Human adipose derived stem cells are superior to human osteoblasts (HOB) in bone tissue engineering on a collagen-fibroin-ELR blend

Esen Sayın a, b, Rosti Hama Rashid c, José Carlos Rodríguez-Cabello d, Ahmed Elsheikh c, Erkan Türker Baran b, Vasilı Hasircı a, b, c, *

a METU, Department of Biotechnology, Ankara, Turkey
b METU Center of Excellence in Biomaterials and Tissue Engineering, Dumlupınar Blvd No: 1, 06800 Ankara, Turkey
c University of Liverpool, School of Engineering, L69 3GJ Liverpool, UK
d BIOFORGE, CIBER-BBN, Campus “Miguel Delibes” Edificio LUCIA, Universidad de Valladolid, Paseo Belén 19, 47011 Valladolid, Spain

1. Introduction

Musculoskeletal disorders are the second biggest reason for long term disability of patients in the world after the mental and behavioral disorders [1]. Fractures or diseases such as osteoporosis lead to a decrease in bone quality and mechanical integrity. Autografts and allografts are used in the conventional therapy of bone defects. Bone autografts are highly preferred over the allografts in bone tissue engineering using synthetic or biological polymers can overcome most of these drawbacks (except mechanical weakness). Besides, degradation products of polyesters such as poly(lactic acid) (PLA) [5], poly(glycolic acid) (PGA) [6] and their copolymers were shown to create an acidic environment. In addition, the synthetic polymers do not possess cell signaling sequences that are naturally present in the structure of biological polymers such as fibronectin, collagen, vitronectin and fibrinogen.

Tissue engineering (TE) can still be an appropriate alternative...
for small and medium sized bone defects because TE can introduce to the scaffolds properties like osteoconduction, osteoinduction and osteointegration. The main constituents of bone tissue engineering are biocompatible and biodegradable porous scaffolds and autologous cells to be seeded into the scaffolds to obtain biologically and mechanically adequate tissue substitutes [7]. Recently, protein-based scaffolds gained importance in load bearing sites in the body such as bone, cartilage, tendon, meniscus, vessels, skin, bladder and cornea [8], but since they lacked the required mechanical strength, additional materials such as hydroxyapatite for bone needed to be added to improve their mechanical strength [9].

Collagen type I has a special place among the natural polymers because it is secreted by a variety of cells including osteoblasts and constitutes the main organic phase of extracellular matrix (ECM) [10] and contributes significantly to the viscoelasticity of bone [11]. In bone, collagen fibrils are reinforced by plate-shaped HAp nanocrystals 10–20 nm in length and 2–3 nm width [12]. However, reconstituted collagen has a much lower mechanical strength than the bone to be substituted [13]. The reason for this is partly the lack of fibrillar arrangement due to hydrolysis during reconstitution. Additionally, collagen denatures during sterilization and this decreases its resistance to enzymes and mechanical strength [14]. An attempt to collagen, silk fibroin has been proposed as protein-based biomaterial for load bearing TE applications due to its unique mechanical properties. Self-assembled Bombyx mori fibroin molecules have a crystalline β-sheet structure which gives its high tensile strength and toughness, and the remaining amorphous region provides the elasticity needed [15]. However, handling of pure silk fibroin scaffolds is an issue and therefore, it is advantageous to use it as a blend with more flexible materials such as collagen, to achieve optimum mechanical strength and ease of use.

Recently, recombinant proteins have become protein materials of choice due to their tailor-made properties. Elastin-like recombinamers (ELRs) are such recombinant proteins coded in a synthetic DNA and expressed via the use of high yield vectors. VPGXG repeating sequences, where X is a natural or modified amino acid except L-proline originates from elastin which is an ECM protein found in many tissues including bone and is responsible for their elasticity. The amino acid sequences are combined in repeating fashion to form the backbone of ELRs [16]. Most ELRs are thermoresponsive materials and aggregate at temperatures higher than their inverse transition temperature (ITT). This feature aids purification of ELRs via solubilization-precipitation [17]. ELRs have earlier been used in bone tissue engineering and shown to be biocompatible [18]. ELRs with special sequences were reported for bone tissue engineering in order to increase cell adhesion [19], to form an antifouling coat [20] or to enhance nucleation of HAp on the implant surfaces [21].

