Self-assembled composite matrix in a hierarchical 3-D scaffold for bone tissue engineering

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ABSTRACT

It is of high clinical relevance in bone tissue engineering that scaffolds promote a high seeding efficiency of cells capable of osteogenic differentiation, such as human bone marrow-derived mesenchymal stem cells (hMSCs). We evaluated the effects of a novel polycaprolactone (PCL) scaffold on hMSC seeding efficiency, proliferation, distribution and differentiation. Porous PCL meshes prepared by fused deposition modeling (FDM) were embedded in matrix of hyaluronic acid, methylated collagen and terpolymer via polyelectrolyte complex coacervation. Scaffolds were cultured statically and dynamically in osteogenic stimulation medium for up to 28 days. Compared to naked PCL scaffolds, embedded scaffolds provided a higher cell seeding efficiency (*t*-test, $P < 0.05$), a more homogeneous cell distribution and more osteogenically differentiated cells, verified by a more pronounced gene expression of the bone markers alkaline phosphatase, osteocalcin, bone sialoprotein I and bone sialoprotein II. Dynamic culture resulted in higher amounts of DNA (day 14 and day 21) and calcium (day 21 and day 28), compared to static culture. Dynamic culture and the embedding synergistically enhanced the calcium deposition of hMSC on day 21 and day 28. This *in vitro* study provides evidence that hybrid scaffolds made from natural and synthetic polymers improve cellular seeding efficiency, proliferation, distribution and osteogenic differentiation.

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1. Introduction

The combination of porous scaffolds with cells capable of osteogenesis has shown promise as effective bone graft substitutes in bone tissue engineering [1–3]. The seeding, proliferation and survival of cells on a suitable scaffold, and the maintenance of the differentiated phenotype of the engineered tissues, are the key steps [3,4]. However, several studies have reported low seeding efficiencies [5–9] and non-uniform cell distribution [5,6,8,10,11] within scaffolds. Furthermore, the limited diffusion of nutrients and metabolites within the scaffold interior is a major barrier to the functioning of bone tissue-engineered implants. Attempts to conventionally culture scaffolds thicker than 1 mm *in vitro* is difficult due to diffusion limits, which range from 200 to 500 μm [12–14].

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Thus, one challenge of bone tissue engineering is to design a porous structure, allowing cell migration and diffusion of nutrients, as well as mimicking the natural biophysical and biochemical properties to serve as a cell delivery vehicle that facilitates adhesion and spreading. Many processing techniques [15–17] have been developed to fabricate natural and synthetic polymeric scaffolds. Naturally derived materials, such as collagen, chitosan and hyaluronic acid, are more versatile than synthetic polymer in providing biological functions (e.g. matrix elasticity). As a physical support for cells, they provide a substrate with specific ligands for cell adhesion and migration, and regulate cellular proliferation and function [18]. They are widely used to mimic the structure and biological functions of native extracellular matrix (ECM) [18,19]. However, they have drawbacks such as difficulties in controlling the batch-to-batch variation, mechanical weakness or limited processability [18]. Synthetic materials such as poly(lactic acid), poly(glycolic acid), polycaprolactone (PCL) and poly(lactide-co-glycolide) (PLGA) are often favored because they can be fabricated for a wide range of biodegradable biomedical applications with a high processability, controlled degradation, good mechanical strength,

and the possibility for modification [20]. Scaffolds from these polymers are not as biologically active as the natural ECM-derived polymers. Furthermore, they exhibit poor cell adhesion due to their intrinsic hydrophobicity [21,22], which is a critical issue for successful in vitro 3-D cell culture and subsequent tissue formation.

Hybrid polymeric scaffolds combining natural and synthetic polymers have attracted increasing interest as a means to overcome these limitations and provide a benign and controllable environment for cellular growth and differentiation [16,23,24]. However, a simple combination of these materials may not lead to successful tissue regeneration. Thus, it is important to contrive a way for biomedical efficiency.

A new concept of polyelectrolyte complex coacervation has been developed for the deposition of numerous objects such as nanoparticles, DNA or proteins [25]. The complex coacervation of two polyelectrolytes, such as methylated collagen and the terpolymer of hydroxylethyl methacrylate, methyl methacrylate and methacrylic acid (HEMA–MMA–MAA), can improve the seeding efficiency and viability of bone marrow-derived mesenchymal stem cells (MSCs) in 3-D scaffolds [26]. Macroporous PCL scaffolds can be embedded within naturally derived gels, e.g. hyaluronic acid, chitosan and collagen, by a freeze-drying procedure to produce a hybrid scaffold for cartilage tissue engineering [27].

In this study, we applied the polyelectrolyte technique to fabricate a hybrid scaffold which combined the advantages of natural and synthetic polymers. We used PCL processed by fused deposition modeling (FDM) as a base scaffold. Biological function was then assigned to base scaffolds with ECM components, such as filamentous hyaluronic acid and fibrillar collagen. It was conducive to the culture of MSCs with the complex coacervation of the polyelectrolytes, hyaluronic acid, methylated collagen and the terpolymer HEMA–MMA–MAA, which self-assembled into membrane meshes within the base scaffold. We aimed to examine whether self-assembled composite matrix and dynamic culture could enhance MSC attachment, differentiation and distribution uniformity, within the base scaffold.

