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Platelet bioreactor: accelerated evolution of design and manufacture

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Abstract

Platelets, responsible for clot formation and blood vessel repair, are produced by megakaryocytes in the bone marrow. Platelets are critical for hemostasis and wound healing, and are often provided following surgery, chemotherapy, and major trauma. Despite their importance, platelets today are derived exclusively from human volunteer donors. They have a shelf life of just five days, making platelet shortages common during long weekends, civic holidays, bad weather, and during major emergencies when platelets are needed most. Megakaryocytes in the bone marrow generate platelets by extruding long cytoplasmic extensions called proplatelets through gaps/fenestrations in blood vessels. Proplatelets serve as assembly lines for platelet production by sequentially releasing platelets and large discoid-shaped platelet intermediates called preplatelets into the circulation. Recent advances in platelet bioreactor development have aimed to mimic the key physiological characteristics of bone marrow, including extracellular matrix composition/stiffness, blood vessel architecture comprising tissue-specific microvascular endothelium, and shear stress. Nevertheless, how complex interactions within three-dimensional (3D) microenvironments regulate thrombopoiesis remains poorly understood, and the technical challenges associated with designing and manufacturing biomimetic microfluidic devices are often under-appreciated and under-reported. We have previously reviewed the major cell culture, platelet quality assessment, and regulatory roadblocks that must be overcome to make human platelet production possible for clinical use [1]. This review builds on our previous manuscript by: (1) detailing the historical evolution of platelet bioreactor design to recapitulate native platelet production *ex vivo*, and (2) identifying the associated challenges that still need to be addressed to further scale and validate these devices for commercial application. While platelets are among the first cells whose *ex vivo* production is spearheading major engineering advancements in microfluidic design, the resulting discoveries will undoubtedly extend to the production of other human tissues. This work is critical to identify the physiological characteristics of relevant 3D tissue-specific microenvironments that

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J.T. is supported by NIH grants R00HL114719 and R01HL130770. J.T. is a paid consultant for, has financial interest in, and is a founder of Platelet BioGenesis, and is an inventor on their patents. B.D. is a paid consultant for Platelet BioGenesis, a company that aims to produce donor-independent human platelets from human-induced pluripotent stem cells at scale. L.B. is an employee of Platelet BioGenesis, and is partially supported by NIH SBIR grant 1R44HL131050-01. The interests of J.T. and B.D. were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict-of-interest policies.

drive cell differentiation and elaborate upon how these are disrupted in disease. This is a burgeoning field whose future will define not only the *ex vivo* production of platelets and development of targeted therapies for thrombocytopenia, but the promise of regenerative medicine for the next century.

Keywords

Biomanufacture; biomedical engineering; bioreactors; hematopoietic stem cells; megakaryocytes; platelets; pluripotent stem cells

Introduction

Platelets are poised to be amongst the first entrants into the field of regenerative medicine because they are anucleate, can be irradiated prior to infusion (alleviating concerns of potential tumorigenesis), are short-lived (10 days), and are easily transplanted. Platelets are produced by megakaryocytes, which are large, polyploid cells that are localized adjacent to sinusoidal blood vessels in the bone marrow, through which they extend and sequentially release platelets into the circulation (Figure 1) [2]. To make platelets *ex vivo*, one must engineer the steps in the supply chain from stem cell, to megakaryocyte, to platelet. The discovery of thrombopoietin in 1994 [3] quickly drove the generation of the first human platelets from hematopoietic stem cells *in vitro* [4]. However, it was the discovery of human embryonic stem cells a few years later [5] that ushered in a new realm of regenerative medicine. Within a decade it was demonstrated that human megakaryocytes [6] and platelets [7,8] could be produced from embryonic stem cells, although their function was somewhat limited compared to their *in vivo* counterparts. Furthermore, translation to the clinic faced additional issues due to the use of animal-derived feeder cells and media components, as well as ongoing ethical opposition to the use of human embryo-derived cellular therapies. The discovery of human induced pluripotent stem cells (hiPSCs) [9,10], together with advances in cell culture techniques [11], have largely resolved these issues and have finally enabled progress toward the scalable generation of megakaryocytes under feeder-free, xenofree conditions [12–14]. The remaining bottleneck involves triggering hiPSC-derived megakaryocytes to produce platelets at yields necessary for clinical/commercial application. Maximizing platelet yield requires exposing platelet progenitors to the architecture and intravascular shear stresses characteristic of their native microenvironment, and this is precisely what platelet bioreactors are designed to accomplish.

