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Modular flow chamber for engineering bone marrow architecture and function

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ABSTRACT

The bone marrow is a soft, spongy, gelatinous tissue found in the hollow cavities of flat and long bones that support hematopoiesis in order to maintain the physiologic turnover of all blood cells. Silk fibroin, derived from *Bombyx mori* silkworm cocoons, is a promising biomaterial for bone marrow engineering, because of its tunable architecture and mechanical properties, the capacity of incorporating labile compounds without loss of bioactivity and demonstrated ability to support blood cell formation. In this study, we developed a bone marrow scaffold consisting of a modular flow chamber made of polydimethylsiloxane, holding a silk sponge, prepared with salt leaching methods and functionalized with extracellular matrix components. The silk sponge was able to support efficient platelet formation when megakaryocytes were seeded in the system. Perfusion of the chamber allowed the recovery of functional platelets based on multiple activation tests. Further, inhibition of AKT signaling molecule, which has been shown to be crucial in regulating physiologic platelet formation, significantly reduced the number of collected platelets, suggesting the applicability of this tissue model for evaluation of the effects of bone marrow exposure to compounds that may affect platelet formation. In conclusion, we have bioengineered a novel modular system that, along with multi-porous silk sponges, can provide a useful technology for reproducing a simplified bone marrow scaffold for blood cell production *ex vivo*.
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1. Introduction

Numerous approaches have been made towards development of an “ideal” bone marrow scaffold for blood cell production for transfusions and tissue repair as well as for drug testing *ex vivo* with tissue engineering solution being one of the greatest emerging option to address these needs [1,2].

The bone marrow is a soft, spongy, gelatinous tissue found in the hollow cavities of axial and long bones, which accounts for approximately 5% of the body weight in adult humans and consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinusoids interspersed within a meshwork of trabecular bone [3,4]. In long bones, one or more arteries pass through the cortical bone entering the marrow cavity obliquely, while in flat bones, the bone marrow is served by numerous blood vessels of various sizes entering the marrow via large and small canals [3]. After entry, the artery splits into multiple branches that give rise to a multitude of small thin-walled arterioles and sinusoids that let cells to enter the bloodstream [4–6]. The main bone marrow scaffolding is represented by the components of the extracellular matrix (ECM), which provide, depending on their composition, localization and stiffness, the ideal microenvironment to support hematopoietic stem cell (HSC) differentiation into committed lineages and release of mature cells into blood [7–9].

Research on the bone marrow is currently achieved by bone marrow histopathology which allow the analysis of mature stages of the blood cells differentiation, but not of dynamic processes such as cell migration and platelet production. Further, the quality of the marrow sections is governed by numerous variables related to specimen collection and processing, including fixation, decalcification, embedding, sectioning and staining, which may affect the

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integrity of the sample [3]. To this regard, laboratory research has intensively investigated *in vitro* culture techniques and animal models to extrapolate invaluable insights into hematopoiesis. However, *in vitro* cultures fail to reproduce the three-dimensional bone marrow architecture, while interspecies differences between humans and common animal models sometimes make it difficult to translate the achieved results.

One of the major challenges for the field is the development of bio-compatible scaffolds that can meet the needs to support platelet formation and functions *ex vivo* [9–11]. To this regard, silk fibroin is a biologically-derived protein polymer purified from domesticated silkworm (*Bombyx mori*) cocoons that has demonstrated excellent properties for biomedical applications, including biocompatibility, robust mechanical strength, and slow, controlled degradation to nontoxic products *in vivo* [12–14]. Further, silk can be processed entirely in aqueous systems using mild, ambient conditions of temperature and pressure, allowing the incorporation of labile compounds, as well as complex fluids, without loss of bioactivity [15,16]. Importantly, silk can be prepared in a range of material formats, including films, hydrogels, microspheres and sponges, already tested in a wide range of tissue engineering challenges, from bone to vessels modeling [17]. In particular, silk sponges are a promising candidate material for development of a bone marrow scaffold because of their demonstrated ability to support megakaryocyte (Mk) function and platelet production [16,18]. Importantly, the low thrombogenicity, non-toxicity and low-immunogenicity of silk provide a unique and versatile system for reconstituting bone marrow properties for blood cell production.

In this study, we developed a modular chamber design, rapid to manufacturer by 3D printing and easy to manage, to hold a silk-based 3D sponge with interconnected and controlled-size pores. The advantages of the system include the presence of inlet and outlet ports for perfusion of medium throughout the scaffold and the tunable properties of the silk biomaterials that can be chemically and mechanically tailored to entrap bioactive molecules while retaining bioavailability. We envision this system as a manageable and versatile tool for culturing cells in a 3D environment under perfusion. The validation of this technology for studying hematopoiesis was achieved by culturing human cord blood-derived Mks within the system and by collecting released platelets through perfusion into a transfusion bag. Importantly, collected platelets displayed comparable structure, granule content, antigen expression and functionality of human peripheral blood platelets. Thus, the silk-based 3D sponge presents major advantages for studying released platelet phenotype and functionality that are not easily captured with conventional 2D static culture systems.

