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Effect of fetal bovine serum on mineralization in silk fibroin scaffolds

Jolanda R. Vetsch a,b, Samantha J. Paulsen a, Ralph Müller b, Sandra Hofmann a,b,c,⇑

∥Institute for Biomechanics, Swiss Federal Institute of Technology Zürich (ETHZ), Vladimir-Prelog-Weg 3, Zurich 8093, Switzerland
∥Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, Eindhoven 5600MB, The Netherlands
∥Institute for Complex Molecular Systems, Eindhoven University of Technology, PO Box 513, Eindhoven 5600MB, The Netherlands

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A B S T R A C T
Fetal bovine serum (FBS) is a common media supplement used in tissue engineering (TE) cultures. The chemical composition of FBS is known to be highly variable between different brands, types or batches and can have a significant impact on cell function. This study investigated the influence of four different FBS types in osteogenic or control medium on mineralization of acellular and cell-seeded silk fibroin (SF) scaffolds. In bone TE, mineralized tissue is considered as the final product of a successful cell culture. Calcium assays and micro-computed tomography scans revealed spontaneous mineralization on SF scaffolds with certain FBS types, even without cells present. In contrast, cell-mediated mineralization was found under osteogenic conditions only. Fourier transform infrared spectroscopy analysis demonstrated a similar ion composition of the mineralization present in scaffolds, whether cell-mediated or spontaneous. These results were confirmed by scanning electron microscopy. This study shows clear evidence for the influence of FBS type on mineralization on SF scaffolds. The suitability of FBS medium supplementation in TE studies is highly questionable with regard to reproducibility of studies and comparability of obtained results. For future TE studies, alternatives to conventional FBS such as defined FBS or serum-free media should be considered, as suggested decades ago.

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1. Introduction

Fetal bovine serum (FBS) is a common media supplement used in tissue engineering (TE) cultures. The chemical composition of FBS typically varies from batch to batch, between different types and among different brands due to biological variance [1,2]. FBS contains many unknown substances with unclear functions on cultured cells that may alter the outcome of cell experiments [1]. Today, various types of defined FBS are commercially available. Defined FBS is chromatographically purified, then individual constituents are separated and recombined into a defined composition.

Biomineralization is a process describing mechanisms of mineralized tissue formation by organisms in nature [3,4]. Biominerals are hybrid structures composed of both minerals and organic components [4]. Mineral deposition starts by the formation of precipitation clusters at a templating surface initiated by local supersaturation of ions [5,6]. The formation of these ion clusters is highly dependent on the fluid surrounding the underlying structure of the inorganic matrix [4,6]. The proteins of the inorganic matrix interact with ions of the surrounding fluid and facilitate the formation of mineralized macromolecules [5].

The most abundant mineralized tissue in the human body is bone [5]. Bone is a composite material consisting of hydroxyapatite (HA) and collagen type I (Col I) fibrils [7]. In vivo bone mineralization is a cell-mediated process. Osteoblasts are responsible for the deposition of bone matrix into the extracellular space. Col I is the major component of bone matrix [8] and is a fibrous protein containing repetitive amino acids (Fig. 1A). Col I builds the three-dimensional (3-D) framework of bone on which bone mineral is deposited [4]. The mineralization process in bone occurs by nucleation of HA out of calcium and phosphate ions in solution. The organic Col I acts as a substrate for the mineralization of HA crystals that mineralize in thin layers between sequences of Col I molecules (Fig. 1A) [9].

Like Col I, silk fibroin (SF) is a fibrous protein. It is synthesized by Lepidoptera larvae [10]. SF protein side chains interdigitate and form antiparallel plated β-sheets (Fig. 1B). Highly and less ordered β-sheets are connected by amorphous network chains [11]. SF is a widely used scaffold material for bone tissue engineering (TE) applications [12–16], due to its excellent biocompatibility [17], controllable degradation [18] and favorable mechanical properties [19]. SF is an interesting scaffold material for bone TE consid-

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er ing its ability to regulate the formation of HA nanocrystals when exposed to simulated body fluid (SBF). SBF mimics ion concentrations of human blood serum [20,21]. It was shown that SF has the potential to mineralize spontaneously in SBF by inducing apatite deposition on its surface and provoking continuous growth and enrichment of HA crystals [21,22]. Marelli et al. [11] have shown that the amorphous connections between the β-sheets of SF act as nucleation sites for HA crystals similar to Col I in bone. Spontaneous deposition of mineralization on SF was also shown in calcium chloride (CaCl₂) solution. Choi et al. [23] managed to promote calcium deficient HA formation on SF particles in CaCl₂ solution, due to electrostatic interactions between the calcium ions and the functional groups of SF [23]. Ion compositions in FBS and SBF are very similar and the pH, an important environmental factor influencing spontaneous mineralization, of both solutions is buffered to 7.4 [20,24].