Historical evolution of platelet bioreactor design

Continuous media perfusion, gas exchange and scaffold composition

The iterative development of platelet bioreactors began with Lasky and Yang's seminal work in 2003 and has accelerated in recent years (Figure 2) [15]. Their first published 3D bioreactor utilized a polyethylene terephthalate (PET) matrix to trap murine embryonic stem cells and direct hematopoietic differentiation using specific cytokines and inhibitors [16]. Subsequently, in 2009, Sullenbarger *et al* reported a second 3D modular bioreactor with

polyester and hydrogel scaffolds coated with fibronectin and thrombopoietin (TPO) that specifically promoted megakaryocyte maturation and proplatelet formation from hematopoietic progenitor cells (Figure 3A) [17]. Two years later, Lasky *et al* introduced operational improvements to the bioreactor, wherein optimization of oxygen concentrations and media perfusion resulted in 3-fold increases in platelet production compared to previous iterations [18]. The bioreactor designs by Lasky's group introduced and furthered the concepts of continuous media perfusion, gas exchange and scaffold composition; however, they did not enable the visualization of platelet production in real time nor did they control shear stress and pressure to properly mimic the fluid dynamics in the bone marrow.

Control of flow rate

The importance of controlling the rate of media perfusion in platelet production was addressed in work by Dominique Baruch's group, who used microscopy to demonstrate that high shear rates (1800 s^{-1}) triggered 30–45% of mature human megakaryocytes immobilized on Von Willebrand factor (VWF) to produce platelets within 20 minutes (Figure 3B, *left panel*) [19] compared to 24–72 hours to reach a maximum of 25% in similar conditions without shear [20]. In a parallel study, we applied $\sim 0.5 \text{ Pa}$ force to murine megakaryocytes over a two hour period to demonstrate continuous proplatelet fission and platelet release under shear [21]. Building on this concept, Blin *et al* developed a bioreactor with VWF-coated micropillars that acted to capture megakaryocytes and used shear rates $>1800 \text{ s}^{-1}$ to produce platelets on a much larger scale (Figure 3B, *right panel*) [22]. However, of the ~ 1 million megakaryocytes that could be captured in the device, only 10–50% exhibited proplatelet extensions, resulting in an average of 3.7 platelets produced per megakaryocyte, compared to 1.1 in control experiments. Additionally, this device had a propensity for clogging, potentially due to megakaryocyte/platelet clumping and activation by VWF and high shear stresses. Nevertheless, the platelets collected from the bioreactor had normal morphology with low baseline activation, and aggregated normally when activated with thrombin receptor-activating peptide (TRAP) [22]. While this design contributes to an understanding of the effects of shear on platelet production and scale, challenges of separating platelets from megakaryocytes, and contact activation of platelets are additional factors that must be considered in future devices.

Bone marrow architecture and extracellular matrix composition

A different bioreactor design from the group of Alessandra Balduini attempted to emulate bone marrow physiology in an effort to maximize platelet production. The bioreactors were built around a biocompatible silk microtube containing media under flow, mimicking a blood vessel. Megakaryocytes were then seeded into the area surrounding the microtube (Figure 3C), which (depending on the bioreactor design) consisted of extracellular matrix-mimicking components such as matrigel, VWF, fibrinogen, stromal cell-derived factor-1 α , and type 1 collagen [23], silk sponges [24], or hyaluronan-based hydrogels [25]. The megakaryocytes migrated to and extended proplatelets through the pores of the silk microtube ($2\text{--}8 \text{ }\mu\text{m}$) and released platelets when exposed to low shear rates (60 s^{-1}) into the lumen at a rate of 200 platelets per proplatelet-extending megakaryocyte [23]. However, only $\sim 7\%$ of megakaryocytes adhering to the microtube extended proplatelets, resulting in an average platelet yield of ~ 14 platelets per megakaryocyte. Although these designs

recapitulate several aspects of the bone marrow niche, remaining challenges include the lack of real-time visualization of platelet production, effective scale-up strategies, and control of platelet activation.

