Structure, Mechanics, and Instability of Fibrin Clot Infected with *Staphylococcus epidermidis*

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ABSTRACT  Health care-associated infection, over half of which can be attributed to indwelling medical devices, is a strong risk factor for thromboembolism. Although most experimental models of medical device infection draw upon isolated bacterial biofilms, in fact there is no infection without host protein contribution. Here we study, to our knowledge, a new model for medical device infection—that of an infected fibrin clot—and show that the common blood-borne pathogen *Staphylococcus epidermidis* influences this in vitro model of a blood clot mechanically and structurally on both microscopic and macroscopic scales. Bacteria present during clot formation produce a visibly disorganized microstructure that increases clot stiffness and triggers mechanical instability over time. Our results provide insight into the observed correlation between medical device infection and thromboembolism; the increase in model clot heterogeneity shows that *S. epidermidis* can rupture a fibrin clot. The resultant embolization of the infected clot can contribute to the systemic dissemination of the pathogen.

INTRODUCTION

Clotting, the result of blood coagulation, is critical to hemostasis (i.e., the control of bleeding). Clots normally appear at a site of injury to prevent bleeding, restore barrier function, and promote wound healing. However, abnormal clot conditions (i.e., thrombosis) can lead to disease. For example, if a thrombus embolizes, it can circulate and block downstream arteries in the lung (pulmonary embolism) or brain (stroke) (1,2).

Bacterial infection is a significant risk factor for thromboembolism (3–5). An important virulence factor of many bacterial strains is their ability to develop biofilms, especially on implanted medical devices. Biofilms are bacterial communities embedded in a protective extracellular polymeric substance composed of polysaccharide, proteins, and DNA (6). Clinically, thrombosis and bacterial biofilms often present simultaneously, especially on implanted medical devices (7,8). Indeed, Lordick et al. (9) found that 12 of 14 patients with central venous catheter-related infection had a preceding central venous catheter-related venous thrombosis.

Most experimental models of bacterial biofilm infection on implanted medical devices utilize isolated, single-species bacterial biofilms generated on a variety of biomaterials (10,11). However, bacterial contamination of medical devices involves the colocalization of bacteria as biofilms, and host proteins as clots. For example, implanted abiotic materials provide a niche for attachment and development of bacterial biofilms while also activating host inflammatory and coagulation cascades that lead to fibrin clots (12). In this article, we develop an experimental model of medical device infection that combines biofilm-forming bacteria and the host clot. The in vitro model of clot in this report is a fibrin network, as polymerized by thrombin. Fibrin is the fundamental structural component of a clot (13–15); it is the primary determinant of clot mechanical properties, stability, and strength (16).

To research infected clots, two limits of a spectrum of possibilities are available for study design. In one limit, the role of bacterial cell growth is minimized by direct introduction of the physiologically relevant concentration of bacterial cells and then we study of the effect of these cells on implanted medical devices (7,8). Indeed, Lordick et al. (9) found that 12 of 14 patients with central venous catheter-related infection had a preceding central venous catheter-related venous thrombosis.

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with the understanding that both limits are of research interest.

We therefore introduce *Staphylococcus epidermidis* cells during the thrombin-induced polymerization of fibrinogen to mimic host clot forming on device surfaces in the presence of this pathogen. *S. epidermidis* is an innocuous commensal microorganism on human skin but can be pathogenic once introduced to the bloodstream or colonized on implanted devices (17). Specifically, it is the leading cause of indwelling medical device contaminations/infection (18) and is commonly associated with infective thrombosis (19). It is coagulase-negative; that is, it does not produce coagulase, an enzyme that enables the conversion of fibrinogen to fibrin in blood or plasma by reacting with prothrombin. The selection of a coagulase negative pathogen is parsimonious as it removes the potential confounder of bacterial enzyme-mediated clot formation and focuses solely on the colocalization of bacteria and thrombus. In addition, despite the ubiquitous presence of coagulase-negative species such as *S. epidermidis* in bloodstream and medical device infections, prior studies of the interaction between bacteria and fibrin has been more focused on coagulase-positive strains (e.g., *Staphylococcus aureus*) (20,21). The bacterial concentrations applied in this study are similar to the biofilm cell density found in a rabbit model of central venous catheter infection (22).

In this work, the structure, the mechanics, and the instability of the infected clot are compared to a cell-free clot. The structure of the fibrin network is characterized by its fiber thickness and mesh size, the latter being the average distance between neighboring strands of fibers. These structural properties determine clot mechanical properties that are critical to both normal function and pathology (15). Clinically, clot elasticity—that is, clot stiffness—is measured to diagnose hemostatic defects, such as abnormalities in clot formation/dissolution kinetics and clot strength (23). Moreover, clot instability—that is, propensity for rupture and embolization—and the effectiveness of medical treatments are a function of clot mechanics. The viscoelastic properties of a thrombus determine whether blood flow causes deformation, rupture, or embolization of the clot (15,24,25).