In this paper, swelling and crystallinity of films of pure collagen, fibroin and their blends were studied. ELR was added to the blend composition to compare the suitability of the stem cells and primary osteoblasts for bone tissue engineering. In this study, an ELR with [[(VPGIG)2 (VPGKG) (VPGIG)2]2 DDDEEKFLRRIGRFG [(VPGIG)2 (VPGKG) (VPGIG)2]2 carrying an HAp nucleating sequence was produced and characterized by Prof. José Carlos Rodríguez-Cabello (Universidad de Valladolid, Spain). The theoretical mass of the ELR was calculated according to recombinamer design and found as 31,877 Da [24]. Briefly, E. coli system was used for the oligopeptide synthesis. Cells were lysed by ultrasonication and protein was purified by applying a series of cold and warm centrifugation steps and dialysis. Purification was carried out by using aggregation of the recombinamer with a lower critical solution temperature (LCST) above its transition temperature. ITT was found to be 32 °C at pH = 7.36 as determined by size measurement between 20 °C and 40 °C by using Nano-ZS (Malvern, Worcestershire, UK). Molecular weight and purity of protein were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with a sharp peak at 31,857.21 Da and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a distinctive band around 30 kDa that was close to the theoretical mass.

### 2. Materials and methods

#### 2.1. ELR expression and purification

ELR ([((VPGIG)2 (VPGKG) (VPGIG)2]2 DDDEEKFLRRIGRFG [(VPGIG)2 (VPGKG) (VPGIG)2]2 carrying an HAp nucleating sequence was produced and characterized by Prof. José Carlos Rodríguez-Cabello (Universidad de Valladolid, Spain). The theoretical mass of the ELR was calculated according to recombinamer design and found as 31,877 Da [24]. Briefly, E. coli system was used for the oligopeptide synthesis. Cells were lysed by ultrasonication and protein was purified by applying a series of cold and warm centrifugation steps and dialysis. Purification was carried out by using aggregation of the recombinamer with a lower critical solution temperature (LCST) above its transition temperature. ITT was found to be 32 °C at pH = 7.36 as determined by size measurement between 20 °C and 40 °C by using Nano-ZS (Malvern, Worcestershire, UK). Molecular weight and purity of protein were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) with a sharp peak at 31,857.21 Da and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a distinctive band around 30 kDa that was close to the theoretical mass.

#### 2.2. Template preparation

The chemical etching method was applied for the production of patterned silicon wafer at Bilkent University (Ankara, Turkey). Microchannel dimensions were 5 μm, 10 μm, and 5 μm, for ridge width, groove width and depth, respectively (Fig. 1c). Negative replicas were produced from poly(dimethylsiloxane) (PDMS) by mixing PDMS prepolymer and curing agent (Sylgard 184 Elastomer Kit, Dow Corning, Midland, Michigan, USA) in 10:1 ratio and heating at 70 °C for 3 h [25] (Fig. 1d).

#### 2.3. Film preparation

Collagen type I was isolated from Sprague–Dawley rat tails and fibroin was purified from silk fibers of Bombyx mori according to previously published methods [26]. Bombyx mori silk threads were gift from Prof. Esra Karaca, Uludag University (Bursa, Turkey). In brief, for collagen type I isolation, tendons were removed and dissolved in cold acetic acid (0.5 M). Filtered solution was dialyzed against phosphate buffer (24 mM, pH 7.2) and centrifuged. Pellets were dissolved in acetic acid (0.15 M) and NaCl (5%) was added to solution. Next day, precipitated collagen was separated via centrifugation and dissolved in acetic acid (0.15 M). After a week of dialysis, solution was centrifuged and collagen was sterilized in ethanol (70%) for 2 days. Following the centrifugation step, collagen was lyophilized for long term storage. For silk fibroin isolation, silk threads (12.5 g) were washed in boiled Na2CO3 (0.02 M) for 30 min and dried at 37 °C. Fibroin was dissolved in 9.3 M LiBr (60 °C) and filtered. Solution was dialyzed against water and lyophilized.
Pure collagen (1.6% (w/v)), pure fibrin (8% (w/v)) and blend of the two (1.6% (w/v) collagen and 0.8% (w/v) fibrin) films were prepared. In order to use for in vitro studies, 2.6% (w/v) collagen-fibrin-ELR blend films were produced in a ratio of collagen:fibrin:ELR 6:3:1. For the production of all types of films, materials were dissolved in 0.5 M acetic acid. Solution (250 μL/cm²) was cast on patterned PDMS replicas (2.55 cm²) and dried at room temperature. Afterwards, the collagen bearing films were crosslinked with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (12.5 mM) and N-hydroxysulfosuccinimide (NHS) (5.2 mM) (Sigma, St. Louis, Missouri, USA) and fibrin films were stabilized in methanol solution (90% v/v) at room temperature as performed at the previous study (Fig. 1d) [26]. After the crosslinking procedure, pattern dimensions (without and with ELR films) were measured with a profilometer (NewView™ 73003D Optical Surface Profiler, Zygo, Middlefield, Connecticut, USA) and compared.

