller. An auxiliary device was specifically designed to facilitate the implantation of the multiphased scaffold into the bone tunnel (Supplementary Figure 2). Micro-CT was used to virtually observe the microstructures of the bone ceramic scaffold after being pressed in the host bone. Since dimensional errors exist in the fabrication of the multiphased scaffold as well as the drilling of the bone tunnel, the actual interference value δ was experimentally calculated by measuring the size of the fabricated scaffolds and the bone tunnels.

2.7 Mechanical property for the initial fixation of the multiphased scaffolds and host bones

To test the mechanical property for initial implant-bone fixation, a clamp was designed to mount the porcine femur joint implanted with multiphased scaffolds onto a tensile mechanical tester (CMTS503). The length of ligament part is 30mm [22] and the loading rate as 0.5 mm/min. The force-displacement curve was automatically recorded until the failure of the implant. Micro-CT was performed to observe the microstructure of the implant-bone construct after mechanical testing.

3. Results and discussion

Figure 3a shows the multiphased scaffolds with three different regions, namely ligament region, bone region and interface region. In the ligament region, PLA microfibers were arranged in parallel to mimic native collagen fibers. The bone region consists of β-TCP scaffolds with predefined microchannels and PCL anchor. The β-TCP scaffolds are expected to enhance new bone regeneration after implantation. Previous studies indicated that porous TCP scaffolds seeded with human bone marrow derived mesenchymal stem cells (hMSCs) exhibited comparable osteoinductive properties to autologous bone grafts *in vivo* [23, 24]. The PCL anchor was successfully fabricated around the bone ceramic scaffold in a predefined manner (Figure 3b-c), which can not only provide initial mechanical fixation between the implant and the host bone, but also tightly interact with bone scaffold to enhance

its compressive property for press-fit demands (Supplementary Figure 3).

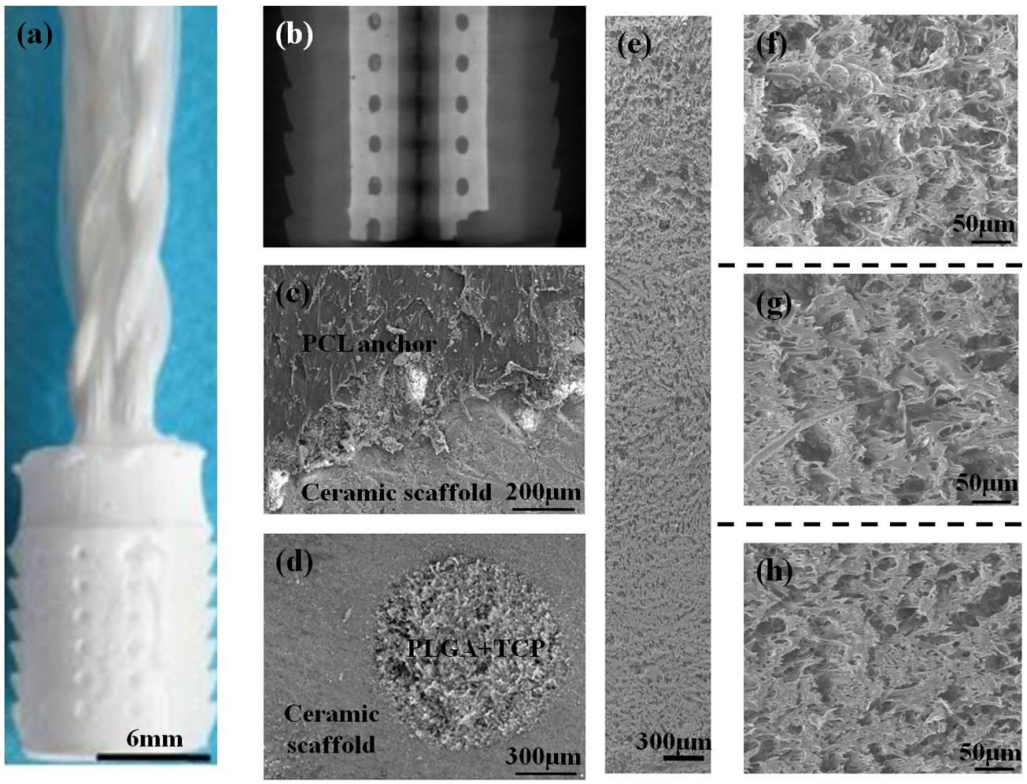

Figure 3 Microstructural characterization of the fabricated multiphased scaffolds. (a) Ligament-bone composite scaffold, (b) Micro-CT image of the bone region, (c) interface of PCL anchor and ceramic scaffold, (d) penetration of zone C into the predefined microchannels of ceramic scaffold, (e) porous microstructure of the triphasic ligament-to-bone interface, (f-h) high-magnification SEM images of zone A, zone B and zone C respectively.