In bone TE applications, the formation of mineralized extracellular matrix by cells seeded on a 3-D scaffold is considered as the final product of a successful culture. Still, obtained results vary highly within and between research groups and reproducibility of studies is often not given. If FBS type (or even batch) has an influence on mineralization due to variations in chemical composition of different suppliers, this effect needs to be quantified or preferably avoided in order to draw conclusions on how the cellular environment influences TE outcomes. There are some studies which investigated the effect of FBS on mineralization focusing on the cellular response, but without having a closer look at the role of the materials used [25,26]. The objective of this paper was to investigate the influence of four different FBS types on mineralized tissue formation in cell-seeded and acellular SF scaffolds. Two conventional and two defined FBS types were compared against each other. Additionally, the influence of osteogenic factor supplementation on mineralized tissue formation was evaluated.

2. Materials and methods

Details of all FBS types used (full label, order number, batch number and detailed supplier information), all experimental conditions (FBS type, medium type and cell use) and all assays performed (including number of samples evaluated for each experimental condition) can be found in Table 1.

2.1. Materials

The four different FBS types used were chosen according to the following criteria. Gibco Standard is the standard FBS currently used in our lab. Like Gibco Standard, PAA Standard is a conventional FBS and was chosen as a direct control for Gibco Standard. PAA Gold and Biochrom Superior are both defined FBS types and were chosen because the suppliers assert no need for batch-to-batch testing. Dulbecco’s modified Eagle medium (DMEM), antibiotic/antimycotic (Anti-Anti) and trypsin were from Gibco (Zug, Switzerland). 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was obtained from abcr chemicals (Karlsruhe, Germany). Methanol (MeOH) was from Merck (Zug, Switzerland) and lithium bromide (LiBr) from Thermo Fisher Scientific (Reinach, Switzerland). Phosphate buffered saline (PBS) was supplied from Medicago (Uppsala, Sweden). All other substances were of analytical or pharmaceutical grade and obtained from Sigma (Buchs, Switzerland). Silkworm cocoons were kindly provided by Trudel Inc. (Zurich, Switzerland).

2.2. Scaffolds

SF scaffolds were produced as previously described [14,27]. Briefly, silk from Bombyx mori L. silkworm cocoons was degummed by boiling in 0.2 M Na₂CO₃ twice for 1 h. Dried silk was dissolved in 9 M LiBr and dialyzed against ultra pure water (UPW) for 36 h using Slide-A-Lyzer cassettes (molecular weight cutoff: 3.5 K; Thermo Fisher Scientific, Reichenach, Switzerland). Dialyzed silk solution was lyophilized (Alpha 1-2, Martin Christ GmbH, Osterode am Harz, Germany) for 4 days and dissolved in HFIP, resulting in a 17% (w/v) solution. 1 ml of dissolved silk was added to 2.5 g NaCl with a granule size of 300–400 μm and was allowed to air dry for 3 days. Silk-salt blocks were immersed in 90% MeOH for 30 min to induce β-sheet formation [28]. NaCl was extracted from dried blocks in deionized water for 2 days. Scaffolds were cut into disks of 5 mm in diameter and 3 mm in height and autoclaved in PBS at 121 °C for 20 min.

2.3. Cell culture

Human mesenchymal stem cell (hMSC) isolation from human bone marrow (Lonza, Walkersville, MD, USA) was performed as previously described [29]. Passage 3 hMSCs were expanded for 7 days. At day 7 hMSCs were trypsinized and half of all scaffolds were seeded with 1 million cells per scaffold, while the remaining scaffolds were left acellular. All scaffolds were incubated in wells of a 24-well plate at 37 °C for 90 min to allow cell attachment. Subsequently, half of all cell-seeded scaffolds and half of all acellular scaffolds were provided with 1 ml control medium (DMEM supplemented with 10% FBS and 1% Anti-Anti) and were incubated at 37 °C and 5% CO₂. The remaining scaffolds were incubated in 1 ml osteogenic medium (control medium supplemented with

