AWARD NUMBER: W81XWH-13-1-0495

TITLE: Platelets as Contractile Nanomachines for Targeting Drug Delivery in Hemostasis and Thrombosis

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REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
December 2015	Final	**30 Sep 2013 - 29 Sep 2015**

4. TITLE AND SUBTITLE

Platelets as Contractile Nanomachines for Targeting Drug Delivery in Hemostasis and Thrombosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0495

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Georgia Institute of Technology, 345 Ferst Drive NW, Atlanta, GA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

For this grant, we proposed to apply different nanocarrier synthesis techniques, modulate fibrinogen density and structure on the nanocarrier surface (which both affect platelet adhesion), the nanocarrier stiffness (which affects the force of platelet contraction and platelet adhesion), and the size of the nanocarrier. In addition, using a novel "endothelial-ized" microfluidic system, we proposed to investigate how external factors such as flow and thrombin concentration affect drug release. The proposed proof-of-concept experiments will validate our concept of platelet contraction-controlled nanocarriers as a novel and potentially paradigm-shifting strategy for targeted drug delivery to achieve hemostasis during bleeding. We have since optimized our nanocarrier synthesis protocol and as well as fibrinogen density and structure on the nanocarrier surface. Finally we determined that physiologic arterial flow conditions optimize the release of our nanocarriers work and have also determined that nanocarrier release is dependent on local thrombin concentration.

15. SUBJECT TERMS

Nothing listed

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	7	

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Introduction

Here we introduce the concept of platelet contraction-controlled nanocarriers as a novel and potentially paradigm-shifting strategy for targeted drug delivery to achieve hemostasis during bleeding. Platelet contraction requires: 1) platelet adhesion to fibrinogen bound to a surface and 2) exposure to soluble thrombin, which is present at sites of active clotting. As such, these parameters function as the two "inputs" of our system. The basic unit of our synthetic nanocarrier is an assembly of fibrinogen-conjugated nanogels, which self-assemble to encapsulate and contain the drug "cargo." After the nanocarriers are infused, circulating platelets adhere to and spread over the fibrinogen-conjugated surface of the nanocarriers, and are primed for activation. Once the circulating platelet-bound nanocarriers enter an area of high thrombin concentration in the circulation, such as sites of bleeding and vascular injury, thrombin activation immediately activates platelet contraction. Platelet contraction then induces a force that ruptures the nanocarrier, enabling the targeted and mechanical delivery of drugs to treat bleeding and achieve hemostasis. Thus, in our system, the patient's own platelets function as "nanomachines" and act as both the sensor and actuator of the "smart" drug delivery process, a paradigm that has not been reported before. For this DOD Discovery Award grant, we will characterize and optimize our platelet-responsive nanocarrier system for targeted drug delivery. We hypothesize that by varying the biochemical and biophysical properties of the nanocarriers, we will be able to tune platelet contraction-driven drug release. Specifically, we will apply different nanocarrier synthesis techniques, modulate fibrinogen density and structure on the nanocarrier surface (which both affect platelet adhesion), the nanocarrier stiffness (which affects the force of platelet contraction and platelet adhesion), and the size of the nanocarrier. In addition, using a novel "endothelial-ized" microfluidic system, we will investigate how external factors such as flow and thrombin concentration affect drug release.

Keywords

platelets, drug delivery, hemostasis, contraction, nanotechnology

Accomplishments

Aim 1: To characterize and optimize the biochemical characteristics of our platelet-contraction activated nanomedicine drug delivery system	**Months**	**Status of completion**
Major Task 1: Synthesizing different types of nanocarriers	1-8	Completed
Major Task 2: Varying fibrinogen structure and fibrinogen density of the nanocarriers	1-8	Completed
Aim 2: To characterize and optimize the physical and material characteristics of our platelet-contraction activated nanomedicine drug delivery system.		
Major Task 1: Varying nanocarrier stiffness	6-15	Completed
Major Task 2: Varying nanocarrier size	6-15	Completed
Aim 3: Proof-of-concept testing of our platelet-responsive nanocarrier drug delivery system	12-18	Completed
Milestone(s) Achieved: Characterization of platelet contraction-controlled nanocarriers as a novel and potentially paradigm-shifting strategy for targeted drug delivery to achieve hemostasis during bleeding;	18	

This past year, we have completed Aim 1 in which we characterized and optimized the biochemical characteristics of our platelet contraction activated nanomedicine drug delivery system. Specifically, we synthesized different types of nanocarriers and selected the platform that yielded the highest performance and we determined the optimum fibrinogen structure and density that yielded the most platelet adhesion.