Figure 3e-h shows the SEM images of microstructures at different zones of interface region. It can be seen that the triphasic zones were porous and no clear material interface was observed among different zone. The pore size gradually decreased from zone A to zone C, which might be due to the gradually increased PLGA and β-TCP concentrations. The average pore sizes in zone A, B and C were 130 ± 30.67μm, 69.5 ± 17.72 μm and 43.1 ± 11.59 μm respectively. Additionally, zone C was found to penetrate into the predefined microchannels to effectively connect the interface region with the bone region (Figure d). When the multiphased scaffolds were implanted *in vivo*, zone A and B are expected to facilitate the formation of non-calcified fibrocartilage and calcified fibrocartilage while zone C is expected to facilitate bone formation.

Figure 4 shows the FEA results by simulating the implantation process of multiphased scaffolds into the host bones with different δ value. It can be seen that with the increase of δ, the maximum von mises stress of the PCL anchor significantly increased. The stress distribution between different sawtooth in the same scaffold is not uniform, which gradually decreased from the first press-in sawtooth to the last one. For the scaffold with δ = 0.7mm, the stress imposed on all sawtooth structures of the scaffold is higher than 60 MPa, which exceeds the maximum compressive strength of PCL scaffold (60 MPa) [18] and would cause PCL anchor failure. The similar phenomenon was also found in the FEA results for ceramic scaffolds. When δ value is below 0.5mm, the maximum stress is lower than the measured compressive strength of the ceramic scaffold (18.86 MPa), which could ensure the intact of the ceramic scaffold during the implantation process. However, when δ = 0.7mm, the maximum stress appears at the top of the scaffold as high as 58.75 MPa, which might cause scaffold rupture.