Industrial cell culture process

To address these limitations, a two-stage process for platelet production from CD34⁺ cells was conceived and patented by Beau Mitchell (Figure 3D, *left panel*), and later built (Figure 3D, *right panel*). The first part conceptualizes a cell culture system that differentiates CD34⁺ cells into mature megakaryocytes, which were then seeded onto woven Nanex membranes (pore size 2–5 μm). The megakaryocyte coated membranes were then introduced to shear rates of 30–70 s^{-1} to induce platelet production, which reached 100 platelets/megakaryocyte [26,27]. Although this system claims platelet production of 1×10^6 platelets per CD34⁺ cell, minimal characterization of the platelets was performed; thus, their quality is uncertain. Furthermore, the bioreactor design precludes real-time visualization of platelet production, making it difficult to verify and study this process [26].

Human pluripotent stem cells as source material

The bioreactors discussed thus far used mouse cells, or human bone marrow-derived or cord blood-derived megakaryocytes to generate platelets. However, the use of human pluripotent stem cells as an unlimited megakaryocyte source would obviate the risks associated with cell collection from human donors and would thus have major clinical ramifications. In 2010, Takayama *et al* differentiated hiPSCs into megakaryocytes via transient over-expression of *c-MYC* alone [12] or in combination with *BM11* and/or *BCL-XL* [13], and demonstrated their ability to create platelets in static conditions. These cells were subsequently used in three different bioreactor designs in attempts to further improve platelet production (Figure 3E) [28]. The first design (Figure 3E, *upper left panel*) adapted Beau Mitchell's bioreactor concept (Figure 3D, *left panel*) by seeding megakaryocytes onto a porous polydimethylsiloxane (PDMS) scaffold with endothelial cells lining the undersurface [28]. Pressure flow perpendicular to the main flow maintained megakaryocytes onto pores, while the main flow through the channel applied shear stresses (0.14–37.5 dyne/cm^2) to produce platelets [28]. The second device (Figure 3E, *right panel*) used PDMS pockets to hold individual megakaryocytes without the use of endothelial cells. Time-lapse visualization showed that higher flow rates correlated with increased proplatelet length, but platelet yield was not significantly improved in either of these two devices [28]. The third device (Figure 3E, *bottom left panel*) utilized smaller, angled PDMS slits (4 μm openings; 60° angle from flow) which led to significantly increased platelet yields (3.6-fold over static conditions) [28]. Overall, the results are promising, but optimization is clearly necessary to further improve platelet yields.

Separation and isolation of platelets

A common problem in many 3D bioreactor designs is that platelet production is accompanied by contaminating megakaryocytes or progenitors. As such, these designs require additional steps to remove any contaminating cells while maintaining platelet structural and functional integrity. A recent advance by Schlinker *et al* involves a spinning-membrane filtration device designed to separate platelets and proplatelets from

megakaryocytes, increasing purity and concentration in preparation for transfusion (Figure 3F) [29]. Importantly, this device also allowed for the exchange of media to platelet storage buffer [29], which is critical for the health of platelets during storage and is approved for transfusions. Furthermore, the megakaryocytes recovered from the initial filtration could be re-used to produce more platelets [29].

Consolidation of design principles

In collaboration with Advanced Cell Technologies (now Astellas) in 2014, we showed that it was feasible to generate functional human megakaryocytes and platelets from research-grade hiPSC cultures [30]. To trigger platelet production, we created an integrated microfluidic platform that combined evolving concepts of bone marrow physiology with biologically-inspired tissue engineering [31] (Figure 3G, *top left and right panels*). Our unique bioreactor design let us leverage the functional advantages of previous approaches in a scalable system that could be adapted for commercial use:

- Bone marrow vascular niche architecture
- Temperature control
- Gas exchange
- Continuous process flow
- Selective trapping of megakaryocyte progenitors from platelet product
- Application and precise control of vascular shear stress
- Selective contact of megakaryocyte progenitors (but not platelets) to relevant ECM proteins
- Low volume perfusion
- Optical clarity of device for real-time visualization of platelet production
- Scalability of bioreactor design and manufacture

By modeling bone marrow architecture *ex vivo* [32,33] and exposing megakaryocytes to shear stresses characteristic of flowing blood, we found that we could reproduce physiological platelet production and increase the overall rate and extent of platelet release far above what had previously been reported [31]. In our bioreactor, platelet production began immediately (reduced from six hours), the percent of platelet-producing megakaryocytes was increased from 40% (mouse) and 10% (human) to more than 90%, and the time to completion of platelet production was reduced from eighteen hours to under two hours. Bioreactor-derived platelets from both mouse fetal liver and human iPSCs were structurally and functionally comparable to donor platelets, establishing proof-of-concept for this approach [31].