2. Materials and methods

2.1. Scaffold manufacturing and surface treatment

PCL (MW = 50 kDa, Perstorp, UK) scaffolds were made by fused deposition modeling at a processing temperature of 100 °C with a BioScaffolder (SYS + ENG GmbH, Germany). Cylindrical scaffolds with a diameter of 10 mm were punched out from a 5 mm thick porous PCL mat using a sterile biopsy punch (Acuderm, Florida). The deposited fibers had an ellipsoidal cross-section with semiaxes of 170 and 120 µm. The thickness of each individual layer was consequently also 120 µm. The center–center fiber distance in each deposited layer was 1.0 mm and the fiber orientation of each consecutive layer was angled 105° and shifted 0.17 mm. The pores of the scaffold were highly interconnected.

To increase surface hydrophilicity to improve cell attachment, the scaffolds were placed in 5 M sodium hydroxide for 3 h, and then 70% ethanol for 24 h for sterilization treatment. The scaffolds were rinsed in sterile water multiple times and dried.

2.2. Methylated collagen and terpolymer preparation

Cationic collagen was prepared by esterification of the carboxyl groups using methanol [28]. The precipitated collagen type I (Sigma, Denmark) was dissolved in 0.1 M HCl containing methanol (J.T. Baker, Denmark) for 6 days, at 4 °C, followed by dialysis to pH 7.4, freeze-drying (FTS, NJ, USA). The concentration of methylated collagen was 3 mg ml⁻¹ in sterile water. The anionic terpolymer

HEMA–MMA–MAA was synthesized and purified [28] and 3% terpolymer was used for the coacervation reaction to embed the 3-D scaffolds.

2.3. Assembly and characterization of the co-polymeric matrix on scaffolds

The scaffolds were immersed in a 4.0 mg ml⁻¹ hyaluronic acid solution (780 kDa, Lifecore Biomedical Inc., Lot P9805-9A) for 24 h in vacuum at room temperature, followed by freeze-drying. Coacervation reaction was done by pipetting 100 µl of 3 mg ml⁻¹ methylated collagen solution into the dried hyaluronic acid pre-coated scaffolds, and then covering this with a final layer of 100 µl 3% terpolymer solution. After 10 min of contact time, the complex coacervation reaction was quenched with phosphate-buffered saline (PBS, Dulbecco, Denmark) at room temperature.

Scanning electron microscopy (SEM, Nova NanoSEM 600, FEI Company) was used to visualize the morphology and distribution of the co-polymer matrix on the scaffold.

2.4. Expansion of hMSC-TERT

A telomerase reverse transcriptase gene-transduced cell population, hMSC-TERT, was used in this study. These cells maintain the functional characteristics of primary MSCs and have the capability to differentiate into certain mesodermal cell types (osteoblasts, chondrocytes and adipocytes) upon specific stimuli [29]. Cells from population doubling (PD) level 262 (passage 45) were seeded at a density of 4000 cells cm⁻² in culture flasks in Dulbecco's modified essential medium (DMEM, Invitrogen, Denmark) containing 10% fetal bovine serum (FBS, Invitrogen, Denmark) and cultivated in a humidified atmosphere of 37 °C and 5% CO₂. After 1 week, cells were washed in PBS, detached with 0.125% trypsin and 5 mM EDTA (Sigma, Denmark) in PBS for 4 min, reseeded and cultured for another week. Cells were trypsinized (PD level 271, passage 47) and resuspended for use (4 × 10⁷ cells ml⁻¹) in DMEM/10% FBS with penicillin (100 U ml⁻¹, Sigma, Denmark) and streptomycin (100 mg l⁻¹, Sigma, Denmark).

2.5. Seeding hMSC-TERT in PCL scaffolds

Scaffolds coated with hyaluronic acid were placed in 1% agarose-coated 6-well culture plates (4 scaffolds well⁻¹). 50 µl of a 2 × 10⁷ cell ml⁻¹ cell suspension in 3 mg ml⁻¹ solution of methylated collagen was dispensed onto scaffolds, giving a seeding number of 1 × 10⁶ cells scaffold⁻¹. Seeded scaffolds were incubated for 2 h and then encapsulated in 50 µl of 3% terpolymer solution in a dropwise manner. After 10 min incubation, 7.5 ml DMEM/10% FBS, 100 U ml⁻¹ penicillin, and 100 mg l⁻¹ streptomycin were added to each well and scaffolds were incubated overnight to allow the cells to adhere. In the control group, naked PCL scaffolds were seeded with 1 × 10⁶ cells scaffold⁻¹.

2.6. Dynamic and static culture for naked and embedded PCL scaffolds

After 24 h, the scaffolds were either moved to new agarose-coated 6-well plates (1 scaffold well⁻¹) or placed in 58 mm diameter dual side-arm spinner flasks (Bellco Glass, Vineland, NJ, USA). An autoclavable stainless steel framework with four needles was constructed and placed in the spinner flasks. Two cell-seeded scaffolds were mounted on each needle, giving a total of eight scaffolds per flask. Spinner flasks containing 120 ml of media each were placed on a Bell-ennium™ five-position magnetic stirrer (Bellco Biotechnology, NJ, USA) with 40 mm magnetic bars rotating at 30 rpm. The spinner flasks' side arm caps were loosely attached to improve oxygenation of the media. From day 1 and thereafter,

the media was DMEM/10% FBS with 10^{-8} M 1,25-(OH) $_2$ -vitamin D $_3$ (Vit D, LEO Pharma, Denmark) for DNA, alkaline phosphatase (ALP) and gene expression analysis. On days 0, 7, 14 and 21, four scaffolds from each group (naked and embedded, static and dynamic) were rinsed in PBS and frozen for DNA quantification; three scaffolds were collected for live/dead staining and histology. On days 1, 7, 14 and 21, four scaffolds were rinsed in cold PBS and frozen in 1 ml Trizol (Invitrogen, Denmark) for gene expression analysis. In addition, on days 7, 14 and 21, four scaffolds were used for ALP activity assay. The cell seeding efficiency was determined as % seeding efficiency = $100 \times \text{DNA amount per scaffold on day 0 (24 h after cell seeding)}/\text{DNA amount of } 1 \times 10^6 \text{ cells (equal to } 6.9 \mu\text{g DNA)}$.