Fig. 1. Comparison of collagen and silk fibroin structures. Both fibrous proteins show a hierarchical structure with repeating amino acids and have been reported to take part in mineralization processes. (A) Structure of collagen with repeating amino acids of glycine (Gly), proline (Pro) and hydroxyproline (Hyp). (B) Structure of silk fibroin with repeating amino acids of glycine (Gly), serine (Ser) and alanine (Ala).
50 μg ml⁻¹ L-ascorbic acid 2-phosphate, 100 mM dexamethasone and 10 mM β-glycerophosphate (β-GP)). Every culture medium was prepared with every type of FBS, resulting in 16 experimental conditions (Table 1).

### 2.4. Calcium assay

A spectrophotometric calcium assay was performed according to the manufacturer’s instruction (Calcium CPC FS, Rolf Greiner BioChemica, Flacht, Germany). After 3, 5 and 7 weeks of culture, 10 scaffolds per group were washed with PBS and disintegrated in 5% aqueous trichloroacetic acid (TCA) using steel balls and a MinibeadBeater™ (Biospec, Bartlesville, OK, USA). Scaffolds were further incubated in 5% TCA at room temperature for 48 h and frozen until evaluation.

For the correlation of calcium assay and micro-computed tomography (μCT) data, 20 scaffolds were analyzed after μCT scanning for calcium amount. After the μCT scans, scaffolds were disintegrated and incubated as described before. For analysis, all scaffolds were centrifuged at 3000 g for 10 min. Supernatant was further diluted in 5% TCA at a ratio of 1:20 (v/v) and the color intensity of the resulting solution was measured with a microplate reader (Infinite 200 PRO, Tecan Group Ltd, Männedorf, Switzerland).

### 2.5. μCT

μCT scans were performed on scaffolds after 7 weeks of culture (N = 10 per group). Scaffolds were washed with PBS, lyophilized overnight (Alpha 1-2, Martin Christ GmbH, Osterode am Harz, Germany) and scanned in air on a μCT 40 (Scanco Medical, Brütisellen, Switzerland). The energy level was set to 45 kVp, intensity to 10 mGy, integration time to 200 ms and a frame averaging of 4 was applied. The scans were executed at medium resolution mode with a nominal resolution of 12 μm. After reconstruction, scans were filtered applying a 3-D constrained Gaussian filter with finite filter support (1 voxel) and filter width (sigma = 1.2). Filtered grayscale images were segmented at a global threshold of 17% of the maximal grayscale value (corresponding to a density value of 125.17 mg HA cm⁻³). Unconnected objects smaller than 50 voxels were removed and neglected for further analysis. For the final evaluation an overall mask (OM) with radius = R₀ and height = H was generated (Fig. 2A). R₀ and H were chosen to fit to the outer boundaries of each scaffold individually due to small differences in scaffold dimensions especially present between mineralized and non-mineralized scaffolds. The resulting 3-D volume was evaluated morphometrically for mineralized tissue volume (BV) and mineralized tissue volume fraction (BV/TV = mineralized tissue volume/total volume), as described previously for bone[30,31]. All scaffolds were grouped into either an “acellular” or a “cell-seeded” group to assess differences in spatial distribution of spontaneously formed mineralization on acellular scaffolds and of cell-mediated mineralization observed on cell-seeded scaffolds. An inner mask (IM) was generated based on the geometry of the OM (Fig. 2B). The percentage of total BV within the IM was calculated based on the BV of the OM. The radius (R(IM)) of the IM equaled 50% × R₀. H was kept constant (Fig. 2B). Bone mineral content (BMC) per scaffold as determined by μCT was correlated with calcium amount per scaffold as measured biochemically.

### 2.6. Fourier transform infrared spectroscopy (FTIR)

After 7 weeks of culture, scaffolds (N = 2 per group) were washed with PBS, frozen at −80 °C overnight and lyophilized for 1 day. For FTIR analysis dried scaffolds were ground with KBr at 4% (w/w). 100% KBr was used as background medium. The ground samples were analyzed using a Bruker Tensor 27 FTIR spectroscope (Bruker Optics GmbH, Fällanden, Switzerland) at a resolution of 4 cm⁻¹. Scans were performed in full range from 4000 cm⁻¹ to 400 cm⁻¹ at a scan time of 100 ms. Measurements were converted to absorbance spectra in OPUS Application Executable 5.0.5 (Bruker Optics GmbH, Fällanden, Switzerland). SigmaPlot 12.2 (Systat Software, Inc., San Jose, CA, USA) was used to determine exact wavenumbers for peaks and Adobe Illustrator CS5.1 (Adobe Systems, Inc., San Jose, CA, USA) was used to mark peaks.