Our bioreactor produced 30–70 platelets per megakaryocyte (1×10^6 platelets per 300 μ L). To achieve a total yield of 3×10^8 platelets, we have since: (1) adapted our original bioreactor from a flat (2D) configuration to a 3D design, (2) introduced a porous membrane between the upper and lower channels of the device to effectively reduce the size of the gaps

through which proplatelets are extended, and (3) increased the functional surface area of the device to support larger numbers of megakaryocytes and thereby increase total platelet yield. Our second-generation (*unpublished*) bioreactor prototype now consists of a “sandwich” design wherein a polycarbonate membrane holding the cells is pressed between two polydimethylsiloxane (PDMS) layers (Figure 3G, *bottom left panel*). While these advancements have laid the groundwork for a commercially scalable platelet bioreactor design, there is still a tremendous amount of work to be done.

Overall, 3D bioreactors have advanced platelet production compared to static conditions *in vitro* by shortening time to production (from hours to minutes) and increasing platelet yields more than ten-fold (from <10 to ~100 platelets/megakaryocyte). It is important to note that while all of these studies attempted to describe some parameters of bioreactor-derived platelet functionality, the thoroughness of testing and paucity of adequate controls in many cases has resulted in remaining uncertainty regarding the extent to which bioreactor-derived platelets can act as *bona fide* functional substitutes for donor platelets.

Major design and manufacturing challenges to be resolved

While the historical evolution of platelet bioreactor design using primary human and murine megakaryocyte cultures has selected for relevant design features that should be preserved in future commercial bioreactor designs, there is still much work to be done. Platelet bioreactor development to-date has mostly focused on the development of low throughput, custom-made, proof-of-concept basic research tools that have been applied to resolve specific questions in platelet production. Laboratory-scale platelet bioreactors are not amenable for high throughput industrial scale production, do not employ standardized manufacture and operation processes that would enable automation, and rates of device manufacture and operation failure remain critically high. Translation to next-generation microfluidic devices for clinical and commercial application presents new manufacturing challenges and the establishment of standardized operational processes and device validation metrics that will meet regulatory requirements and not be cost-prohibitive [34]. For platelet bioreactors, the next major milestones include:

Automation of flow control

Commercial scale platelet bioreactors must be able to be run independently of direct human supervision, in parallel, and over continuous process conditions. These need to account for pressure differentials that develop over larger device architectures that will be required to meet projected scale requirements.

Bioreactor computational fluid dynamics modeling

This is necessary to resolve flow lines during device operation and identify actual forces at play on cells at different positions on the device. The goal in bioreactor design optimization should be to achieve uniform cell distribution and agonist (*e.g.*, shear stress, extracellular matrix protein) exposure of all cells at all positions of the device. This would allow conditions to be focused more precisely to identify ‘sweet spot’ ranges for individual drivers of platelet production.

Use of biocompatible (non-PDMS) scalable materials

Crucial to the further development of commercially scalable platelet bioreactors is the transition away from PDMS and into biocompatible FDA-approved thermoplastics. While PDMS is attractive for microfluidic device prototyping because of its optical clarity, gas and vapor permeability, ability to tune hydrophobicity and bonding properties of its surface, material elasticity, and affordable manufacture using soft lithography [35], there are major limitations to implementing the material in commercial biomedical devices. Principal amongst these are that PDMS is known to leach uncross-linked oligomers from the curing process into solution [36], which can be cytotoxic; and will absorb small molecules [36,37], which can affect critical cell signaling dynamics and drug concentration studies. PDMS also severely limits scalability of device manufacture since it cannot be used for high-throughput methods such as injection molding, rolling and embossing [38]. Next-generation microfluidic devices will need to begin exploring alternative scalable materials such as thermoplastic and thermosetting polymers. For platelet bioreactors, these could include acrylic, cyclic olefin copolymer, polycarbonate, polyvinyl chloride, polyolefin, polyethylene, polyurethane, polypropylene, or graphene oxide.