For calcium deposition measurement, seeded scaffolds were cultured in DMEM/10% FBS for 1 week. The medium was then replaced with osteogenic stimulation media (DMEM/10% FBS with 100 nM dexamethasone, 290 μM ascorbic acid, and 5 mM β -glycerophosphate) (all from Sigma, Denmark). On days 14, 21 and 28, four scaffolds from each group were collected for calcium assay and one scaffold for von Kossa staining. Media in all cultures were changed every 3–4 days.

2.7. DNA quantification

The total cell number in the 3-D cellular scaffold was estimated by quantifying the double-stranded DNA (dsDNA) content in each scaffold using the Quant-iTTM PicoGreen[®] dsDNA assay (Invitrogen, Denmark). Scaffolds were thawed and sonicated at intervals of 1 s on/5 s off for a total of 1 min. 3 mg of collagenase (Sigma, Denmark) were added to each DNA sample and the samples were incubated in a 37 °C waterbath for 3 h. One milligram proteinase K (Sigma, Denmark) was then added and the samples were incubated overnight in a 45 °C waterbath. Sample volume was diluted 1:10 in Tris–EDTA buffer and vortexed to release DNA from scaffold debris. From each sample, $2 \times 50 \mu\text{l}$ were drawn, $50 \mu\text{l}$ of PicoGreen (diluted 1:200 in TE buffer) was added, and the mixture was incubated for 5 min in the dark, and measured in a 96-well plate using a microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark). Samples were excited at 480 nm, and the fluorescence emission intensity was measured at 520 nm. Standards were prepared according to the manufacturer's instruction (lambda DNA, concentration range: 0–1 $\mu\text{g ml}^{-1}$). Technical duplicates were used for each biological sample.

2.8. Live/dead staining

The scaffolds were rinsed in PBS and stained with 2 μM calcein AM and 4 μM ethidium homodimer (EthD-1) in PBS (LIVE/DEAD[®] Viability Kit, Molecular Probes) for 30 min in the dark. Non-fluorescent calcein AM is converted to highly fluorescent calcein by intracellular esterase activity and it stains viable cells green, whereas EthD-1 enters cells with injured membranes and stains dead cells red by binding to DNA. Images were immediately acquired using a laser scanning confocal microscope (510 Meta, Zeiss Microimaging GmbH, Germany). The confocal settings (excitation, laser power, detector gain and pinhole size) were identical for all acquired images. Separate channels and filters were used for each selected wavelength. Excitation/emission wavelengths were 494 nm/517 nm for calcein and 528 nm/617 nm for EthD-1.

2.9. Alkaline phosphatase activity assay

ALP activity was determined using a colorimetric endpoint assay measuring the enzymatic conversion of *p*-nitrophenyl phosphate (Sigma, Denmark) to the yellowish product *p*-nitrophenol in the presence of ALP. Absorbance of *p*-nitrophenol was measured

by a microspectrophotometer at wavelengths of 405 and 600 nm. Standards were prepared from *p*-nitrophenol (concentration range: 0–0.2 mM). Technical duplicates were used for each biological sample.

2.10. Calcium content assay

Calcium contents of cell-seeded scaffolds were quantified by colorimetric endpoint assay based on the complexation of one Ca^{2+} ion with two Arsenazo III molecules to a blue-purple product (Diagnostic Chemicals Limited, Charlottetown, PE, Canada). Briefly, the calcium deposition is dissolved in 1 M acetic acid by placing in a shaker overnight. The samples were diluted 1:50 with double-distilled (dd)H $_2$ O and aliquots of 20 μl were transferred to a 96-well plate. Arsenazo III solution (280 μl) was added and incubated for 10 min at room temperature. A standard dilution series of calcium ranging from 0 to 50 $\mu\text{g ml}^{-1}$ was prepared and Ca^{2+} concentration was quantified spectrophotometrically at 650 nm. Calcium content was expressed as micrograms of Ca^{2+} per scaffold.

2.11. von Kossa staining

Scaffolds were gently rinsed with PBS and fixed for 5 min in 4% (w/v) formaldehyde solution (pH 7.0, BDH, Denmark), then washed with ddH $_2$ O, incubated with a 2.5% silver nitrate solution (Sigma, Denmark) for 20 min in the dark, and subsequently developed by adding 0.5% hydroquinone (Merck, Denmark) for 2 min. Finally, surplus silver was removed using sodium thiosulfate (Sigma, Denmark) for 5 min. Scaffolds were dried under vacuum and pictures taken. The scaffolds were embedded in Technovit[®] 7100 (Ax-lab, Vedbaek, Denmark), and cut into 25 μm sections using a sawing microtome (KDG 95, Meprotech, Heerhugowaard, the Netherlands). Sections were counterstained with 0.1% toluidine blue (Fluka, Denmark) at pH 7. Sections were viewed and photographed on a BX50 fluorescence microscope with a digital camera (Camedia C-5060) (All products from Olympus, Denmark).