2.4. Swelling test

The swelling degrees of pure collagen, pure fibrin and collagen-fibrin blend films were determined via gravimetric method. Pre-weighed, crosslinked films were incubated in 10 mM PBS for 24 h at 37 °C, rinsed with distilled water and blotted lightly. Swelling was calculated as follows:
\[
\text{Swelling} = \frac{w_S - w_D}{w_D} \times 100
\]

where \(w_S\) is the swollen weight and \(w_D\) is the dry weight.

2.5. Differential scanning calorimetry

Glass transition temperatures of crosslinked collagen, fibroin and collagen-fibroin blend films were determined by differential scanning calorimetry (DSC) (Perkin Elmer Diamond, Waltham, Massachusetts, USA). Samples were heated under nitrogen gas at a rate of 10 °C/min in the range 0–250 °C.

2.6. ADSC isolation and characterization

Human fat tissue was obtained from consenting patients at Cag Hospital (Ankara, Turkey) and the project protocols were approved by Middle East Technical University Ethical Committee (B.302.2.DTD.0.AH.00.00/126/95-1585). Lipoaspirate tissue was processed according to the literature [27]. In brief, the tissue was washed with PBS (pH 7.4, 10 mM) and digested with collagenase type 1 (150 μg/mL, Gibco, Waltham, Massachusetts, USA) in HBSS (Lonza, Basel, Switzerland) on a shaker at 250 rpm for 1 h (37 °C). 10% FBS was added and 160 mM NH₄Cl (pH 7.2) was used to lyse the red blood cells. Bicoll separating solution (Biochrom, Darmstadt, Germany) was used to separate cell layer. Cells were resuspended in PBS and filtered through cell strainers (100 and 40 μm, BD Biosciences, Franklin Lakes, New Jersey, USA). As culture medium, low glucose DMEM (Biochrom, Darmstadt, Germany) supplemented with 40% FBS, 1% penicillin-streptomycin, 250 ng/mL amphotericin B and 10 ng/mL epidermal growth factor (EGF) was used for 1 day. Then floating cells were discarded and FBS ratio was changed to 10%.

For phenotype characterization cells were detached with 0.25% trypsin–EDTA (5 min, at 37 °C) and centrifuged (5 min, 3000 rpm). ADSCs were counted with NucleoCounter (ChemoMetec A/S, Allerod, Denmark). ADSC phenotype was confirmed as reported earlier [26].

2.7. HOB isolation and characterization

Bone samples were obtained from the Gulhane Medical Military Academy (GATA) (Ankara, Turkey) with the approval of GATA (50687469-1491-262-14/1648.4-553) and the Middle East Technical University (28620816/203-598) Ethical Committees and consent of the patient. HOBs were isolated from the healthy bone tissue by the surgeon during the elective joint replacement surgery. HOBs were incubated with anti-collagen type I and anti-osteopontin antibody solutions (final antibody concentration 10 μg mL⁻¹ in 0.1% BSA in PBS) for 2 h at 37 °C. Then, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Catalog no: A11029, Invitrogen, Waltham, Massachusetts, USA) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (Catalog no: A11034, Invitrogen, Waltham, Massachusetts, USA) as secondary antibody (final anti-body concentration 20 μg mL⁻¹ in 0.1% BSA in PBS) for 1 h at 37 °C to complete the immunostaining of collagen type I and osteopontin markers, respectively. Cell nuclei were stained with DAPI. Samples were visualized with fluorescence microscopy (Zeiss, Jena, Germany). HOBs with passage numbers up to 5 were used in in vitro studies.