### 2.7. Scanning electron microscopy (SEM)

After 7 weeks of culture, scaffolds (N = 2 per group) were washed in PBS and fixed in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 4 h. After rinsing in 0.1 M...
A second fixation step was performed using 0.04% aqueous osmium tetroxide in 0.1 M cacodylate buffer at room temperature in the dark. Scaffolds were again rinsed in 0.1 M cacodylate buffer, dehydrated for 10 min in serial EtOH baths of 37%, 67% and three times 96%, frozen overnight in 100% EtOH at \(-80^\circ C\) and lyophilized. Scaffolds were coated with gold using a sputter coater (Balzers SCD 030, former Balzers Union Ltd, Liechtenstein) for 90–120 s at a current of 40 mA and imaged at 850 \(\times\) and 5000 \(\times\) magnification in an SEM (Leo 1530 Gemini, Zeiss, Oberkochen, Germany) operated at a voltage of 5 kV to investigate scaffold surface morphology.

### 2.8. Statistical analysis

Quantitative data are represented as mean ± standard deviation. IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA) was used for the evaluation of statistically significant differences for calcium assay and quantitative \(\mu CT\) data. Under the assumption of normally distributed data, analysis of variance was performed followed by post hoc assessment using the Bonferroni method. Differences between groups were considered statistically significant at a level of \(P < 0.05\). Images in Fig. 4 represent the upper median BV/TV sample of each group.
3. Results

3.1. Calcium assay

Calcium assays were performed to assess total calcium content of the scaffolds, as a method to represent the total mineralized tissue volume per scaffold (Fig. 3A–C). As expected, cells responded to the different culture media used. Cells seeded on scaffolds cultured in control medium remained undifferentiated, whereas cells seeded on scaffolds in osteogenic medium underwent osteogenic differentiation and started to form mineralized tissue. Scaffolds cultured in osteogenic medium exhibited the highest calcium contents after 7 weeks (Fig. 3C). Interestingly, considerable amounts of calcium were detected for acellular scaffolds with certain FBS types starting as early as week 3 up to week 7. Elevated calcium levels were observed particularly for acellular scaffolds cultured with Biochrom Superior and Gibco Standard (Fig. 3A–C).

Cell-seeded scaffolds cultured in osteogenic medium showed significantly increased calcium contents compared to cell-seeded scaffolds cultured in control medium at each time point \( P < 0.05 \). No differences were observed between cell-seeded scaffolds cultured in control medium at any time point. Acellular scaffolds cultured in osteogenic medium with Biochrom Superior showed significantly increased calcium deposition compared to cell-seeded scaffolds cultured in control medium with the same FBS type at week 3 \( P < 0.05 \); Fig. 3A). In week 5 a similar result was observed for acellular scaffolds cultured in control and osteogenic medium with Biochrom Superior with significantly higher calcium levels compared to their corresponding cell-seeded scaffolds cultured in control medium \( P < 0.05 \); Fig. 3B). The same result was observed for acellular scaffolds cultured in control and osteogenic medium with Gibco Standard at week 7 \( P < 0.05 \); Fig. 3C).

3.2. \( \mu \)CT

\( \mu \)CT measurements of BV/TV (Fig. 3D) confirmed calcium assay data of week 7 (Fig. 3C). Cell-seeded scaffolds cultured in osteogenic medium exhibited the highest BV/TV values compared to cell-seeded scaffolds cultured in control medium and all acellular scaffolds \( P < 0.001 \). Acellular scaffolds cultured with Biochrom Superior and Gibco Standard showed significantly increased calcium levels compared to their corresponding cell-seeded scaffolds cultured in control medium \( P < 0.05 \).

