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# Contact activation of blood coagulation on a defined kaolin/collagen surface in a microfluidic assay

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ABSTRACT

Generation of active Factor XII (FXIIa) triggers blood clotting on artificial surfaces and may also enhance intravascular thrombosis. We developed a patterned kaolin (0 to 0.3 pg/μm$^2$)/type 1 collagen fibril surface for controlled microfluidic clotting assays. Perfusion of whole blood (treated only with a low level of 4 μg/mL of the XIIa inhibitor, corn trypsin inhibitor) drove platelet deposition followed by fibrin formation. At venous wall shear rate (100 s$^{-1}$), kaolin accelerated onset of fibrin formation by ~100 sec when compared to collagen alone (250 sec vs. 350 sec), with little effect on platelet deposition. Even with kaolin present, arterial wall shear rate (1000 s$^{-1}$) delayed and suppressed fibrin formation compared to venous wall shear rate. A comparison of surfaces for extrinsic activation (tissue factor TF/collagen) versus contact activation (kaolin/collagen) that each generated equal platelet deposition at 100 s$^{-1}$ revealed: (1) TF surfaces promoted much faster fibrin onset (at 100 sec) and more endpoint fibrin at 600 sec at either 100 s$^{-1}$ or 1000 s$^{-1}$, and (2) kaolin and TF surfaces had a similar sensitivity for reduced fibrin deposition at 1000 s$^{-1}$ (compared to fibrin formed at 100 s$^{-1}$) despite differing coagulation triggers. Anti-platelet drugs inhibiting P2Y$_1$, P2Y$_{12}$, cyclooxygenase-1 or activating IP-receptor or guanylate cyclase reduced platelet and fibrin deposition on kaolin/collagen. Since FXIIa or FXIa inhibition may offer safe antithrombotic therapy, especially for biomaterial thrombosis, these defined collagen/kaolin surfaces may prove useful in drug screening tests or in clinical diagnostic assays of blood under flow conditions.



## Introduction

Contact pathway can be strongly triggered by negatively charged surfaces such as glass, kaolin and celite [1]. Zymogen factor XII (FXII) is activated to FXIIa upon contacting with anionic surfaces and leads to a multistep cascade, whereby thrombin (FIIa) forms as a potent platelet activator and trigger of fibrin polymerization [2,3]. The pathophysiology of contact pathway is not fully elucidated. While tissue factor triggered extrinsic pathway prompts major response to vascular injury, contact pathway likely has a minor role in hemostasis since factor XII deficiency is not associated with a bleeding defect. However, recent experiments revealed that FXII-mediated fibrin formation is essential for thrombus stability in a mice model [4–6]. In contrast, FXI-deficient (hemophilia C) patients display little spontaneous bleeding but at elevated risk of bleeding post-injury or post-operative, especially at sites with high fibrinolysis [7]. It has been suggested that pharmacological inhibitors of FXIIa or FXIa may be drugs useful for limiting thrombosis with reduced risk of bleeding side effects [8–10].

Many studies of contact pathway have been conducted for the purpose of investigating unfavorable thrombosis on blood-contacting medical devices. Most of these studies mainly focus on the activation mechanism of FXII. The approach often eliminates blood flow and cellular components, which then allows contact activation in static tubes with plasma [11–13]. However, flow and cellular constituents are both present in human blood vessels, fundamentally altering reaction dynamics as compared to a cell-free system under static conditions. Flow based studies designed to intentionally trigger and measure contact pathway are less common. Glass capillary flow reactor has been used to study plasma coagulation via artificial surface activation [14]. Kaolin-activated thromboelastography (TEG) has been applied as a predictive test for post-operative bleeding to assess clotting factors (i.e. rate, strength, and stability) under non-flow condition [15,16]. Typically, citrate is used as an anticoagulant which allows recalcification immediately prior to an experiment. However, FXIIa can be formed under calcium-free conditions and the resting time in citrate is often an uncontrolled variable. Similar to the older practice of using "light heparinization" of blood ex vivo to study platelet function in the presence of triggered thrombin production [17], we use "light CTI" as a versatile tool to study contact pathway in the context of clotting ex vivo. CTI only inhibits βFXIIa and does not inhibit αFXIIa. Even at high CTI (40–100 μg/mL), unactivated human blood will clot ex vivo after a period of time of 40 to 100 minutes [18]. This clotting time becomes even shorter when the following three conditions are imposed: first, CTI is lowered to very low levels such as 4 μg/mL; second, a platelet

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stimuli is used (collagen); and third, a potent contact activator is used (kaolin). While no perfect container exists to hold blood ex vivo prior its perfusion over a thrombotic patch, the use of low CTI at 4 μg/mL and use of the blood within 20 min of phlebotomy allows controlled and repeatable ex vivo study of the contact pathway at a precise location of interrogation.

Microfluidic devices allow the study of thrombotic events by perfusion of whole blood over well-defined prothrombotic surfaces [19–22]. Microfluidics enables precise control of flow condition and real-time observation of thrombus structure. In this paper, we describe a prothrombotic surface composed of collagen and kaolin that is capable of activating blood coagulation via the contact pathway, independent of tissue factor (TF). This surface also serves as a biologically important substrate for anchoring activated platelets and polymerized fibrin. Engagement of contact pathway was evaluated by dynamic accumulation of localized platelets and fibrin on the collagen/kaolin surface. This microfluidic assay allowed a controlled study of the sensitivity of contact pathway function to wall shear rate.