2.12. Histology

Scaffolds were fixed in 70% ethanol, Technovit[®] 7100 (Ax-lab, Vedbaek, Denmark) embedded and cut in 25 μm sections using a KDG 95 sawing microtome. Sections were taken from the peripheral and the central part of the scaffold, stained for 10 min at 37 °C with 1 $\mu\text{g ml}^{-1}$ Hoechst 33258 (Sigma–Aldrich, Denmark) in PBS and mounted. Sections were viewed and photographed on a BX50 fluorescence microscope equipped with a WU 330–385 nm filter and a digital camera (Camedia C-5060). To generate an overview of the entire scaffold height, three adjacent images were stitched together using Photoshop CS3 and transformed into a black background so that white dots represented cell nuclei.

2.13. RNA extraction and real-time PCR

Scaffolds were vortexed thoroughly in Trizol for 30 s; chloroform (Sigma, Denmark) was added and samples vortexed again for another 30 s. RNA was precipitated with isopropanol (Merck, Denmark), washed with 75% ethanol and dissolved in RNase-free, DEPC-treated water (Ambion, Cambridgeshire, UK). RNA amount, purity and integrity were assessed using UV/vis spectrophotometry (absorbance at 260 and 280 nm) and 1% agarose gel electrophoresis. First-strand cDNA was synthesised from 2 μg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Naerum, Denmark).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed under standard enzyme and cycling conditions on a 7500 fast real-time PCR system using TaqMan[®] gene expression assays

(Applied Biosystems) for TATA box binding protein (TBP, Hs00427621_m1), β -2-microglobulin (B2 M, Hs99999907_m1), glucuronidase β (GUSB, Hs99999908_m1), Runx2 (Hs00231692_m1), ALP (Hs00758162_m1), collagen type I alpha I (Col I, Hs00164004_m1), osteocalcin (OC, Hs01587813_g1), osteonectin (ON, Hs00277762_m1), bone sialoprotein I/osteopontin (BSP-I/OP, Hs00167093_m1), bone sialoprotein II (BSP-II, Hs00173720_m1), SOX9 (Hs00165814_m1), Aggrecan (AGC, Hs00153936_m1), Collagen type X (Col X, Hs00166657_m1), and a custom-designed TaqMan[®] assay for human RNA polymerase II (RPII). These assays all include two unlabeled primers and one FAM[™] labeled TaqMan[®] MGB exonuclease probe spanning an exon–exon boundary. Ampli-con sizes were all less than 170 bp. Template cDNA corresponding to 40 ng of RNA was added to each PCR reaction and each biological sample was run in technical duplicates for each gene. Data analysis was performed using 7500 Fast System Sequence Detection Software version 1.3. Expression levels of the genes of interest were normalized to a BestKeeper index [30], calculated for each sample based on a geometric mean of threshold cycles (Ct) from TBP, B2 M and GUSB. The following equation was used for each sample:

$$\text{Relative gene of interest expression} = 2^{\text{Ct}(\text{BestKeeper}) - \text{Ct}(\text{gene of interest})}$$

2.14. Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD) for $n = 4$ biological replicates. Statistics were assessed using Stata 10.0 (College Station, TX). The data of ALP activity, calcium content and gene expression were determined using two-way ANOVA (time $\times$ treatment). When significant main effects or an interaction between main effects were found, specific comparisons were made with Student's *t*-tests (equal variance) or the Wilcoxon rank-sum test (non-equal variance). Differences between means were considered statistically significant when *P*-values were < 0.05 .

3. Results

3.1. Characterization of the co-polymeric matrix on scaffolds

SEM micrographs of the PCL scaffolds (Fig. 1) showed how the scaffolds were formed by the fusion of approximately 180 μm wide PCL fibers. With a center–center fiber spacing of 1 mm, the edge–edge distance between fibers in each layer is 640 μm . The fibers in Fig. 1A showed the individual fiber surfaces scattered with 20–50 μm wide pits and shallow crevices, which were structures created during polymer cooling and crystallization.

After scaffolds were embedded by polyelectrolyte complex coacervation, the hyaluronic acid–methylated collagen–terpolymer complex formed around the scaffold (Fig. 1B–D). The co-polymer complex formed a delicate foam with nanometer-sized features. The foam was supported and distributed evenly inside the macroporous PCL scaffold.

3.2. Cell seeding efficiency

Assuming that no cellular division occurred during the first 24 h after seeding, the cell seeding efficiency was determined as % seeding efficiency = $100 \times \text{DNA amount per scaffold on day 0 (24 h after cell seeding)} / \text{DNA amount of seeded cells}$. The cell seeding efficiency was calculated to be 49.6% for the naked PCL scaffolds and 70.7% for the embedded PCL scaffolds (*t*-test, $P = 0.0001$, $n = 4$) (data not shown).

3.3. DNA quantification

The DNA amount was assumed to be proportional to the cell number. Thus, cell proliferation over time could be followed by extracting DNA from the scaffolds (Fig. 2). During the culture period, increasing amounts of DNA were observed in all cultures. For

Fig. 1. SEM micrographs of the naked and embedded PCL scaffolds. (A) PCL fibres in the naked scaffold with highly interconnected pores. (B) Co-polymeric matrix of hyaluronic acid, methylated collagen and terpolymer embedded in PCL scaffold. (C) Vertical section of co-polymeric matrix embedded in PCL scaffold. (A–C) 400 $\times$ magnification. (D) Higher magnification of co-polymeric matrix filling the pores of the PCL scaffold (3000 $\times$ magnification).