Mineralized tissue was located primarily on scaffold edges and the top of the scaffolds in cell-seeded scaffolds. Acellular scaffolds cultured with Biochrom Superior and Gibco Standard showed substantial mineralization (Fig. 4A and B). Very little mineralization was observed in acellular scaffolds cultured with PAA Gold or PAA Standard or in cell-seeded scaffolds cultured in control medium (Fig. 4A–C). Cell-seeded scaffolds cultured in osteogenic medium displayed highly mineralized structures with every type of FBS (Fig. 4D). For the evaluation of differences in spatial distribution of acellular scaffolds and cell-seeded scaffolds only mineralized scaffolds (BV of OM > 0.001 mm\(^3\)) were considered. Acellular scaffolds \( (N = 77) \) showed significantly increased BV percentage in the IM \((18.87 \pm 18.32\%)\) compared to cell-seeded scaffolds \( (N = 52) \) that displayed \( 10.66 \pm 11.16\% \) of the total BV in the IM \( (P < 0.05); \) Fig. 5). BMC per scaffold correlated highly with calcium amount per scaffold \( (R^2 = 0.96; \) Supplementary Fig. S.1A). Both measurement methods were in agreement and no systematic differences could be observed between calcium assay and \( \mu \)CT data (Bland Altman Plot; Supplementary Fig. S.1B).

3.3. FTIR

FTIR was performed to analyze differences in phosphate and carbonate precipitates on the scaffolds (Fig. 6; Supplementary Table S.1). Typical absorption spectra of the underlying SF scaffold were observed between 1700 and 1000 cm\(^{-1}\) for every culture condition [32]. All spectra showed features of different apatite
precipitates. Carbonate was present in all samples [11,33] and phosphate bands were visible between 1200 and 900 cm\(^{-1}\) [34–37]. Cell-seeded scaffolds cultured in osteogenic medium displayed a characteristic peak for phosphate ions (PO\(_4^{3-}\)) at 1038 cm\(^{-1}\), indicating a higher amount of mineralized tissue formation (Fig. 6D) [11,34]. Overlapping phosphate bands between 1200 and 900 cm\(^{-1}\) present in spectra of acellular scaffolds might have masked less prominent phosphate peaks.

3.4. SEM

SEM micrographs confirmed the results of the calcium assay and \(\mu\)CT data (Fig. 7; Supplementary Fig. S.2). Cell-seeded scaffolds showed extracellular matrix covering the surface of the scaffolds (Supplementary Fig. S.2I–P). No mineralization clusters were visible on acellular scaffolds cultured in osteogenic medium scaffolds with PAA Gold (Fig. 7A and C). In contrast, clearly visible mineralization clusters were observed with Gibco Standard under the same culture conditions (Fig. 7B and D).

4. Discussion

FBS has been used for more than a century as a cell culture additive for most cell types cultured in vitro [38,39]. It has been previously shown that differences in serum composition lead to statistical differences in experimental outcomes [40]. The disadvantages of FBS, such as undefined composition or batch-to-batch variations, have been known for decades. Alternatives to FBS have been proposed and include options such as sera from other species, reduced serum media, chemically defined FBS or chemically defined serum-free media [38,39]. Using defined FBS would eliminate the necessity to test every new batch of FBS and would minimize experimental differences due to different FBS batches or types. Nevertheless, if correct batch testing is performed, experimental differences could already be reduced. It is important that every FBS batch is tested, preferably under real culture conditions.

Very few publications can be found discussing differences in experimental outcomes provoked by FBS. The exact ordering and batch number of the FBS used in experiments is not required for publications and therefore a direct comparison of experiments from different laboratories seems impossible. It is very difficult to assess whether experimental outcomes occur due to the experimental conditions or if they are attributed to the FBS type or batch. Differences in FBS composition may be a major reason why different labs are not able to reproduce data published in the literature.

In this study, the effect of four different FBS types on mineralized tissue formation on SF scaffolds was investigated. The influence of FBS on mineralization is not new and has been addressed earlier. For example, mineral precipitation on decalcified newborn rat tibia in the absence of cells has been shown to occur when culture media were supplemented with FBS and osteogenic factors (IGF) [26], but not with FBS-containing control media only as shown with this study. Evident effects of FBS on mineralization, alkaline phosphatase activity and osteogenic marker expression based on
serum conditions have also been shown for human adipose stem cells, but the results were not compared to acellular culture conditions and therefore the influence of the material could not be determined [25]. The results observed in the present study revealed that the FBS type influences mineralization formation on acellular and cell-seeded SF scaffolds significantly. The addition of osteogenic factors did not have an influence on mineralized tissue formation in acellular scaffolds, but led to osteogenic differentiation with cell-seeded scaffolds.