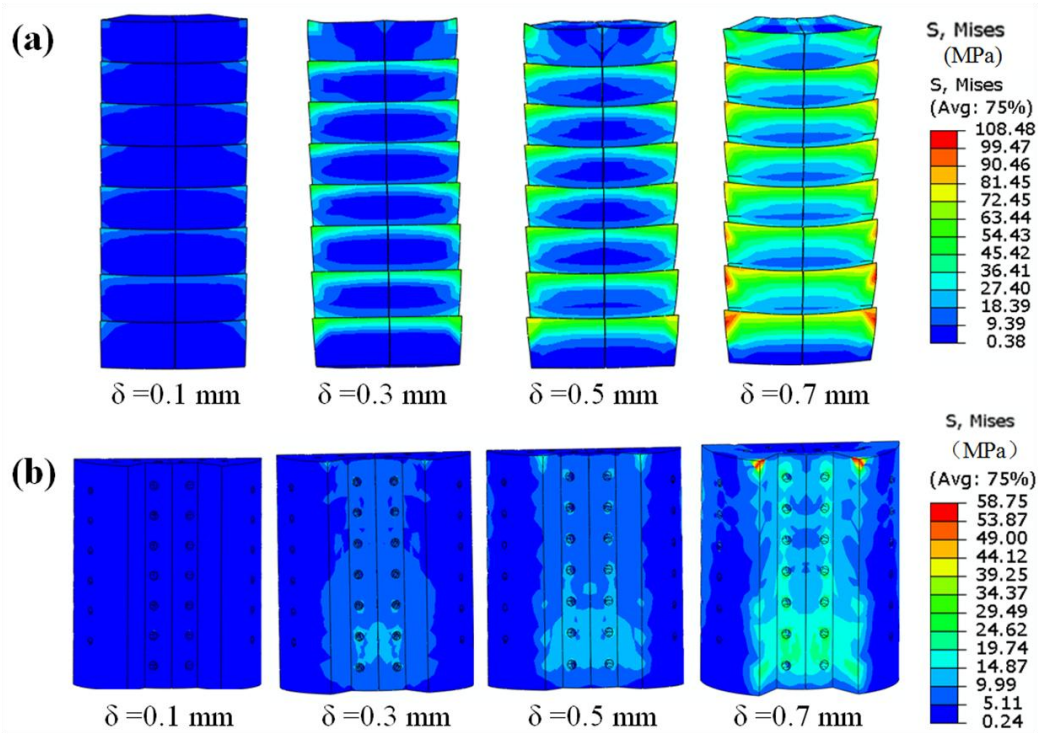

Figure 4 FEA results of compressive stress imposed on the PCL anchor as well as ceramic scaffolds when the multiphased scaffold was pressed into the host bone tunnel. (a) Stress distribution of PCL anchor with δ = 0.1, 0.3, 0.5 and 0.7mm, (b) stress distribution of ceramic scaffolds with δ = 0.1, 0.3, 0.5 and 0.7mm.

To investigate the mechanical fixation of the multiphased scaffold and host bone, the multiphased scaffold was implanted into fresh porcine joint *in vitro* as shown in Figure 5a-b. Since the implant was press-fit with the bone tunnel, a stainless steel device was fabricated to facilitate the implantation process. Figure 5c shows the overall micro-CT image of the femur bone after implantation. It is clear to see that the multiphased scaffold was accurately implanted in the pre-drilled bone tunnel and exhibited similar grey value with surrounding host bone. Figure 5d demonstrates the cross-sectional images of the bone tunnel at different positions. From position 1 to position 3, the grey value in the bone tunnel gradually changed due to the increase of β-TCP concentration at the interface region of the scaffold. The Micro-CT images at position 4 and 5 clearly demonstrate the microchanneled ceramic scaffold in the bone region, which maintained intact and tightly contacted with the host bone. The small bone debris caused by the drilling process was filled in the bottom of the bone tunnel as shown in Figure 5d.

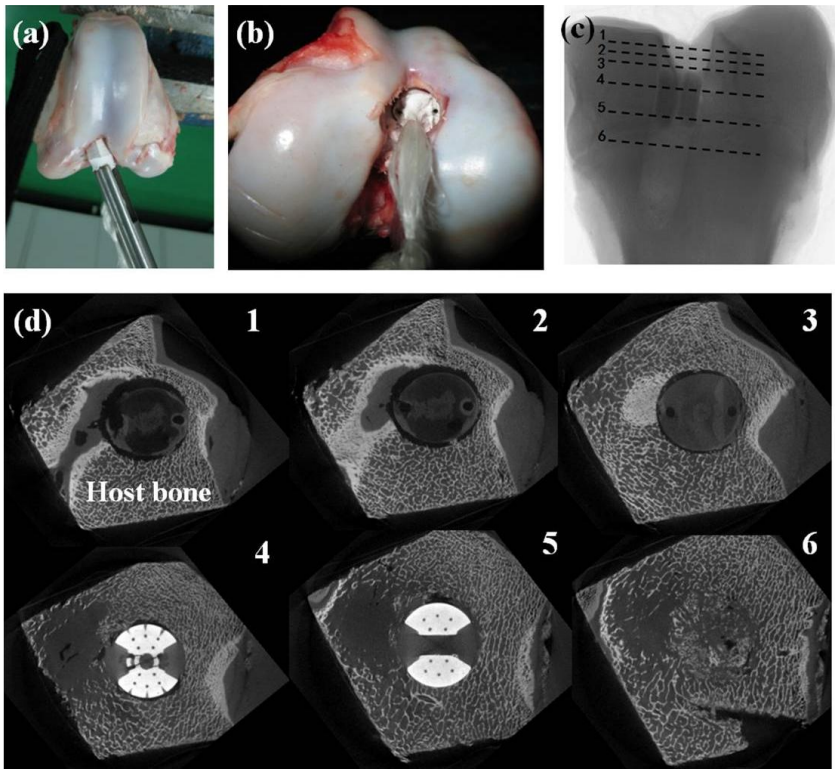

Figure 5 In-vitro implantation of the multiphased scaffold into the porcine femur joint. (a-b) Implantation of the scaffolds into the bone tunnel, (c) Micro-CT images of overall host bone after implantation, (d) the cross-sectional Micro-CT images of bone tunnel.