Fig. 2. Quantification of DNA from hMSC-TERT cultivated on the scaffolds by Picogreen assay. Cells were seeded on the scaffolds with or without co-polymeric matrix embedded and were cultivated under either static or dynamic conditions for 7, 14 and 21 days. The amount of DNA is expressed as mean $\pm$ SD ($n = 4$). *Significant difference between naked and embedded scaffolds within the same culture condition and time point ($P < 0.05$). #Significant difference between static and dynamic culture within the same treatment of scaffold and at the same time point ($P < 0.05$).

static culture, embedded scaffolds had significantly higher DNA levels than naked scaffolds on day 7 ($P = 0.001$) and day 14 ($P = 0.003$). For dynamic culture, the DNA levels of the naked scaffolds were higher than those of the embedded scaffolds on day 7 ($P = 0.006$) and day 14 ($P = 0.013$). The DNA levels were higher in dynamic culture compared with static culture for naked scaffolds on day 14 ($P = 0.0001$) and day 21 ($P = 0.0000$), the same for embedded scaffolds on day 14 ($P = 0.0001$) and day 21 ($P = 0.0000$).

3.4. Live/dead staining and confocal microscopy

The distribution of viable cells on the surface of scaffolds was qualitatively observed by confocal microscopy after staining by the live/dead fluorescent marker method (Fig. 3). On day 0, the seeding resulted in a slightly irregular dispersion of cells on both embedded and naked scaffolds. By day 7, this was overcome in static cultures by cells proliferating and migrating to cover the whole scaffold surface for both embedded and naked scaffolds. However, the embedded scaffolds showed a more homogeneously dispersed population of cells on the surface than the naked scaffolds at all the time points in static culture. For dynamic culture, a regularly dispersed cell layer was obtained on top of the scaffolds for both naked and embedded on day 14 and day 21. The naked scaffold showed a more confluent cell layer compared with embedded scaffold on day 14, but no difference on day 21. To compare static culture and dynamic culture, more confluent cell layers were obtained in the static culture.

3.5. ALP activity

The ALP activity of the embedded scaffolds was significantly higher than that of naked scaffolds for both static and dynamic culture at all the time points except on day 21 under static culture (Fig. 4). In static culture, the ALP activity of the embedded scaffolds was 1.99 times and 2.58 times higher than that of naked scaffolds on day 7 and day 14, respectively; in dynamic culture, the ALP activity of the embedded scaffolds was 3.29, 2.32 and 1.53 times higher than that of naked scaffold on days 7, 14 and 21, respectively.

For static culture, the ALP activity of naked and embedded scaffolds increased from day 7 to 21 in static culture; in dynamic culture, however, ALP activity decreased from day 7 to day 21 for both naked and embedded scaffolds.

3.6. Mineralization

To assess the extent of matrix mineralization on the scaffolds, the calcium contents were determined. Fig. 5A shows total calcium content per scaffold after 14, 21 and 28 days culture. Calcium deposition in the embedded scaffolds was significantly higher than in the naked scaffolds on days 21 and 28 for dynamic culture. There was no difference between naked and embedded scaffolds in the static culture at any time point. Dynamic culture resulted in a significant increase in calcium deposition compared to the static culture for both naked and embedded scaffolds on days 21 and 28. It was 2.2 times and 3.5 times higher in the dynamic cultured than the static cultured for naked group on days 21 and 28, respectively. With embedding, the increase was 4.6 times and 5.3 times higher.

Qualitative results of von Kossa staining supported the quantitative measurement of calcium deposition. We observed that the black color of von Kossa staining increased with culture time from day 14 to day 28. The staining color was darker in the dynamic cultured than the static cultured scaffolds on both days 21 and day 28. The embedded scaffolds had a more uniform black color throughout the scaffolds than the naked ones under static culture on day 28 (Fig. 5B).

In toluidine blue-stained sections, we observed that viable cells were present in the interior of the embedded scaffolds regardless of the culture method. Cells penetrated deeper into the embedded scaffolds than in the naked scaffolds for both static and dynamic culture. Additional von Kossa staining showed that scaffolds under dynamic culture had a more pronounced black staining than that under static culture. The intense black color of the matrix in the embedded scaffold under dynamic culture suggested a higher degree of matrix mineralization (Fig. 5C).

3.7. Histology

Cellular distribution within the scaffold was evaluated by fluorescent staining of cell nuclei on histological sections. Pictures were taken from top to bottom of the scaffold across the central part of the whole scaffold section and merged using Photoshop CS3 (Fig. 6). After 7 days of culture, cells penetrated into the interior of the scaffolds and migrated to the fibers at the bottom. New ECM formation was observed on day 7 in the static and dynamic scaffolds, but most pronounced in the embedded scaffolds under dynamic culture. After 14 days of culture, cells had proliferated and migrated throughout both naked and embedded scaffolds. In the naked scaffolds, cells proliferated and accumulated around the initial attachment sites. This clustering of cells was especially apparent under dynamic culture. In the embedded scaffolds, cells were more spread out and filled in the pores between the fibers. A clear difference between embedded and naked scaffold was seen on day 21 of dynamic culture, where cells were better dispersed in the inter-fiber space across the embedded scaffold compared to the naked.

3.8. Quantitative RT-PCR

The transcript levels of osteogenesis-related genes Runx2, ALP, Col I, OC, ON, bone sialoprotein I (BSP-I/OP), and bone sialoprotein II (BSP-II) were assessed by qRT-PCR. We furthermore chose to include chondrogenesis-related genes (SOX 9, AGC and Col X) in the analysis (Fig. 7).

Fig. 3. Confocal micrographs of whole scaffold surface (cell seeding side) after live/dead staining. The distribution of viable cells on the surface of constructs was qualitatively observed by confocal microscopy after staining by the live/dead fluorescent marker method on days 0, 7, 14 and 21. Pixels, representing viable cells, were transformed into black pixels for cell distribution analysis. The number of dead cells was negligible on all scaffolds.