Ultimately, we discovered that polyelectrolyte-based capsules are the ideal nanocarrier. Polyelectrolyte capsules were fabricated via layer-by-layer deposition method onto calcium carbonate cores by altering positive and negative polyelectrolytes, followed by core removal. The polyelectrolytes used were poly-L-lysine (PLL) and poly-L-glutamic acid (PLG). Briefly, calcium carbonate cores were prepared by vortexing equal volumes of 0.33 M aqueous solutions of sodium carbonate and calcium chloride for 30 seconds at room temperature. The mixture was left to stand for 10 minutes, and the precipitate was then filtered, washed three times with deionized (DI) H2O, and dried under vacuum. To deposit polyelectrolyte layers onto cores, the cores (2% w/v) were suspended in a 0.5 M NaCl aqueous solution (pH 6.5) of 2 mg/mL polyelectrolyte (PLL as first layer) and placed on a shaker for ten minutes. The solution was pelleted (200 g for five minutes) and washed (four minutes on shaker) three times with a 0.5 M NaCl (pH 6.5) aqueous solution before the next later (PLG) was deposited. The last layer containing fibrinogen was deposited using a 1:1 solution (2 mg/mL total) of PLG and fibrinogen in 0.5 M NaCl aqueous solution (pH 6.5). Once the desired number of layers were deposited, the cores were removed by resuspending the particles in a 0.2 M ethylenediaminetetraacetic acid (EDTA) aqueous solution (pH 7.5) for 30 minutes, followed by three rounds of pelleting (8,000 g for five minutes) and washing (shaking for four minutes) with the same EDTA solution. The capsules were then pelleted and washed four times with DI H2O and stored at 4 °C in DI H2O.

Polyelectrolye capsules as targeted platelet contraction-drive drug delivery vehicles. Once it was established platelet contractile forces were sufficient to significantly destroy droplet structure, we focused on developing a capsule that is easily fabricated, can carry an aqueous-soluble cargo such as a thrombolytic drug, display fibrinogen on the surface exterior while retaining its biological activity, rupture by mechanical force, and is biocompatible. The optimized system accommodates those requirements and is a polyelectrolyte (PE) capsule (~3-8 microns in diameter containing an aqueous core) fabricated via layer-by-layer (LbL) deposition of poly-L-lysine (PLL), poly-L-glutamic acid (PLG), and fibrinogen (Figure 1). The polyelectrolytes are deposited onto a calcium carbonate core of tunable size in a LbL method and then exposed to EDTA, which chelates out the calcium leaving behind a capsule with an aqueous core.[8]

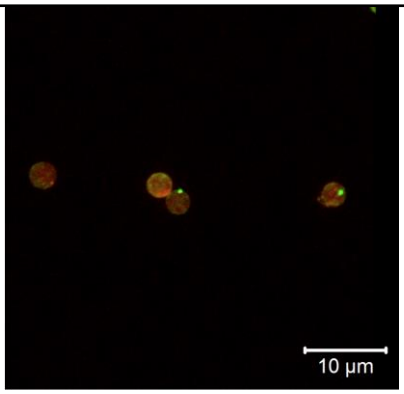

Figure 1. 3D confocal micrograph of polyelectrolyte capsules (PLL – red and fibrinogen – green).

As polyelectrolyte capsules were suspended in platelet-rich plasma, which was subsequently exposed to 1 U/mL of thrombin, the capsules successfully targeted target thrombotic sites via integration into the forming fibrin networks and binding to activated platelets. The delivery vehicles successfully adhere to platelet aggregates, as shown by confocal microscopy (Figure 2), indicating fibrinogen's biological activity on the capsule surface is preserved. We have begun tuning the mechanical stiffness and elasticity of the capsules by varying wall thickness and capsule size to achieve rupture during clot contraction and allow long-term storage.

