Biomaterials 178 (2018) 122–133

Contents lists available at ScienceDirect

Biomaterials

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

Multi-channel silk sponge mimicking bone marrow vascular niche for platelet production

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ARTICLE INFO

Article history:
Received 5 June 2018
Accepted 12 June 2018

Keywords:
Silk
Bone marrow
Haematopoiesis
Megakaryocyte
Platelet
Computational Fluid Dynamic

ABSTRACT

In the bone marrow, the interaction of progenitor cells with the vasculature is fundamental for the release of blood cells into circulation. Silk fibroin, derived from *Bombyx mori* silkworm cocoons, is a promising protein biomaterial for bone marrow tissue engineering, because of its tunable architecture and mechanical properties, the capacity to incorporate labile compounds without loss of bioactivity and the demonstrated ability to support blood cell formation without premature activation. In this study, we fabricated a custom perfusion chamber to contain a multi-channel lyophilized silk sponge mimicking the vascular network in the bone marrow niche. The perfusion system consisted in an inlet and an outlet and 2 splitters that allowed funneling flow in each single channel of the silk sponge. Computational Fluid Dynamic analysis demonstrated that this design permitted confined flow inside the vascular channels. The silk channeled sponge supported efficient platelet release from megakaryocytes (Mks). After seeding, the Mks localized along SDF-1α functionalized vascular channels in the sponge. Perfusion of the channels allowed the recovery of functional platelets as demonstrated by increased PAC-1 binding upon thrombin stimulation. Further, increasing the number of channels in the silk sponge resulted in a proportional increase in the numbers of platelets recovered, suggesting applicability to scale-up for platelet production. In conclusion, we have developed a scalable system consisting of a multi-channeled silk sponge incorporated in a perfusion chamber that can provide useful technology for functional platelet production *ex vivo*.

1. Introduction

The bone marrow (BM) is a complex organ that is responsible for blood cell homeostasis [1]. It is a densely cellularized, gel-like tissue that fills the spongy cavity of bones [2]. The trabecular structure of the cancellous bone, together with the fine mesh of Extracellular Matrix (ECM) proteins provide structural organization, while the extensive BM vascular system constitutes the interface between the hematopoietic tissue and the blood, through which 10^{11}-12^{12} new blood cells are released every day into circulation. The spatial localization of these elements, provides the biochemical and mechanical stimuli for hematopoietic stem cell (HSC) maintenance and differentiation into the different blood lineages [3].

Megakaryocytes (Mks) in the BM are responsible for the continuous production of platelets in the blood [4]. Mks associate with the BM vasculature and extend proplatelets that protrude through the vascular endothelium into the lumen where they release platelets into the bloodstream [5–8]. There are countless human pathologies caused by alterations in platelet production or function, yet for many of these, pathogenesis and the required targeted therapies remain unknown, resulting in palliative treatments [9–11]. Platelet transfusion from donors still constitutes the standard of care for many of these conditions. However, the increasing demand for platelet units together with their extremely short shelf time (~5 days), often results in platelet supply shortages.

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https://doi.org/10.1016/j.biomaterials.2018.06.018
0142-9612/

For this reason, scientific and clinical communities are actively searching for new ways to generate functional platelets *ex vivo* to address clinical needs and for insight into fundamental studies of mechanisms [12,13]. It is hypothesized that the vascular niche, namely basement membrane components, vascular endothelium and shear, play a pivotal role in directing megakaryopoiesis [6–8,14].

Engineered BM models that allow *ex vivo* blood cell production hold promise for regenerative medicine. Nevertheless, developing suitable systems for BM engineering presents multiple challenges in terms of material choice, scalability and design [12,13]. 3D cell cultures are widely used in tissue engineering and regenerative medicine. 3D scaffolds can reproduce the architecture, ECM composition and cellular interactions of native tissues, as well as off extended surface area on which cells adhere and grow, compared to conventional 2D cultures [15,16]. However, reproducing and maintaining complex tissues *in vitro* in the absence of a vascular system is limited by mass transfer. Bioreactors can be designed to overcome these limitations and provide efficient diffusion of nutrients and oxygen in 3D scaffolds, and can also be used to introduce mechanical stimuli to the engineered tissues [17–19]. Perfusion bioreactors are particularly interesting for BM engineering, since they allow continuous media exchange and enhanced mass transfer through the scaffolds, while also providing flow-induced shear stress to the cells.

Silk fibroin is a natural protein polymer that can be processed into different formats (films, sponges, gels, fibers, microparticles) for many applications, from tissue engineering to drug delivery and regenerative medicine [20–22]. Silk can be processed entirely in water at low temperature/pressure conditions, which makes it suitable for large-scale batch production [23]. Because of the well-documented biocompatibility, mechanical properties and controlled degradability into non-toxic byproducts, silk-based devices have received approval from the Food and Drug Administration (FDA) for medical and cosmetic applications.