Expression of Runx2 was higher for static than dynamic cultivation at all time points. For static culture, the expression reached a peak from day 14. For dynamic culture, the expression increased by embedding and peaked on day 21. The expression of ALP was higher for static than dynamic cultivation at all time points. The expression was higher in embedded than in naked matrix regardless of the culture methods. The expression rose during culture in statically cultivated scaffolds, whereas a stable expression was observed in dynamically cultured ones. The Col I expression increased significantly at day 21 for dynamically cultivated scaffolds from stable day 1 levels for both cultures. OC expression was higher for static than dynamic cultivation from day 7. For static culture, OC peaked at day 7 and subsequently decreased. OC was upregulated by embedding at most time points, from day 7 in dynamic culture, and from day 14 in static culture. ON expression was higher for dynamic culture than for static culture. Embedding downregulated ON expression at day 7 and day 14, but not at day 21 in both cultures. The expression of BSP-I (OP) decreased from day 1 in both static and dynamic cultures. For static culture, embedding always upregulated the expression. For dynamic

culture, embedding upregulated the expression only at day 1. BSP-II expression was highest at day 1 and decreased dramatically afterwards in both static and dynamic cultures.

The expression of SOX 9 was higher for statically cultured scaffolds than dynamically cultured scaffolds at all time points. For static culture, there was no difference between naked and embedded scaffolds and the expression peaked at day 14. For dynamic culture, the expression increased in embedded scaffolds until day 7 and then remained stable. Expression of AGC was higher in static culture than in dynamic culture, and embedding reduced AGC expression in both statically and dynamically cultivated scaffolds. Col X was highly expressed in dynamically cultured scaffolds from day 7 and peaked at day 21, while the Col X expression was low in statically cultured scaffolds at all time points. The expression was higher in naked than in embedded matrix regardless of the culture methods.

4. Discussion

This study evaluated the effects of a novel scaffold embedding and seeding system on cell seeding efficiency, proliferation, distri-

Fig. 4. Alkaline phosphatase (ALP) activity of hMSC-TERT per scaffold normalized by μg DNA per scaffold. Cells were seeded on the scaffolds with or without copolymeric matrix embedded and were cultivated under either static or dynamic conditions for 7, 14 and 21 days. The activity is expressed as mean $\pm$ SD ($n = 4$). Activity is indicated in $\text{nmol } \mu\text{g DNA}^{-1} \text{ min}^{-1}$. *Significant difference between naked and embedded scaffolds within the same culture condition and time point ($P < 0.05$). #Significant difference between static and dynamic culture within the same treatment of scaffold and at the same time point ($P < 0.05$).

bution and differentiation. The embedding system was a blend of both naturally derived and synthetic polymers and was applied to the scaffold along with the cells using a three-step process. We examined a PCL-based scaffold embedded with hyaluronic acid, methylated collagen and terpolymer via polyelectrolyte complex coacervation using an immortalized MSC population cultured under two different methods: dynamic and static culture. Porous FDM PCL scaffolds were selected as the scaffold model because of their availability, biodegradability and easy sectioning, and because they are a good candidate for clinical bone tissue engineering [31].

4.1. Naked PCL scaffolds

By analyzing the scaffolds using fluorescence microscopy, we qualitatively observed increased cell ingrowth and cell density at the interior of these PCL scaffolds, compared to previous reports [12]. This might be due to the different geometry of the scaffolds. In our study, we fabricated the PCL scaffolds using the FDM technique. These scaffolds can be made with bigger pore sizes than scaffolds fabricated by other techniques, e.g. salt leaching or thermally induced phase separation. Ishaug et al. showed that the mineralized matrix reached a maximum penetration depth of 240 μm from the top surface of PLGA foams [13]. A higher penetration depth of 2 mm from the surface of honeycomb-like PCL-TCP scaffolds was reported by Zhang et al. [32]. We observed that in static culture, cells had penetrated all the way through the 5 mm thick, 10 mm diameter scaffolds and all porosities had been filled with cells and matrix on day 21 (Fig. 6). This suggests that scaffolds manufactured by the FDM method provide better cell viability and penetration than those produced by salt leaching. However, the dynamic culture resulted in inhomogeneous cell distribution within the naked scaffolds, though these problems can be solved by our embedding method. This finding is inconsistent with previous studies in our group, even though we used the same cell type and the same dynamic set up. Stiehler et al. have shown that cellular distribution appeared homogeneous within the dynamic culture in the long

term [33]. In an earlier study, Mygind et al. also showed that dynamic culture improved the cells in the central part of the scaffolds [34]. This might partly be caused by the differences in material (polymer vs. hydroxyapatite) because cell migration depends not only on the adhesion surface, but also on the different geometry of the scaffolds. The bigger distances between the strands, and hence the larger pores, also increase the fluid convection through the scaffold during dynamic culturing. Thus it is important to optimize the flow speed for different scaffold geometries.

4.2. Embedded PCL scaffolds

Our study showed that hyaluronic acid-methylated collagen-terpolymer embedding significantly improved all the outcome variables, and furthermore seemed to have a dispersive effect on cell distribution within the porous scaffold owing to the increase of scaffold surfaces. The embedding process filled the larger pores of the PCL scaffold with a microporous matrix that had a delicate secondary pore structure. In addition, hyaluronic acid and collagen embedding can be seen as a polymer surface modification. They acted as a coating rich in ligands for integrins and CD44 on the PCL surface. The effects of the embedding may come mainly from its crude mimicking of the natural ECM in the immediate surroundings of the cells. Hyaluronic acid and collagen type I were used for this purpose as they are the major constituents of ECM [18].