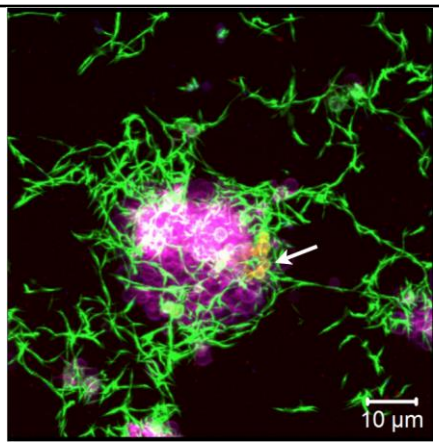

Figure 2. 3D confocal micrograph of polyelectrolyte capsules (red outlined in green) (indicated by arrow) incorporated into a platelet (purple) aggregate (purple) within a fibrin (green) network).

Successful platelet-contraction mediated controlled release with cargo-loaded polyelectrolyte capsules

Capsule rupture has successfully been achieved in static fibrin gels comprised of a high concentration of platelets (Figure 3). Only capsules incorporated into the fibrin network and near an activated, contracting platelet undergo structural deformation and rupture (Figure 3A,C). Furthermore, when capsules loaded with FITC-Dextran (average MW 68 kDa) were used, release of cargo was seen after capsule rupture.

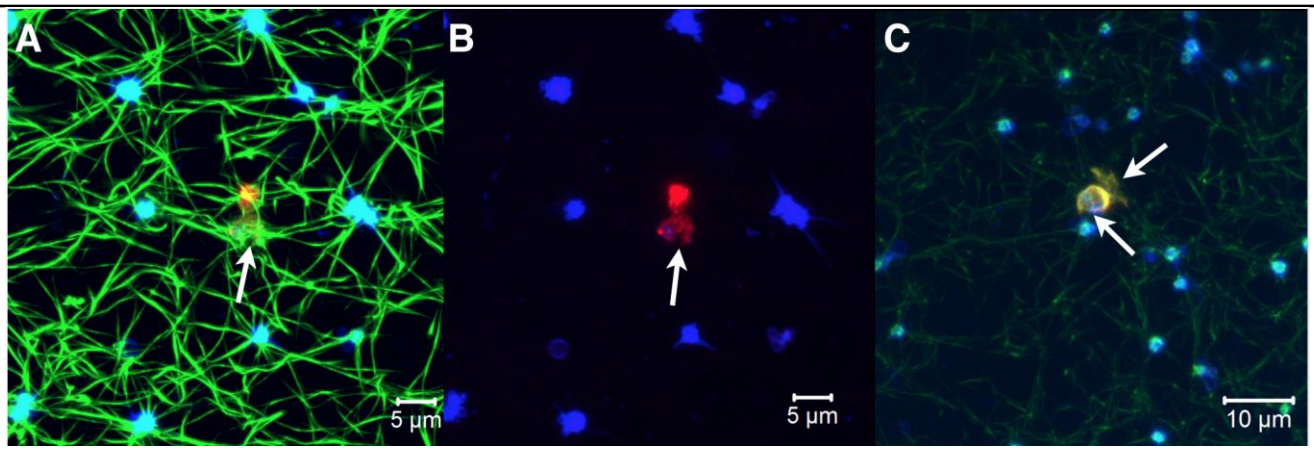

Figure 3. 3D confocal micrographs of PE capsules (PLL red) in clot-like environments of fibrin (green) and activated platelets (blue). Arrow indicates capsule rupture (A-C) and diffusion of loaded FITC-Dextran (green) (C). Micrograph B is the same as A with the green channel removed to highlight capsule rupture.

This work demonstrates that targeted drug delivery from PE capsules occurs only in platelet aggregates and controlled release is driven by the chemomechanical action of activated platelets. As thrombi in myocardial infarctions and strokes are platelet-rich, this is an ideal system to achieve high concentrations of thrombolytics only in areas of those thromboses.

In addition, we have determined that the optimized fibrinogen-coated polyelectrolyte nanocarrier size is 3-8 microns in diameter, which subsequently results in optimized rupture/delivery. Finally, we have successfully modified our “endothelialized” microfluidic system to function as a “bleeding” model that is ideal to assess hemostatic agents such as our fibrinogen-coated polyelectrolyte nanocarrier.

Impact
Nothing to report during this period

Changes/Problems
N/A

Products
Nothing to report during this period

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Special Reporting Requirements
N/A

Appendices
N/A