2.8. ADSC and HOB proliferation

Films were sterilized in 70% (v/v) ethanol for 2 h and then air dried inside the laminar flow hood. 10,000 cells were seeded on the films and after one day in growth medium, films were placed into new wells. ADSCs were cultured in the growth medium for 1 week and then the medium was changed with osteogenic medium (high glucose DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, 250 ng/mL amphotericin B, 100 mM dexamethasone, 10 mM β-glycerophosphate and 50 μM L-ascorbic acid) in which ADSCs were cultured for the next 3 weeks. Since HOBs are originally bone cells no osteogenic medium was used. Proliferation of ADSCs and HOBs were measured with 10% Alamar Blue test (Invitrogen, Waltham, Massachusetts, USA) in colorless DMEM (HyClone, Logan, Utah, USA) on Days 1, 7, 14, 21 and 28. Cells were incubated with Alamar Blue solution for 1 h at 37 °C and optical densities were determined at 570 and 595 nm in a multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA). Absorbances were converted into percent reduction values and cell numbers were determined from a calibration curve.

2.9. Scanning electron microscopy (SEM)

Morphologies of ADSCs and HOBs on film surfaces were examined with SEM on Day 28. Additionally, surfaces of unseeded films were examined on Days 1 and 28. Films were washed with PBS and 0.1 M cacodylate buffer (pH 7.4) and then, cells were fixed with glutaraldehyde solution (2.5% in cacodylate buffer) for 2 h at room temperature. Films were stained in 1% osmium tetroxide (in cacodylate buffer) for 1 h. Samples were dehydrated in graded series of ethanol (50–95%). After final incubation in pure ethanol for 15 min, dehydrated films were freeze dried (Sanyo MDF-US3865, Osaka, Japan), coated with Au-Pd and analyzed with SEM and EDX (FEI, Quanta 400F, Eindhoven, Holland).

2.10. Cell alignment

On Day 28, cell seeded samples were stained with Phalloidin Alexa Fluor 532 (Invitrogen, Waltham, Massachusetts, USA). Cells were fixed and permeabilized as explained earlier, incubated in blocking solution (1% BSA in PBS) at 37 °C for 30 min and in Phalloidin solution (118 μg mL⁻¹ final concentration prepared in PBS with 0.1% BSA) at 37 °C for 1 h. Images were obtained with Confocal Laser Scanning Microscopy (Zeiss LSM 9100, Lena, Germany).
2.11. Mineralization

Quantity of calcium in samples was measured with a colorimetric assay based on o-cresol phthalain complexone that forms a violet colored complex with calcium. Films of Day 28 were washed with PBS and calcium was extracted from them by immersing in 0.6 N HCl overnight at 4 °C. Supernatant (10 μL) was added to a solution (190 μL) containing equal volumes of calcium binding reagent (0.024% o-cresol phthalain complexone and 0.25% 8-hydroxyquinone in water) and calcium buffer (500 mmol/L 2-amino-2-methyl-1,3 propanediol in water). All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Absorbance was measured at 570 nm with a multiwell plate reader. The amount of calcium in each sample was determined with a standard curve. Normalized calcium calculations were performed by subtracting the background calcium reading, unseeded normalized calcium calculations were performed by subtracting the background calcium reading, unseeded normalized calcium readings from the calcium readings of the seeded samples.

2.12. Tensile testing of ADSC and HOB seeded films

ADSC and HOB seeded collagen-fibroin-ELR blend films were tested after 28 days of culture to investigate the effect of ECM secretion of cells on the tensile behavior of blend films. None of the samples were fixed. Films (n = 3–5) were cut into 4 mm × 10 mm strips and tested at room temperature by Instron 3366 Uniaxial Testing Machine (Instron, Norwood, MA, USA) with a 10 N load cell. Films were placed in a longitudinal direction parallel to the microchannel axis with custom made clamps in a Perspex chamber, which was filled with PBS. Preload (0.01 N) was applied in uniaxial tension mode to prevent loose layout of films between clamps. Preload rate was 0.4 mm/min and no data was recorded during preload. After preload, uniaxial tension was applied up to a maximum load of 10 N at an elongation rate of 0.04 mm/min which corresponded to 1.0%/min strain rate. Testing was continued until failure.