Hyaluronic acid is a very large glycosaminoglycan present in most tissues. The size and hydrophilicity of the molecule are important properties for its function of adding turgor and mechanical resilience to tissues, especially those of the musculoskeletal system. Hyaluronic acid is abundant in ECMs where it contributes to tissue hydrodynamics, modulation of cell attachment strength, motility and proliferation. It participates in a number of cell surface receptor interactions, notably those including its primary receptor, CD44 [35]. Pathways that determine cell morphology and survival [36] can also be triggered by CD44 receptor–ligand interactions. This is analogous to the effects of the integrin receptors and both receptor families converge on the activation of Ras and Rac.

Single cells in suspension retain a pericellular coat that is rich in hyaluronic acid. This coat mediates a fast, integrin-independent adhesion to conventional substrates such as glass or plastic tissue culture dishes [37,38]. If the coat is enzymatically removed using hyaluronidase, the cells will detach even in the presence of integrin ligands. However, if both the substrate and the cell are covered by hyaluronic acid, no integrin-dependent attachment will take place and the cell will also detach. This was why the cells were seeded as a suspension in a collagen solution rather than in a hyaluronic acid solution.

The rationale behind the embedding procedure is that we tried to form a coacervation complex between the opposite charges. The initial layer of deposition on the scaffold was negatively charged hyaluronic acid which could interconnect the macroporous PCL scaffold and provided an increased surface area for the cell seeding. The cells were mixed with the positively charged methylated collagen solution and seeded into the hyaluronic acid-precoated scaffold. Collagen solution with corresponding cell spreading would aid in bonding the cells to the hyaluronic acid, and thus cells dispersed not only on the fibers, but also in the pores of the scaffolds. The final step was the application of the negatively charged terpolymer, which could help to form a loose network with a relatively even distribution of the coacervation complex [39].

4.3. Static culture

For a comparable DNA amount, the statically cultured, embedded scaffolds displayed a more homogeneous and dispersed cell

Fig. 5. (A) Calcium contents per scaffold on days 14, 21 and 28. The amount of calcium is expressed as mean $\pm$ SD ($n = 4$). *Significant difference between naked and embedded scaffolds within the same culture condition and time point ($P < 0.05$). #Significant difference between static and dynamic culture within the same treatment of scaffold and at the same time point ($P < 0.05$). (B) von Kossa staining of naked and embedded scaffolds under static and dynamic culture on days 14, 21 and 28. Each scaffold is shown viewed from the cell seeding side, the vertical side and the bottom side. (C) Vertical sections of corner parts of the scaffolds with von Kossa and toluidine blue staining after 28 days of culture. Scale bars represent 1 mm.

Fig. 6. Hoechst staining of cell nuclei on histology sections. Each picture is composed of three images stitched together and shows the total height of the central part of the sectioned scaffolds. Scale bars represent 1 mm.

Fig. 7. RT-PCR results for osteogenic and chondrogenic markers on days 1, 7, 14 and 21 of culture. The expression of the genes of interest was normalized to the geometric mean of the threshold cycles of three housekeeping genes (HKGs), TBP, B2 M and GUSB, using the BestKeeper index. The results are expressed as mean $\pm$ SD ($n = 4$). *Significant difference between naked and embedded scaffolds within the same culture condition and time point ($P < 0.05$). #Significant difference between static and dynamic culture within the same treatment of scaffold and at the same time point ($P < 0.05$).

distribution than naked scaffolds, especially at the earliest time points (Figs. 3 and 6). The migrating and dividing cells on the naked scaffolds will only have the ECM components available that

are synthesized de novo [40]. On the embedded scaffolds, cells had nearly the same expression levels of, for example, collagen type I (Fig. 7) but because of the pre-coating, their surroundings are more

conductive for cell migration from the start [41]. This ultimately increases the migration distance on embedded scaffolds and gives rise to a more dispersed cell distribution compared to the naked scaffolds. The delaying of cell sheet formation on the top surfaces of the statically cultured embedded scaffolds could likewise be attributed to the cells' motility caused by the embedding (Fig. 3).

ALP is an enzyme produced by active osteoblasts involved in the early initiation of mineralization of newly formed bone tissue [42]. There was a significant increase in ALP gene expression (Fig. 7) and ALP activity (Fig. 4) for the cells on the embedded scaffolds. Osteogenic differentiation is associated with a preceding assembly of focal adhesions and cytoskeletal rearrangement, which in turn are promoted by a substrate rich in RGD-containing proteins [43]. The complex coacervation procedure may contribute to the precipitation of methylated collagen into a functional integrin-binding conformation. This could have an advantage over adsorption-based protein coating techniques, which are more sensitive to the surface chemistry of the native substrate [44].

The calcium deposition assay revealed an equal mineralization between the statically cultured embedded and naked scaffolds (Fig. 5). The quantitatively similar degree of mineralization, despite the higher ALP activity for the cells on the embedded scaffolds, is because the transport of Ca^{2+} ions and β -glycerophosphate for both groups is diffusion limited. It is no longer enzyme activity but rather the supply rate of hydroxyapatite building blocks that forms the bottleneck.

PCR results showed that in the statically cultured scaffolds, there was an increased expression of osteogenesis-related genes, compared to dynamically cultured scaffolds. Apart from collagen X, chondrogenesis-related genes are also expressed more in static culture. This could be explained by the existence of steep nutrient gradients within the statically cultured scaffolds by which zones with distinct environments can be formed [45]. Hypoxia, in particular, favors chondrocytic differentiation [46].