2. Materials and methods

2.1. Materials

B. mori silkworm cocoons were supplied by Tajima Shoji Co., Ltd. (Yokohama, Japan). Pharmed tubing was from Cole-Parmer (Vernon Hills, IL, USA). Transfer bags for platelet collection were from Fenwal (Mont Saint Guibert, Belgium). Thrombin was from Sigma Aldrich (Saint Louis, MO, USA and Milan, Italy). AKT inhibitor VIII, isozyme-Selectine, AKTI-1/2 was from Calbiochem (La Jolla, CA). Convulxin was from Enzo Life Scinces (Rome, Italy). Type I collagen was purified as described previously [19]. Immunomagnetic separation system was from Miltenyi Biotech (Bergisch Gladbach, Germany and Bologna, Italy). Recombinant human thrombopoietin (TPO) and interleukin 11 (IL-11) were from Peprotech (London, UK). TruCount tubes, human fibronectin and PAC-1 FITC were from Becton Dickinson (S. Jose, CA, USA). 5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was from BioLegend (London, UK). The following antibodies were used: mouse monoclonal anti-CD61, clone SZ21, from Immunotech (Marseille, France); rabbit monoclonal anti-β1-tubulin was a kind gift of Prof. Joseph Italiano (Brigham and Women's Hospital, Boston, USA); rabbit polyclonal anti-human von Willebrand Factor (Dako, Milan, Italy); goat polyclonal anti-CD61 (clone C-20) (Santa Cruz Biotechnology, CA, USA); PE mouse monoclonal anti-human CD42b (clone HIP1), FITC mouse monoclonal anti-human CD61 (clone PM6/13) and mouse monoclonal anti-Thrombospondin (clone A4.1) (Abcam, Cambridge, UK); mouse monoclonal anti-CD42b (clone SZ2) (Beckman Coulter, Milan, Italy); rabbit polyclonal anti-fibronectin and mouse monoclonal anti-phosphotyrosine (clone 4G10) (Millopre, Milan, Italy); mouse monoclonal anti-fibronectin (clone EP5) (Santa Cruz Biotech, TX, USA); mouse monoclonal anti-β-actin (clone AC-15) and mouse monoclonal anti-α-tubulin (clone DM1A) (Sigma Aldrich, Milan, Italy); Alexa Fluor-conjugated secondary antibodies and Hoechst 33258 were from Life Technologies (Monza, Italy); anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, California, USA); anti-mouse and anti-rabbit HRP-conjugated secondary antibody and Precision Plus protein standard (Bio-Rad, Milan, Italy). Enhanced chemiluminescence reagents (ECL) were from Millipore (Milan, Italy).

2.2. Cell culture

Human umbilical cord blood was collected following physiologic pregnancies and deliveries upon informed consent of the parents. All human samples were obtained in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki. Mks were differentiated from human $CD34^+$ hemopoietic progenitor cells using previously described methods [20,21]. Briefly, $CD34^+$ cells were separated by an immunomagnetic beads technique and cultured for 13 days in Stem Span medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 10 ng/mL TPO, IL-11, 1% penicillin-streptomycin and 1% L-glutamine, at 37 °C in a 5% CO_2 fully-humidified atmosphere.

2.3. Peripheral blood platelet sample preparation

Human whole blood was collected from healthy volunteers, in accordance with the ethical committee of the IRCCS Policlinico San Matteo Foundation and the principles of the Declaration of Helsinki, using citric acid-citrate-dextrose (ACD) as anticoagulant. Human peripheral blood platelets were isolated from whole blood that was centrifuged at 200 × g for 10 min to obtain platelet-rich plasma (PRP). Platelet were finally washed in Tyrode's buffer (134 mM NaCl; 0.34 mM Na_2HPO_4; 2.9 mM KCl; 12 mM $NaHCO_3$; 20 mM HEPES; 5 mM glucose) in presence of 0.2 U/ml apyrase and 1 μM PGE 1 (Sigma, Milan, Italy) and allowed to rest at room temperature for 1 h, before being used. Morphology and functionality of peripheral blood platelets were analyzed by microscopy, western blotting or flow cytometry, as subsequently described. For immunofluorescence microscopy, whole blood was smeared on a glass microscope slide, air dried and then stain, as subsequently described.

2.4. Modular flow chamber design and 3D printing

A modular flow chamber has been designed to re-create the characteristic features of the bone containing spongy marrow. The chamber consists of one well of 19.8 × 36.0 × 8.2 mm having a hollow cavity of 3.4 × 20 × 5 mm, enclosed in a block of 35.4 × 51.6 × 21.58 mm and connected to the outside of the

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