2.13. Statistical analysis

Significant differences between groups were examined with one-way ANOVA followed by Tukey’s test and p ≤ 0.05 was taken as statistically significant. Two-way ANOVA was performed for the cell proliferation results. Student’s t-test was employed for the statistical analysis of the normalized mineralization assay results.

3. Results and discussion

3.1. Swelling test

ECM is known for its ability to carry water needed for the metabolism [28] and therefore, scaffolds also need to contain a comparable amount of water to create an appropriate microenvironment for the cells. Swelling ratio (water uptake capacity of the films per unit sample weight) was determined for collagen, fibroin and collagen-fibroin blend films. Collagen film gained significantly more water than fibroin film (163% vs 47%) (Fig. 2a and c). It is known that the amorphous regions of the scaffold swell more than crystalline regions due to fewer contact points between the polymeric chains allowing liquid influx [29]. The blend swelled less than the pure collagen film because of the fibroin in the composition of the blend. The presence of collagen twice as much as fibroin led to a significantly higher swelling capacity (139%) than pure fibroin.

3.2. Thermal analysis

Glass transition temperature (Tg) provides information about the organization of a material. The thermal properties of the collagen and fibroin reported by several studies. Lu et al. found higher Tg value with the increase in the β-sheet structure of the fibroin after stabilization by the use of water annealing process [30]. Additionally, formation of crystalline structure was stimulated by the application of EDC crosslinking to collagen-fibroin blend hydrogels which in turn elevated the Tg of the blend material [31]. DSC revealed that Tg of pure collagen (55.5 °C) was significantly lower than that of pure fibroin (81.5 °C) (Fig. 2b and c) due to the β-sheet rich content of fibroin [32]. The Tg of the blend (58.8 °C) on the other hand is closer to the collagen due to the high collagen content in the blend and much more smaller than fibroin because the crystallinity of fibroin is decreased due to the more amorphous collagen [33]. In the rest of the study, collagen-fibroin-ELR blend was employed since collagen-fibroin blend has superior properties such as optimum water uptake due to collagen and enhanced mechanical strength owing to presence of fibroin.

3.3. Pattern dimensions

The depth and width of the grooves and ridges of the microchannels were determined as 4.3 ± 0.1 μm, 10.0 ± 0.3 μm and 7.4 ± 0.3 μm, respectively for the collagen-fibroin blend film by using a profilometer (Fig. 2d). For the ELR-added films, dimensions were 4.6 ± 0.2 μm, 10.3 ± 0.4 μm and 6.8 ± 0.3 μm (Fig. 2e) showing that the presence of ELR does not lead to a distinct change in the dimensions of micropatterns. In addition, both films had the same topography with the template showing the fidelity of the process of microchannel patterned film production.

3.4. Characterization of HOB

Human osteoblasts (HOBs) were isolated from the tissues of patients who underwent joint replacement surgery. HOBs were smaller in size in comparison to ADSCs as the phase contrast microscopy images show (Fig. 3a and b). According to literature, HOBs can be half the size of ADSCs but a definite comparison is not possible due to the heterogeneity of the size of ADSCs [34,35]. Additionally, HOB phenotype was confirmed by immunostaining for common human osteoblast markers secreted by HOB: anti-collagen type I for collagen type I and anti-osteopontin for osteopontin [36,37]. These proteins constitute different components of the extracellular matrix in bone. The isolation of HOBs in this study is considered successful because no cells were observed with DAPI-only staining; this indicates that contaminating cells like fibroblasts originating from the isolation step are not present (Fig. 3c and d). Immunostaining also indicated that HOBs preserved their phenotype during passaging up to 5 which was the highest number of passage used in this study.