4.4. Dynamic culture

Dynamic culture in spinner flasks significantly improved osteogenic differentiation on the embedded scaffold, compared to static culture. We observed that the embedded scaffolds under dynamic culture had the highest level of ALP activities of all on day 7 and the highest calcium content on day 28.

Dynamic culture in itself appears to antagonize the formation of cell sheets on the surface of the scaffold (Fig. 3). The constant fluid turbulence around the scaffold makes it more difficult for cells to bridge gaps. Since the overall number of cells is not reduced compared to static culture (Fig. 2), a larger fraction of cells are probably migrating to the deeper parts of the scaffolds in dynamic culture.

Fig. 4 showed that the ALP activity increased from day 7 to day 21 for the static culture, while it decreased with culture time for the dynamic culture. This suggested that the cells started to initiate mineralization earlier in dynamic culture than in static culture. The PCR results for ALP did not show the same early peak for the embedded dynamic scaffold as seen from the ALP activity measurement. We suspect that for embedded dynamic scaffolds the peak gene expression for ALP took place between day 1 and day 7, leading to the detected protein expression peak.

Several studies have shown that fluid shear stress upregulates anabolic factors relating to proliferation and differentiation of bone cells [47,48]. The improved mineralization of hMSCs in dynamic cultured environment over a static culture system is in line with previous findings [49–51]. However, the naked scaffolds in dynamic culture showed lower osteogenic gene expression and ALP activity, compared to the naked ones in static culture, which implies that fluid shear stress is not a guarantee of osteogenic differentiation. Again, the increased calcium deposition in the naked

scaffold, compared to both of the statically cultured scaffold groups, showed that high ALP activity by itself is not sufficient for mineralization when transport of Ca^{2+} ions and β -glycerophosphate is impaired (Fig. 5A).

It can be seen that the gene transcription patterns for the naked dynamic cultured scaffolds were remarkable for, for example, Col X, Runx2, and ALP (Fig. 7). Collagen type X is a specific marker for chondrocyte hypertrophy, which is the stage before the chondrocyte-to-osteogenic differentiation that occurs in endochondral ossification [52]. In the naked dynamic group, the finding of elevated expression of collagen X together with the comparatively low expression of Runx2 conflicts with the established mechanism of collagen X regulation where Runx2 is a positive factor [53].

Rowlands et al. have described how stem cell differentiation can be directed by substrate stiffness and the presence of specific matrix proteins. They found that collagen type I in combination with a stiff substrate generated the highest levels of Runx2 expression, which was an indicator for osteogenic differentiation [54]. In our study, the combination of shear stress and absence of embedding could push a majority of the seeded MSCs towards chondrogenic differentiation at an early time point. Alternatively, chondrocyte differentiation could be caused by the generation of a high density cell clusters at the conjunction of scaffold fibers in the absence of embedding. This may have mimicked the chondrogenic effects seen in pellet culture [55], which was then augmented by the mechanical stimulation [56].

BSP-I and BSP-II are non-collagenous glycoproteins that facilitate nucleation of hydroxyapatite crystals during the initial stages of mineralization. They have been used as markers for osteogenic differentiation [57]. We saw that both proteins had very pronounced gene expression peaks after 1 day of culture followed by a stable low-level expression during the remaining culture period. This is an interesting finding in light of previous results in our group [34], where quantitative real-time RT-PCR was first performed after 7 days and thus might have overlooked a potential peak. It is also notable that the highest contrast between all four groups was present so early. The results suggest that early regulation of BSP-I is mainly dependent on substrate properties, which is apparent from the increased expression in the embedded groups, compared to the naked groups. This is in line with previous studies using murine preosteoblasts on controlled surface chemistries [58]. It also implies that shear stress has a positive effect on BSP-I expression. BSP-II, on the other hand, appears to be highly regulated by mechanical stimulation and indifferent to embedding. This finding is difficult to explain from existing literature and raises an interesting topic for further study.

There was a remarkable contrast in the expression of two major non-collagenous bone matrix proteins, osteonectin and osteocalcin. These proteins appear to control apatite crystal growth after mineralization has been initialized [59]. Both static cultures have elevated expression of osteocalcin, compared to dynamic cultures, while it is the other way around for osteonectin expression. Ninomiya et al. found that osteonectin is 21 times more abundant in human trabecular bone compared to cortical bone while osteocalcin is 30 times more abundant in cortical bone compared to trabecular bone [60]. Consequently, the results, especially those for osteocalcin, suggest that fluid shear stress plays an important role in determining the morphology of engineered bone tissue.

5. Conclusion

The present in vitro study described a new concept of scaffold modification procedure and dynamic culture technique. The results showed that the embedding and dynamic culture synergistically enhanced the osteogenic differentiation of hMSC and calcium

deposition. Embedded scaffolds had a higher cell seeding efficiency, higher number of differentiated cells and more homogeneous cell distribution. Qualitatively, the embedding decreased the extent of cell sheet formation on the scaffold surfaces, which limits nutrient transport. The ALP activity, calcium contents and the expression of several osteogenic markers (e.g. ALP, OC and BSP-I) were significantly higher in the embedded scaffolds. Dynamic culture was better than static culture in terms of higher ALP activity and calcium deposition, which are important for bone tissue engineering.

The study was carried out on scaffolds fabricated in our laboratory, but the procedure is applicable to commercialized macroporous scaffolds to further improve their osteoconductivity and clinical potential.

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