We previously demonstrated that silk-based scaffolds support Mk differentiation and allow functional platelet recovery [24–26]. Building upon our experience, we proposed the rational design of a perfusion bioreactor system that incorporates a BM vascular architecture, mechanical forces and controlled shear (Fig. 1). The advantage of this system is the possibility to control scaffold architecture and properties and to model the flow behavior to obtain maximum platelet yield. In addition, this approach is highly flexible and can be easily scaled for high-volume production of platelets.

2. Materials and methods

2.1. Materials

Bombyx 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). Immunomagnetic separation system was from Miltenyi Biotech (Bergisch Gladbach, Germany and Bologna, Italy). Recombinant human thrombopoietin (TPO), interleukin 11 (IL-11), Stromal Derived Factor (SDF)-1α were from Peprotech (London, UK). TruCount tubes 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). FITC monoclonal anti-human CD41 (clone HIP8) was from BioLegend. FITC mouse monoclonal anti-human CD61 (clone PM6/13) and PE mouse monoclonal anti-human CD42b (clone HIP1) were from Abcam (Cambridge, UK). Alexa Fluor-conjugated antibodies and Hoechst 33258 were from Life Technologies (Monza, Italy). Anti-human β1-tubulin antibody was kindly provided by Prof. Joseph Italiano Jr.

2.2. Silk solution preparation

The silk fibroin solution (hereafter referred to as silk) was prepared according to published methods [23]. Silk was extracted from *Bombyx mori* cocoons after sericin removal, by boiling the fibers for 30 min in 0.02 M sodium carbonate. The degummed silk fibers were dried in a fume hood for 48 h and subsequently dissolved in LiBr 9.3 M for 4 h at 60 °C at 25% w/v ratio. The solution was dialyzed using SnakeSkin tubing (3500 MWCO, Thermo Fisher) for 72 h, to remove the LiBr. The silk concentration was calculated as the ratio of dry/wet weight of a known volume of silk solution.

2.3. Custom molding system fabrication

The custom molding system was designed using AutoCad software (AutoDesk, San Rafael, CA) and machined at Tufts University. The molding system is composed of 3 parts, the base, machined out of corrosion-resistant aluminum (McMaster-Carr, Atlanta, GA), the

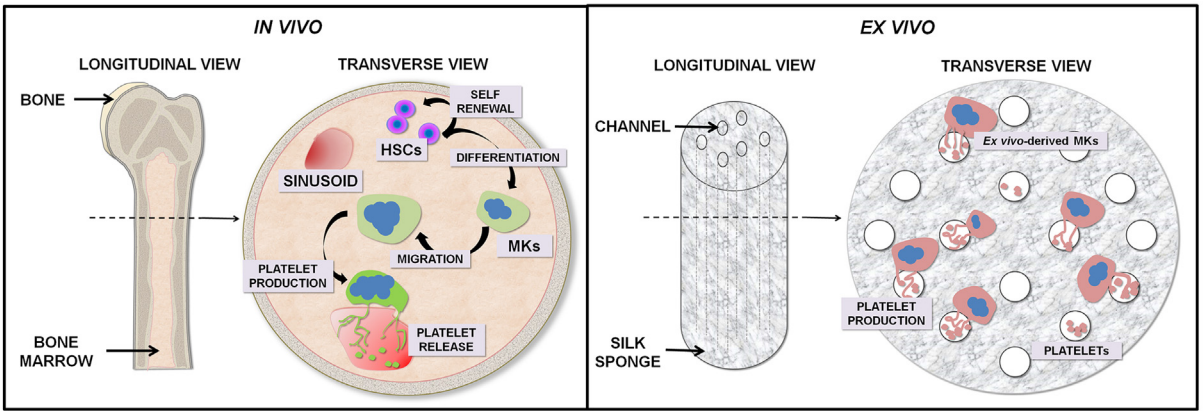

Fig. 1. Schematic representation of *in vivo* megakaryopoiesis *VS ex vivo* modeling using the multichannel silk sponge. Bone marrow is a spongy tissue composed by a network of sinusoids and ECMs. Within this context hematopoietic stem cells (HSCs) undergo self-renewal as well as differentiation into all committed lineages. Specifically, thrombopoietin (TPO) promotes differentiation into megakaryocytes (Mks) that are in close contact with sinusoids, where they extend multiple long pseudopods, called proplatelets that assemble platelets at their terminal ends. The release of mature platelets can be attributed to blood hydrodynamics that promote their final shedding. In order to mimic these functions, we designed a multi-channel silk sponge to allow Mk culture and perfusion of medium in order to recover *ex vivo*-released platelets.