To verify the FEA results, the multiphased scaffolds with different δ value were implanted into the host bones. Due to the fabrication errors, the actual δ values were determined by measuring the dimension of the multiphased scaffolds and bone tunnel, which were 0.27, 0.47 and 0.68mm in corresponding to the designed δ values of 0.3, 0.5 and 0.7mm. Figure 6a-c show the Micro-CT images of the multiphased scaffolds with different δ value implanted into the host bones. It can be seen that the ceramic scaffold maintained relatively well and intact when δ is smaller than 0.47mm while the scaffold with δ=0.68mm was fractured at the top region. These findings are in well agreement with previous FEA results. Since the crack of the ceramic scaffold might affect the fixation of the implant, the multiphased scaffolds with δ= 0.27 and 0.47mm were used for further mechanical testing.

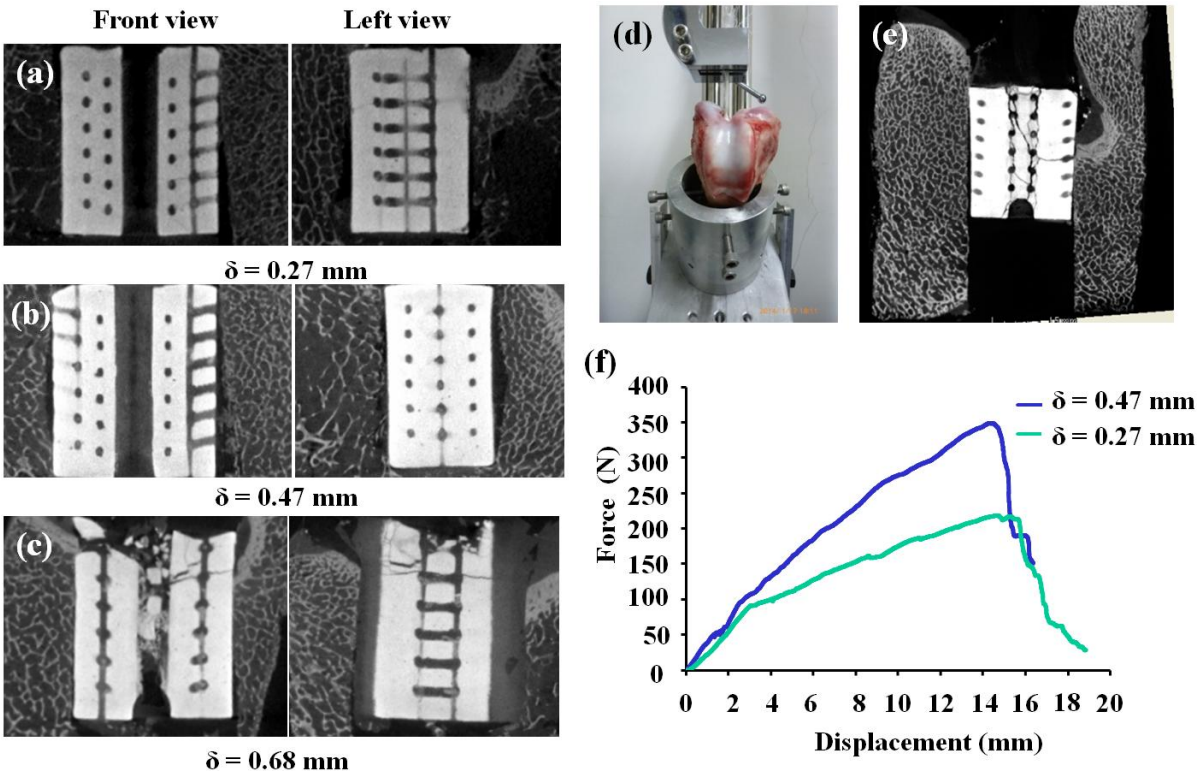

Figure 6 Morphology and mechanical characterization of the implanted multiphased scaffolds with different δ value. (a) Micro-CT morphology of the implant with δ=0.27mm, (b) Micro-CT morphology of the implant with δ=0.47mm, (c) Micro-CT morphology of the implant with δ=0.68mm, (d) mechanical fixation testing, (e) Micro-CT morphology of the implant with δ=0.47mm after mechanical testing, (f) the force-displacement curve for the implants with δ= 0.27 and 0.47mm respectively.