3.5. Proliferation of ADSC and HOB on collagen-fibroin-ELR blend films

Alamar Blue assay was performed to study cell proliferation on Days 1, 7, 14, 21 and 28. The osteogenic differentiation of the ADSCs was induced with the introduction in differentiation medium to drive the ADSCs into osteoblast phenotype. The attachment and proliferation rate of the ADSCs and HOBs were compared for their potential to form an engineered bone tissue on biodegradable films. Collagen-fibroin-ELR blend films supported ADSC adhesion and growth (Fig. 3e). Application of osteogenic medium on Day 7 slowed down the increase in the ADSC proliferation rate and ended...
in a plateau (on Days 14 and 21) on both TCPS and collagen-fibrin-ELR blend surfaces possibly due to differentiation of stem cells [38]. No osteogenic medium was used for HOBs since they already possessed osteoblast phenotype (Fig. 3c and d) and they showed a proliferation trend as the ADSCs on film. HOBs tested on the same surfaces showed 2-fold lower adhesion on both surfaces than ADSCs (Day 1). As the incubation time went by, the cell number of both HOBs and ADSCs were higher on TCPS (3.9 cm²) than on film (2.55 cm²) surfaces. The larger surface area of the TCPS along with its cell adhesive chemistry can explain the higher cell attachment and proliferation extent. It can be suggested that wider surface of TCPS provided a space for cell attachment and division which in turn might have enhanced Day 1 results of HOBs and ADSCs. Interestingly, number of ADSC on films was approximately 2-fold higher than that of the HOB at each time point. It can therefore be stated that ADSCs proliferate on collagen-fibrin-ELR blend films more than HOBs, despite the low rate due to differentiation. Previously, HOBs were shown to proliferate more on silk fibrin coated PCL - biphasic calcium phosphate (BCP) scaffolds than uncoated ones [39]. Additionally, Gronthos et al. showed that HOBs adhered on the collagen type I surface as well as surfaces coated with other ECM components (collagen types IV, V, fibronectin, laminin, and vitronectin) [40]. According to these studies, HOBs perform well on both collagen and fibrin materials and this is an advantage of the blend film. Similarly, another study pointed out that while ADSCs attached on collagen scaffolds at a higher rate than silk fibrin scaffolds, bone marrow stem cells (BMSCs) attached on both scaffolds without any particular preference for either surface [41].

3.6. Surface characterization and morphology of ADSCs and HOBs on collagen-fibrin-ELR blend films

Collagen-fibrin-ELR blend films patterned with microchannels were produced with predetermined groove and ridge topographies as SEM micrographs showed and crosslinking did not cause any swelling in the physical cues which can be the case in a water based crosslinking medium (Fig. 4a). Features were mainly preserved after crosslinking with EDC/NHS in methanol. Blend films could withstand 27 days of cell culture conditions (Fig. 4b). However, some holes in the films could be seen due to film degradation during culture.

HOBs were lower in number than ADSCs and both HOBs (Fig. 4c)

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**Table:**

| Samples                  | Degree of Swelling (%) | Tg (°C) |
|--------------------------|------------------------|---------|
| Collagen                 | 103                    | 55.5    |
| Fibrin                   | 47                     | 81.5    |
| Collagen-fibrin blend    | 139                    | 58.8    |

**Fig. 2.** Characterization of pure and blend films. (a) Degree of swelling of collagen, fibrin and collagen-fibrin films. Statistically significant differences were determined between collagen film and other groups (*P < 0.05, **P < 0.01, ***P < 0.001) and also between fibrin and collagen-fibrin blend films (*P < 0.05, **P < 0.01, ***P < 0.001). (b) DSC spectra of films. Tg values for each sample are marked with arrows on the DSC spectra. (c) Swelling ratios (%) and Tg of unseeded films. Pattern dimensions of (d) collagen-fibrin, and (e) collagen-fibrin-ELR blend films with microchannel patterns were studied with a profilometer (x20).
and ADSCs (Fig. 4d) were guided on collagen-fibroin-ELR blend film surfaces after 28 days of cell culture. Energy Dispersive X-Ray Spectrometry (EDX) showed no Ca and P atoms on the unseeded (Days 1 and 28) and HOB seeded (Day 28) films. ADSCs, on the other hand, deposited calcium and phosphorus containing compounds on the collagen-fibroin-ELR blend films with a Ca/P ratio of 0.47 after 28 days of cell culture. This value is lower than that of HAp (1.67); its in vivo precursor octacalcium phosphate (OCP) has this value as 1.33 [9] and tricalcium phosphate as 1.5. The reason for the 0.47 needs to be further investigated.

