Acta Biomaterialia 13 (2015) 277–285

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Effect of fetal bovine serum on mineralization in silk fibroin scaffolds

Jolanda R. Vetsch [a], Samantha J. Paulsen [a], Ralph Müller [a], Sandra Hofmann [a,b,c,*]

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

ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 5 November 2014
Accepted 13 November 2014
Available online 20 November 2014

Keywords:
Fetal bovine serum
Silk fibroin
Scaffolds
Bone tissue engineering
Spontaneous mineralization

ABSTRACT

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.

1. Introduction

Fetal bovine serum (FBS) is a nutritional serum supplement used for most cell cultures. It contains important basic proteins such as growth factors and hormones for maintaining cell survival, growth and division. 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 prenucleation 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-

* Corresponding author at: Technische Universiteit Eindhoven, P.O. Box 513, 5600MB Eindhoven, The Netherlands. Tel.: +31 40 247 3494; fax: +31 40 247 3744.
E-mail address: S.Hofmann.Boss@tue.nl (S. Hofmann).

http://dx.doi.org/10.1016/j.actbio.2014.11.025
1742-7061/

ering 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 ($CaCl_2$) solution. Choi et al. [23] managed to promote calcium deficient HA formation on SF particles in $CaCl_2$ 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 between 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.

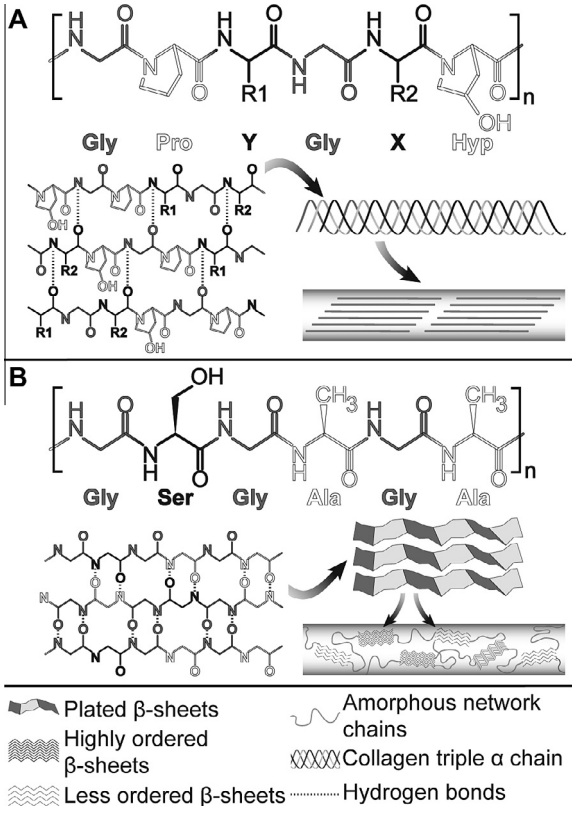

Fig. 1. Comparison of collagen and silk fibroin structures. Both fibrous proteins show a hierarchical structure with repeating amino acids and have been reported to take part in mineralization processes. (A) Structure of collagen with repeating amino acids of glycine (Gly), proline (Pro) and hydroxyproline (Hyp). (B) Structure of silk fibroin with repeating amino acids of glycine (Gly), serine (Ser) and alanine (Ala).

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 Na_2CO_3 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, Reinach, 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% CO_2. The remaining scaffolds were incubated in 1 ml osteogenic medium (control medium supplemented with