In this study we first use rheometry to show the mechanical influence of *S. epidermidis* on fibrin clot formation and its elasticity. Second, using confocal microscopy, we evaluate changes in the microstructure of fibrin clots after the addition of *S. epidermidis* cells. Third, we connect the changes in infected clot elasticity to the changes in fibrin network structure using multiple particle tracking (MPT) micro rheology. Fourth, through microscopic image analysis, we describe the time variation of fibrin filament area, bacteria-fibrin overlap, and network mesh size in an infected fibrin clot. Finally, by combining microscopy and time-lapse photography, we show mesoscopic and macroscopic instability of the infected clot structure at long times.

### MATERIALS AND METHODS

#### Bacterial strain and culture conditions

*S. epidermidis* RP62A, a biofilm-forming strain, was obtained from American Type Culture Collection (ATCC 35984; Manassas, VA). One colony from a Lysogeny broth plate was inoculated in a flask (125 mL) containing 32 mL of tryptic soy broth with 1 wt % glucose (TSB) and cultured at 37°C at 200 rpm of continuous shaking for ~9 h to an optical density (OD) of 600 nm (OD600) = 1.35 ± 0.1.

#### Fibrin clot and infected fibrin clot constitution

Citrate-free thrombin from human plasma (605206-100U) and fibrinogen (Fg) from human plasma (341576-100MG) were purchased from EMD Millipore (Billerica, MA). Fibrinogen solutions were made by diluting the stock solution with Hank’s Balanced Salt Solution (HBSS, 1 x, with calcium, magnesium, without Phenol Red, 1 g/L D-glucose; Thermo Fisher Scientific, Waltham, MA) at 37°C.

After reaching the prescribed OD600, the bacterial suspension was allowed to stand quiescently at room temperature for 1 h so large cell clusters could sediment. An INCYTO C-Chip Disposable Hemoctometer (Thermo Fisher Scientific) was used to determine the cell concentration. A target number of cells were resuspended in the Fg solution to achieve the desired final cell concentration. The introduced cell concentrations for bulk rheology measurements were 1 × 10⁹, 2 × 10⁹, 4 × 10⁹, and 6 × 10⁹ cells/mL. These values are similar to the 10⁵ CFU/cm² cell density found in a rabbit model of central venous catheter infection (22). A cell concentration of 4 × 10⁹ cells/mL was used for the remaining experiments. Specifically, a volume of the bacterial culture was centrifuged (15,000 g, 40 s) and washed twice with HBSS; the resulting bacterial pellet was suspended in Fg solution. The Fg solution or the cell-Fg mixture was kept on ice for 3 min before clot initiation by adding thrombin (0.5 U/mL as final concentration). Then the samples were loaded to either the rheometer (5°C) or a Nunc Lab-Tek II chambered cover glass dish (Thermo Fisher Scientific), then kept on ice. The rheometer temperature was increased to 37°C after loading, and the chambered cover glass was held in a 37°C incubator. The chambered cover glass was sealed with paraffin to prevent desiccation. The effect of cold fibrin initiation is discussed in Fig. 51 and Supporting Materials and Methods.

#### Clot elasticity

Clot elasticity was characterized by measuring the linear elastic modulus, $G'$, on a mechanical rheometer (model No. AR-G2, cone and plate fixture with a 2° cone angle and a 40-mm diameter; TA Instruments, New Castle, DE). All tests were performed at a frequency $\omega = 0.1$ Hz under an imposed strain $\gamma = 0.01$ at 37°C in a humid atmosphere. Clot desiccation was further prevented by coating the sample edge with a thin layer of mineral oil (14). Loading the sample in the rheometer at 5°C delayed clotting until the instrument was prepared for measurement. After loading, the rheometer reached 37°C in ~1 min; measurements were initiated 30 s later. Experiments were stopped after steady state was reached, defined as the variability in $G'$ in any 2-min interval being no more than 0.5% for 20 min. The steady-state elastic modulus was taken as the average of all the data within the steady-state region with error bars denoting SD of all replicates.

#### Microstructure visualization and MPT

Image volumes (3D) and time series (2D) of fibrin and bacterial cell microstructure were acquired using a model No. A1Rsi confocal laser scanning microscope with a 100×, 1.45 NA, oil immersion objective (Nikon, Melville, NY). Specimens were prepared in a Nunc Lab-Tek II chambered cover glass dish (Thermo Fisher Scientific). Image volumes for...
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