Scaled design that supports commercial application

Platelet bioreactors being developed for clinical application must first be scaled up to support sufficient numbers of megakaryocytes to enable preclinical studies. Using the most recent published yields of ~100 platelets/megakaryocyte [26,31], the bioreactor must support at least 3×10^6 megakaryocytes to produce 3×10^8 platelets, approximately the number needed to assess platelet quality/safety *in vitro* and perform function studies in mice. Eventually, the bioreactors must be scaled up several orders of magnitude further to accommodate 3×10^9 megakaryocytes, enough to produce 3×10^{11} platelets, the equivalent of one apheresis platelet unit.

Scaled manufacture and assembly of devices

Laboratory-scale manufacture of microfluidic devices is a highly specialized activity that is particularly sensitive to inter-user and inter-batch variability. Next-generation platelet bioreactors, therefore, need to be designed to support large-scale reproducible manufacture and automated assembly of devices in the industrial setting, while supporting flexibility in production to adapt to expected future scientific advances and market changes. Although unit requirement needs for the existing platelet bioreactors are low given the largely preclinical stage of the field, it is critical that design principles supporting scalable manufacture and assembly of platelet bioreactors such as microinjection molding, rolling, or embossing techniques be introduced at this stage to reduce inter-batch device variability during testing and avoid the potentially prohibitive cost of device re-design and re-validation in the future.

Media volume reduction and product concentration

Support of media recirculation loops in microfluidic device operation should be introduced to the bioreactor design to minimize operational volume, reduce media/cytokine cost, and further concentrate the cell product. In the case of platelet bioreactors, the introduction of

peristaltic pumps is especially attractive as they allow recapitulation of physiological (~1 Hz) pulsatile flow and can keep resultant platelets agitated and in suspension, which is necessary to preserve their functionality [39,40].

Conclusion

The future of regenerative medicine depends on bringing academic discoveries to market. Since our seminal work just three years ago, we have formed a company (Platelet BioGenesis Inc.) to translate platelet bioreactor technology to the clinic, and have begun repeating our proof-of-concept studies with current Good Manufacturing Practice (cGMP)-compliant human induced pluripotent stem cell lines that we intend to advance for investigational new drug approval and first-in-human clinical trials. This work is not trivial [1]. Platelet production needs to support a market that transfuses more than 15 million platelet units annually and requires the further scale up of platelet production to meet demand through continued enhancements in platelet bioreactor design and operation. Introduction of automation of flow control, bioreactor computational fluid dynamics modeling, biocompatible (non-PDMS) scalable materials, scaled design, manufacture and assembly of devices, media volume reduction, and product concentration into next-generation platelet bioreactors will greatly increase megakaryocyte trapping and total platelet yield, decrease device manufacture cost at commercial scale by >10x, reduce lead time, reduce total operating volume, concentrate the platelet product, and more accurately mimic biological conditions of platelet formation. Platelet bioreactors are currently poised to become among the first stem cell-derived tissue engineering platforms advanced for clinical use and represent a major first step toward a sustainable, donor-free blood system. The platelet bioreactor field has advanced tremendously in the last 10 years, and the implications of these next 10 years will define the promise of regenerative medicine for the next century. This is a thrilling and rapidly evolving story with an increasing number of protagonists, and we are excited to have a part in its telling.

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Figure 1.

Human platelets are produced by megakaryocytes in the bone marrow. Figure adapted from Machlus and Italiano (2013) [41] and Zhang et al (2012) [42].

Figure 2.
Number of platelet bioreactor manuscripts published each year since 1990. Figure highlights the inception of this field.

Figure 3.

Historical Evolution of Platelet Bioreactor Design, 2009–2016. Panel A adapted from Sullenbarger et al (2009) [17]. Panel B adapted from Dunois-Larde et al (2009) [19] and Blin et al (2016) [22]. Panel C adapted from Pallotta et al (2011) [23]. Panel D adapted from Mitchell (2011) [27] and Avanzi et al (2016) [26]. Panel E adapted from Nakagawa et al (2013) [28]. Panel F adapted from Schlinker et al (2015) [29]. Panel G adapted from Thon et al (2014) [31].