3.7. ADSC and HOB alignment

Guidance of bone cells is important because it helps an anisotropic ECM deposition by aligned cells. On Day 28, actin filament staining of seeded ADSCs and HOBs showed that cytoskeletons of these cells were aligned along the microchannel direction on patterned collagen-fibroin films (Fig. 5a and b). It can be stated that ADSCs and HOBs were aligned on ridges and grooves smaller than the cells as seen in different examples involving other cell types and materials [43–45]. BMSCs were also a supporting example for cell
alignment on a similar sized microchannels composed of ther-
moreponsive poly(N-isopropylacrylamide) films with ELR adsor-
bed on the surface [46]. Additionally, the guidance of HOBs was
reported by Biggs et al. with the best alignment on microchannels
of poly(methylmethacrylate) (PMMA) with the grooves of 10 μm
size amongst the ones that had 25 and 100 μm width [47]. Previ-
ously, ADSCs were also reported to align on graphene oxide (ridge:
30 μm, groove: 15 μm) and collagen-fibroin (ridge: 7.4 μm, groove:
10 μm) microchannels with increased osteogenic differentiation in
regard to smooth surfaces [26,48].

3.8. Quantification of mineralization

The amount of mineral deposited on the HOB and ADSC seeded
collagen-fibroin-ELR blend films were quantified with o-cresol
phthalein assay on Day 28 in order to get a chemical measuremen.
Unseeded collagen-fibroin-ELR blend film, incubated for 28 days,
was employed as control. Calcium was totally extracted from the
film structure via HCl treatment; controls were used for back-
ground level check. There was no statistically significant difference
between unseeded film of Day 28 (14 μg) and HOB seeded film on
Day 28 (24 μg) (Fig. 5c). The low levels of calcium was also sup-
ported by EDX analysis. When HOB seeded film was compared with
unseeded film on Day 28, it can be said that HOBs deposited cal-
cium however, the amount was not significantly higher. The cal-
cium content of ADSC seeded ELR blend films on Day 28 (274 μg)
was significantly higher than on similar films seeded with HOB.
Calcium amount was normalized to cell number and ADSCs were
shown to deposite significantly higher amounts of mineral than
HOBs (Fig. 5d). This result indicated that ADSCs produced more
minerals than HOBs.

3.9. Mechanical testing

Films that were used in this study were designed to mimic bone
lamellae and were tested along the microchannel direction to
investigate the contribution of ECM synthesized by the seeded
HOBs and ADSCs to the tensile properties. Amruthwar et al. studied
the effect of ELR addition to collagen hydrogels for bone tissue
engineering. Improvement in the UTS and E were observed from
0.34 MPa to 0.99 MPa and 4.06 MPa to 11.41 MPa with the addition
of ELR (25 mg) to collagen (8 mg) hydrogel due to the more
concentrated protein content in the scaffold [18]. These values are
higher than the UTS and E values measured in this study because of
the lower polymer concentration in collagen-fibroin-ELR blend
film. Causa et al. reported that addition of 13% (v/v) HAp to PCL
scaffolds enhanced the UTS from 0.93 MPa to 2.19 MPa [42]. As far
as we know the mechanical properties of bone tissue engineered
scaffolds has been enhanced by concentrated solutions of synthetic
polymer or with the addition of ceramics. These results in our study
showed the importance of seeded cell type on the mechanical
strength of bone tissue engineered scaffolds for the
first time.