For mechanical testing, the host bones implanted with the multiphased scaffolds were mounted on the specific-designed clamp as shown in Figure 6d. The clamp angle and position could be flexibly adjusted to ensure the force direction along with the bone tunnel. Figure 6e shows the Micro-CT morphology of the scaffold with δ= 0.47 mm after mechanical testing. The failure of the mechanical fixation mainly results from the crack of the ceramic scaffold. The maximum pull-out forces for the implants with δ= 0.27 and 0.47 mm were 211.08 ± 18.16 N and 360.31 ± 97.51 N respectively. Although the initial fixation force for the multiphased scaffold is still lower than the maximum force (454 N) of human ACL in daily life [25], it might be significantly improved by reinforcing the ceramic scaffold using PLA polymer [18].

In our previous study, non-degradable polyetheretherketone (PEEK) anchors were used to mechanically fix the silk-TCP constructs with host bone for the regeneration of ACL in porcine models. The results demonstrated that new bone tissues could rapidly form in the ceramic TCP scaffold and biomimetic interfacial tissues were also regenerated between silk ligament grafts and TCP scaffolds as well as regenerated bone tissues [26, 27]. Here biodegradable PCL anchor was used to replace PEEK anchor for initial mechanical fixation and triphasic zones were produced between ligament region and bone region. Figure 7 schematically shows the potential time-dependent evolution of the multiphased scaffolds *in vivo*. At the early stage of implantation, the implant was mechanically fixed with the host bone via the PCL anchor. With the increase of implantation time, new bone tissues are expected to firstly grow into the predefined microchannels of the ceramic scaffold. The PCL anchor as well as ceramic scaffold will gradually degrade and extensive bone tissues formed to occupy of the degraded site. In this way, the fixation of the implant with the host bone gradually changed from mechanical fixation to biological fixation. Additionally, the interface region is expected to facilitate the regeneration of non-calcified and calcified tissues between the ligament graft and regenerated bone, which might provide a promising way to finally realize the biological fixation between ligament graft and host bone. However, *in-vivo* experiment should be

further conducted to verify the efficiency of the multiphased scaffolds for long-term fixation of ligament grafts.

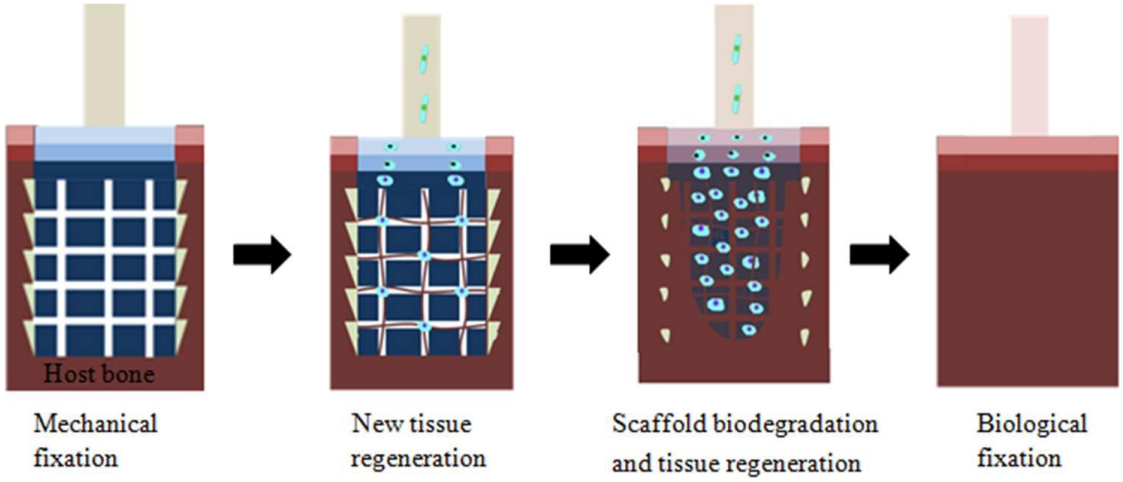

Figure 7 Schematic of the time-dependent evolution of the multiphased scaffolds after implantation *in vivo*.