Acellular scaffolds showed an unexpected and completely different mineralization pattern compared to cell-seeded scaffolds. Scaffolds cultured with Biochrom Superior and Gibco Standard show strong evidence of spontaneous mineralization on acellular SF scaffolds presenting calcium values up to one third of the highest calcium level measured overall. The amount of spontaneous mineralization of acellular scaffolds was therefore clearly dependent on the FBS type used. Interestingly, spontaneous mineralization occurred even under control conditions. This result is contradictory to previous results where mineral precipitation in cell-free conditions only occurred with FBS containing media supplemented with β-GP [26].

The ion concentration was stated for Biochrom Superior only (Supplementary Table S2). Hamlin and Price [26] hypothesized that there exist one or more nucleators of bone mineralization in serum that escaped from the bone matrix to the blood. For future studies it would be helpful to identify key components that contribute to mineralization.

Cell-seeded scaffolds showed mineralization when cultured with osteogenic medium only (Figs. 3 and 4). It is known that hMSCs differentiate towards the osteogenic lineage with the addition of osteogenic factors to the medium [41]. The presence of mineralization in these scaffolds shows a successful differentiation of the hMSCs towards osteoblasts. The amount of mineralization formed was dependent on the FBS type, especially at early time points (week 3 and 5; Fig. 3A and B), which is in agreement with previous results [25].

In cell-seeded scaffolds, we assume that cells prevented spontaneous mineralization by either changing the ion concentration of the solution surrounding the substrate [21]. Differences in spontaneous mineralization between the four FBS types used can be explained by differences in elemental composition. It is known that sera exhibit significant differences in composition between different types and batches [2,40]. The elemental composition of FBS, or sera in general, is not defined. Only some common components are defined for all FBS types (Supplementary Table S2). Basic properties like pH or osmolality, primary components like Hemoglobin or proteins and the absence of bacteria, fungi and mycoplasma were tested for all the four FBS types used.

The formation of spontaneous mineralization under control conditions on acellular scaffolds compared to cell-seeded scaffolds, however, is not fully understood. One possibility is that cells cultured with control medium take up ions from the surrounding medium like cells cultured under osteogenic conditions. In contrast, cells subsequently are not able to form mineralized tissue, because they are not differentiated towards the osteogenic lineage. Another possible mechanism inhibiting spontaneous mineralization in cell-seeded scaffolds under control conditions could be an active inhibition of mineralization by the hMSCs itself. It is known...
that some cell types are able to inhibit mineralization [42]. So far, active inhibition of mineralization by hMSCs could not be shown and further investigation would be needed to test this hypothesis. Spontaneous mineralization of acellular SF has been described before in SBF or CaCl₂ [21–23] but, to our knowledge, it was never shown in control or osteogenic medium or on other scaffold materials.

The underlying silk structure is thought to provide an ideal environment for spontaneous mineralization, because its chemical structure is very similar to Col I (Fig. 1). Comparisons of mineralization on different scaffold materials are therefore critical, because some materials could induce spontaneous mineralization more effectively than others because of their different chemical structure. Therefore, it is necessary to include acellular control groups to test spontaneous mineralization potential of each scaffold material with the respective culture media used.

It is known that in static cell cultures seeded cells tend to concentrate on the outer scaffold surface, leading to a poor nutrient and waste exchange to the center of the scaffold [43]. Because of this effect the cell-mediated mineralization is expected to be located predominantly at the outer scaffold surface in the presented study setup. This effect was confirmed by μCT scans showing mineralized tissue formation located mostly at the edges and the top of the scaffolds (Fig. 4D). Ions are small enough to diffuse throughout the whole scaffold volume easily. Because spontaneous mineralization is dependent on local ion concentration [21], the spatial distribution of spontaneous mineralization on acellular scaffolds was expected to be more homogeneous compared to cell-seeded scaffolds. Quantitative evaluation of spatial distribution of mineralization volume confirmed this hypothesis and showed a significantly increased percentage of total BV in the center of acellular scaffolds (Fig. 5).

SEM provided additional visual confirmation of the results detected with the calcium assay and μCT (Supplementary Fig. S2). The absence of cells in acellular scaffolds was confirmed, and indicated that the mineralization in acellular scaffolds is purely attributable to the spontaneous mineralization phenomena.