## Materials and Methods

### *Fluorescent labeling of kaolin particles*

For imaging of kaolin on collagen, fluorescent labeling of kaolin particles was carried out in a two-step reaction [23]. Kaolin was mixed with 3-mercaptopropyl-trimethoxysilane in 80% methanol (50 mL methanol/g kaolin) in a 3:1 mass ratio. The mixture was stirred at room temperature for 6 hr to activate kaolin surface by converting surface hydroxyl groups to thiol groups, filtered, and washed 3 times with 80% methanol. The residue was collected and vacuum-dried for 12 hr. Powdered kaolin was then dried at 80 °C for 5 hr. Labeling solution was prepared by adding 5 mg fluorescein-5-maleimide into 120 mL phosphate buffered saline (PBS). Activated kaolin (125 mg) along with 50 mL ethanol was mixed with labeling solution for 1 hr. Kaolin was centrifuged (5000 g, 1 min) and supernatant was discarded. The pellet was resuspended in 1 mL PBS buffer. Centrifugation and resuspension were repeated several times until supernatant was clear. Fluorescent kaolin pellet was vacuum-dried (12 hr) and stored to avoid light and moisture.

### *PS/PC liposomes*

Liposomes were prepared according to a previous reported technique [24]. L-α-phosphatidylcholine (PC) and L-α-phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL) were vacuum-dried in an 80:20 molar ratio. The dried film was resuspended in 1 mL HEPES buffered saline (HBS) at 2.3 mg-lipid/mL. A size extruder generated <100 nm diameter liposomes.

### *Thrombin biosensor on platelet surface*

Soluble thrombin was detected under flow conditions using a platelet-linked thrombin biosensor [25]. A total of 4 μL of anti-human CD61 antibody (5 mg/mL, Biolegend, San Diego, CA) was mixed with 8 μL of 900 μM DBCO-sulfo-NHS ester (Click Chemistry Tools, Scottsdale, AZ) in 28 μL of HBS buffer. The mixture was incubated at room temperature for 30 min. A volume of 2.5 μL of Tris–HCl (1 M, pH 8) was then added to quench the DBCO linking of anti-human CD61. Diluted peptide thrombin sensitive peptide (4 μL of 4 mM) was added into the reaction to initiate labeling reaction and incubated in the dark at room temperature for 4 hr. The thrombin sensor was then gel filtrated with P6-Gel beads (hydrated in HBS buffer) yielding approximately 100 μL of platelet binding thrombin sensor (5 μg/mL).

### *PDMS patterning and flow devices*

The microfluidic patterning device and the 8-channel microfluidic flow device were fabricated with poly(dimethylsiloxane) (PDMS, Ellsworth Adhesives, Germantown, WI) as previous described [22]. The protein patterning device has a single channel (250 μm in width, 60 μm in height) and two outlets at both ends of the channel allowing protein infusion for coating. The flow device has 8 cylindrical reservoirs connecting to 8 evenly spaced channels that merge to a single outlet. Both devices have a vacuum groove that allows them to be reversibly vacuum bonded onto glass slides.

### *Kaolin/collagen and TF/collagen surfaces*

Glass slides were rinsed with ethanol for 15 sec followed by DI water for 30 sec and were dried with compressed filtered air. The patterning device was vacuum bonded onto a cleaned glass slide. A volume of 5 μL of acid insoluble collagen type I (Chronolog Corp, Havertown, PA) followed by 20 μL bovine serum albumin (0.5% BSA in HBS) perfusion through the channel forming an immobilized thin matrix of well aligned collagen fibrils. Kaolin suspension (50 mg/mL HBS) was centrifuged briefly (500 g, 15 sec) to remove aggregates and supernatant was mixed with prepared PS/PC liposomes in a 3:1 volume ratio. Kaolin surface concentration can be varied via changing the centrifugation time: 5 sec centrifugation gives highly packed kaolin surface; 30 sec centrifugation gives sparse kaolin deposition while 15 sec centrifugation gives a medium density of localized kaolin on collage fibrils. A volume of 10 μL of kaolin/lipids suspension or Dade Innovin recombinant human tissue factor (50% in HBS, VWR Corp, Radnor, PA) was pulled through the channel and allowed to settle over collagen for at least 30 min before rinsing with 10 μL BSA to remove excessed kaolin, TF or lipids.

### *Characterization of kaolin/collagen surface*

For calibration, fluorescent kaolin was suspended in HBS buffer to five concentrations (0, 10, 20, 30, 40 mg/mL) and allowed to completely fill the main channel of the patterning devices and to settle overnight at 65 °C forming five dried fluorescent kaolin (without collagen) films with surface concentrations from 0–2.4 pg/μm$^2$. Fluorescent intensity was measured by imaging. A fluorescent intensity vs. mass curve was then constructed (Supplemental Fig. 1). Four fluorescent kaolin/collagen surfaces with zero, low, medium and high amount of kaolin were made. Their surface mass concentrations were extrapolated from the mass vs. fluorescent intensity curve. Surface coverage of kaolin/collagen surface was calculated with thresholding tool in imageJ (NIH). A calibration curve was made by relating surface concentration to surface coverage.

### *Blood collection and preparation for microfluidic assay*

Blood was collected via venipuncture from health donors (who were free of alcohol and medication for 72 hr prior to experiments) into corn trypsin inhibitor (CTI, 4 μg/mL WB, Haematologic Technologies, Essex Junction, VT). All donors were consent under approval of University of Pennsylvania Institutional Review Board. First 5 mL of blood was discarded to avoid tissue factor contamination. Blood was treated with anti-human CD61 antibody (BD Biosciences, San Jose, California) for platelet detection and Alexa Fluor 488 fluorescent fibrinogen (Life Technologies, Grand Island, NY) for observation of fibrin generation. All experiments were initiated within 5 min after venous phlebotomy. For antithrombotic therapy tests, platelet thrombin biosensor was added into blood in 1:9 ratio for the measurement of thrombin level. Anti-human CD41a antibody and Fluor 647 fluorescent fibrinogen were added for platelet and fibrin detection, respectively.