4. Conclusion

Here we present a multi-material strategy to fabricate composite scaffolds potentially for ligament-to-bone fixation. The scaffold was designed to have ligament region, interface region and bone region. The interface region exhibited triphasic zones to mimic native non-calcified and calcified fibrocartilage. The bone region consisted of PCL anchor and microchanneled ceramic scaffolds, which respectively provide initial mechanical fixation and potential biological fixation for the construct and the host bone. A specific fabrication approach was proposed to successfully fabricate the predefined scaffolds and the multiple phases were effectively integrated during the fabrication process. FEA results demonstrated that the multiphased scaffolds with interference value smaller than 0.5mm should be used to avoid ceramic scaffold fracture, which was validated by *in-vitro* implantation of the multiphased scaffolds into porcine joints. Pull-out experiments showed that the initial fixation between the multiphased scaffolds with 0.47 mm interference and the host bone could withstand the maximum force of 360.31 ± 97.51 N. It is envisioned that the multiphased scaffolds could potentially induce the regeneration of new bones as well as interfacial tissues with the gradual biodegradation of the scaffold and subsequently induce the fixation of the implant with the host bone from initial mechanical to long-term biological fixation.

Acknowledgement

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Supplementary Information

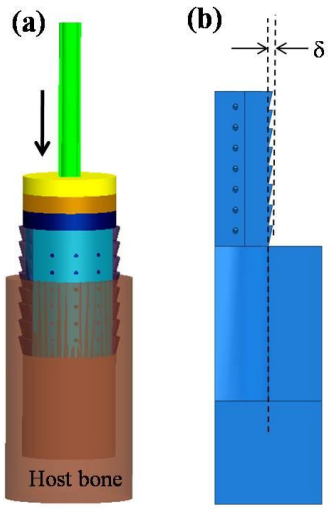
Fabrication of biomimetic multiphased scaffolds for ligament-to-bone fixation

Jiankang He [a*], Wenyou Zhang [a,b], Yaxiong Liu [a], Xiang Li [c], Dichen Li [a] and Zhongmin Jin [a]

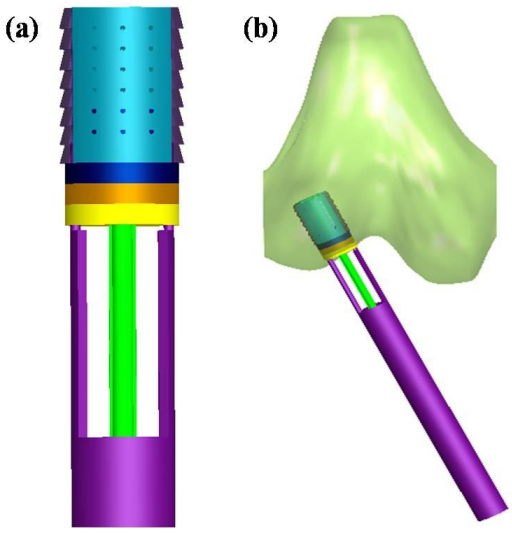
[a] State key laboratory for manufacturing systems engineering, Xi'an Jiaotong University, Xi'an 710049, China

[b] Qingdao R&D Institute, Xian Jiaotong University, Qingdao 266300, China

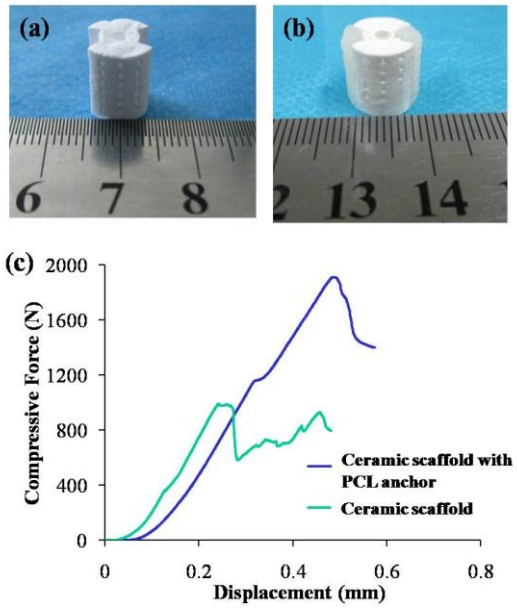
[c] Department of Health Sciences and Technology, ETH Zurich, Switzerland



Supplementary Figure 1 FEA model for the implantation simulation of the multiphased scaffolds.



Supplementary Figure 2 Schematic of the implantation process of the multiphased scaffolds into host bone.



Supplementary Figure 3 Enhanced compressive property of the ceramic scaffold with PCL anchor.

Responses to Technical Check Results

Technical Comments:

1. All the listed references should be cited in the text completely and sequentially.

All the references in the manuscript have been carefully checked and were cited in a complete and sequential manner.