FTIR revealed that the mineralization formed in all of the 16 experimental groups, whether cell-mediated or not, consisted of similar phosphate and carbonate precipitates. Unfortunately, FTIR lacks the possibility to clearly distinguish between all different precipitates present, especially when they are available in small amounts. FTIR gives information about the chemical composition of the samples only. In addition to FTIR, X-ray diffraction could be used to determine crystallinity (size and orientation) to compare the mineralization formed to in vivo bone mineralization. In addition to that, transmission electron microscopy can be performed to investigate the orientation of mineral crystals relative to the orientation of collagen fibrils. To characterize mineral mechanical properties of the formed mineralization atomic force microscopy can be used [44]. The solubility behavior of a mineral phase could reveal further information about the composition of the solid phase [45].

With respect to clinical applications it is of great importance that the effects of FBS on cell cultures are known and animal components are avoided. Human serum is an interesting alternative to animal-derived serum. It seems attractive for the culture of tissue-engineered grafts for future implantation and clinical cell therapy applications. Because of the clinical relevance of human serum, it would be interesting to investigate its effect on the mineralization on acellular and cell-seeded SF scaffolds. Nevertheless, results that have been observed with human serum are still conflicting. The use of human serum, however, makes sense only if enough autologous serum is available. Due to the limited availability and high variability within different donors, human serum is not a reasonable option for large-scale cell cultures in clinical applications [46] and therefore it is highly recommended to look for serum-free alternatives for in vitro cultures.

The use of conventional FBS for cell culture is questionable and should be considered with reservation. Not only cell growth, but also cell viability, cell attachment, cell density, geometrical environment (2-D/3-D) and scaffold material should be considered for every FBS type used [38]. Chemically defined FBS, like PAA Gold, could prevent excessive and time-consuming batch-to-batch testing. Ideally, the use of defined FBS is to be preferred over conventional FBS.

It should be noted that the results of this paper are valid only for the described example of bone TE with hMSCs and 3-D SF scaffolds prepared by the methods described herein. However, it can be assumed that similar results could be expected for any other output parameter in any other type of static cell culture. The effect of different FBS types in dynamic cell cultures needs to be investigated more precisely. Results are expected to be different for dynamic cultures because the scaffold-surrounding medium is moving, which could highly influence total ion concentration in the scaffold and therefore the process of spontaneous mineralization. This study is also limited to the four different FBS types used and batch variations for each individual FBS type were not investigated. It has to be expected that other FBS types or batches show different mineralization amounts and patterns on SF scaffolds.

It is important that these differences will be revealed in the near future. Hopefully, cell culture will soon be performed using serum-free medium if possible. If, for any reason, serum-free medium cannot be used, suitable FBS pre-testing needs to be performed and it should be a matter to state at least FBS type and batch used in publications. Alternatively, standards for batch testing procedures for specific applications should be determined, similar to cell characterization protocols. If these recommendations are followed in the future, it is possible that the complexity of TE cultures can be reduced, leading to more robust and more comparable results.

5. Conclusions

The objective of this study was to investigate the effect of different FBS types on TE cultures; in this example, on bone TE cultures of hMSCs on 3-D SF scaffolds. The results provide clear evidence of the strong influence of FBS type on mineralization in SF scaffolds using different evaluation methods. Unexpectedly, spontaneous mineralization was observed in acellular scaffolds dependent on FBS type. Only two of the four FBS types used induced spontaneous mineralization under acellular conditions. The addition of osteogenic factors to the medium did not influence spontaneous mineralization. No mineralization was present in cell-seeded scaffolds cultured with control medium, which indicates that cells are involved in the prevention of spontaneous mineralization. The observed results emphasize a clear dependence of experimental outcomes on FBS type. The use of FBS in TE cultures is therefore highly questionable and alternatives to conventional FBS should be considered, as already suggested decades ago. It is important to solve these issues now in order to reduce variability and improve experimental comparability to promote the entire field of TE.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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Appendix A. Figures with essential colour discrimination

Certain figure in this article, particularly Fig. 6 is difficult to interpret in black and white. The full colour images can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.11.025.

Appendix B. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.11.025.

References


[34] Drouet C. Apatite formation: why it may not work as planned, and how to conclusively identify apatite compounds. Biomol Int 2013;2013:490946.