Tensile properties of unseeded collagen-fibroin-ELR blend films
on Days 1 and 28 and ADSC and HOB seeded collagen-fibroin-ELR
blend films on Day 28 were determined. UTS, E (Fig. 5e) and EB
were calculated and the average values are presented in Fig. 5f. No
significant difference could be observed for UTS and EB for all
groups. On Day 28, unseeded collagen-fibroin-ELR blend film had
higher UTS, E and EB than unseeded film on Day 1. This result could
be due to collagen degradation being faster than fibroin [26]. This

Fig. 4. SEM micrographs of unseeded collagen-fibroin-ELR blend films on Days (a) 1, (b) 28 and (c) HOB and (d) ADSC seeded collagen-fibroin-ELR blend films on Day 28. Magnification: ×5,000; ×25,000 (inset).
selective degradation could expose a stronger and crystalline structure of fibroin (β-sheet) after 28 days of cell culture. The higher β-sheet level of fibroin was supported by its low swelling degree and high Tg which are indications of the superior crystallinity of fibroin over collagen film. Similar to our results, the positive relationship between higher crystallinity level of more crosslinked polycaprolactone fumarate and increased tensile modulus was reported by another study [49]. Hu et al. employed films formulated with recombinant human like collagen and fibroin for hepatic tissue engineering purposes. They showed EB elevated from 28.7% to 30.9% with the increase of fibroin content by 10% (w/w) in films [50]. In our work, on Day 1, E was 0.58 ± 0.13 MPa for unseeded collagen-fibroin-ELR blend film. This value is higher than the literature value of 502 ± 575 kPa which was measured from crosslinked and microchannel patterned HAp nucleated ELR membrane after 7 days of incubation in simulated body fluid [51]. By taking this result into account, it can be suggested that collagen and fibroin promoted the mechanical strength of the film.

ADSC seeding contributed to the UTS and E which indicated ECM secretion however, HOB seeding decreased these properties substantially and led to highest EB. Significantly enhanced E in ADSC seeded film (1.21 MPa) when compared to HOB seeded film (0.41 MPa) could be explained by matrix metalloproteinase-2 (MMP-2) secretion, by HOBs in vitro [52]. A relevant work also

![Fig. 5. HOBs and ADSCs were tested for their ability to align, deposit calcium minerals and contribute to tensile properties. Fluorescence micrographs of actin filament stained with Phalloidin (green) on Day 28 shows the cell alignment along the macrochannel direction (white arrow). (a) HOB and (b) ADSC on collagen-fibroin-ELR blend films (scale bar: 250 μm). (c) Calcium amounts of unseeded collagen-fibroin-ELR blend films, HOB and ADSC seeded collagen-fibroin-ELR blend films were quantified by o-cresol phthalein complexone method on Day 28. Statistical differences were determined and differences between the groups were analyzed. (d) Calcium amounts of HOB and ADSC seeded films were normalized to cell number. (e) Young’s modulus of the unseeded and seeded films are presented with the statistical analysis (*p ≤ 0.05, **p < 0.01; #: unseeded film on Day 1 vs other groups, #: unseeded film on Day 28 vs HOB and ADSC seeded films on Day 28). (f) The average ultimate tensile strength (UTS), Young’s modulus (E) and elongation at break (EB) values of unseeded and seeded collagen-fibroin-ELR blend films (n = 3–5).]
showed that MMP-2 is downregulated during osteogenic differentiation of ADSCs [53] and therefore, degradation by MMP-2 might have lowered E significantly for HOB seeded film by breaking the protein chains and leaving them more stretchable. In return, this effect could lead to increase in the EB up to 2-fold (71.32%) when compared to other films. Additionally, Ascenzi et al. applied tensile test in longitudinal direction for wet human fully calcified osteon and measured EB as 6.84% [54] which is five-fold lower than that of Day 28 film on which ADSCs were seeded (35.53 ± 19.18%).

4. Conclusions

In this study, swelling test and DSC experiments showed that the reason of higher mechanical strength of unseeded film incubated for 28 days when compared to Day 1 film was the increase in fibron fraction relative to collagen as a result of the polymer degradation. Guided HOBs and ADSCs on microchannel patterned collagen-fibroin-ELR blend films mimicked the naturally aligned bone tissue. Furthermore, higher rates of cell adhesion, proliferation, mineralization and mechanical properties were obtained by ADSC seeding when compared to HOB due to enhanced ECM secretion. Potential of ADSC over HOB was proven by these means are vital for bone tissue engineering substrates. Osteogenically differentiated ADSCS improved the stiffness and tensile strength of the collagen-fibroin-ELR films after 28 days of incubation.

Conflict of interests

We declare that authors have no competing financial interest.

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