Controlling Bioprocesses with Inorganic Surfaces: Layered Clay Hemostatic Agents

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Received May 29, 2007
Revised Manuscript Received July 11, 2007

The sometimes dramatic response of biomolecules and biomolecular processes to their interface interactions with inorganic surfaces is evident in biomineralization,[1] biomolecular chromatographic separations,[2] supported enzyme activity and lifetime,[3,4] and protein folding and denaturation.[5] The development of advanced wound-dressing materials that are capable of arresting hemorrhage due to traumatic injury is another emerging important application using materials chemistry to control bioprocesses.[6−9] Advanced wound-dressing materials are estimated to have saved hundreds of lives in the current military conflicts[6] and are finding increasing civilian use.[10]

Here, we report in vitro whole blood coagulation studies of an aluminosilicate structural family, the layered clays. We have identified how changes in structural and surface properties influence the blood-clotting response, providing useful information for future aluminosilicate-based clotting agent design. Furthermore, our studies revealed that some layered clays may be excellent alternatives to the hemostatic agents that are currently in use.

One of the most effective wound dressing materials currently available is QuikClot (QC), a zeolite 5A composite material. QC conferred the highest rates of survival in a swine model of fatal femoral injury when compared with other advanced wound dressing materials and gauze.[11] Although the mechanism of QC has not been experimentally confirmed,[6] the effectiveness of QC for stopping hemorrhage has been attributed to local dehydration and/or release of heat upon contact with blood.[11] The layered clays investigated in this study were fully hydrated and do not release heat upon contact with blood. Therefore, the tendency of these clays to elicit a procoagulant response depends on the structural and surface properties of the clay.

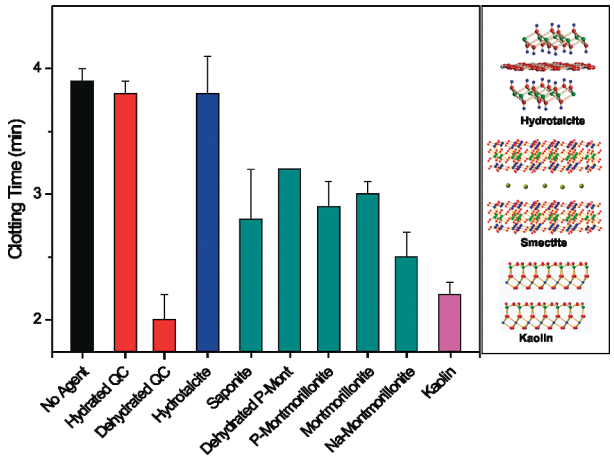

Figure 1. (a) Clotting times induced by QC and layered clays in porcine whole blood (concentrations of 42 mg/mL). Abbreviations: QC = QuikClot, P-Mont = pillared montmorillonite. (b) Types of structures of the clays used in the study: hydrotalcite in blue, the clays from the smectite family in green, and kaolin in magenta.

Clays can be made with a wide range of chemical and physical properties, including variable swelling capacities, particle morphologies, surface charge, and the ability to control the local electrolyte balance through ion exchange. To compare the hemostatic efficacy of clays, we synthesized and selected layered clays (structures in Figure 1b) with specific properties that are thought to strongly influence their hemostatic performance. For example, we selected synthetic hydrotalcite because it contains positively charged magnesium aluminum hydroxide layers, which might limit hemorrhage via adhesion to tissues and red blood cells.[12] The smectite family of clays, composed of an octahedral alumina sheet sandwiched by two sheets of tetrahedral silica, are promising hemostatic materials because of their ability to readily absorb water and swell upon hydration, thereby possibly thickening the blood and restricting blood flow. Pillared smectite was tested to determine the effects of surface area and local blood dehydration on blood coagulation kinetics. The smectites have a net negative surface charge, which can serve as a platform for protein activation. Several negatively charged materials, such as kaolin, have been shown to activate the contact pathway of coagulation,[13] and kaolin is routinely used as a clotting initiator in clinical assays.[14] Kaolin layers consist of a sheet of silicon tetrahedrally bonded to oxygen atoms, and a sheet of aluminum octahedrally bonded to oxygen atoms and hydroxyl groups, which give kaolin a negative surface charge at the pH of blood.

The clotting activities of clay minerals and QC were measured using thrombelastography (TEG) in porcine whole

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10.1021/cm071457b CCC: $37.00
Published on Web 08/14/2007

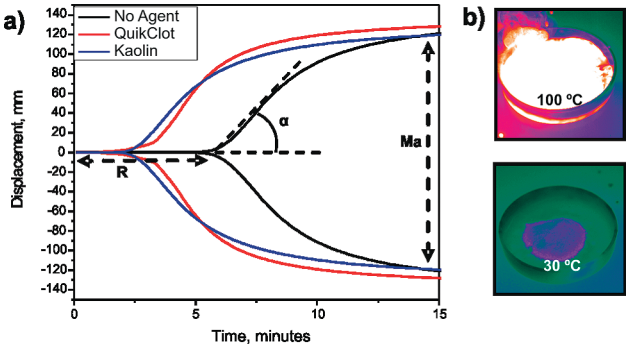

Figure 2. (a) Thrombelastograph showing clotting characteristics of kaolin and QC in porcine whole blood. (b) Thermal images of QC (top) and kaolin (bottom) captured at the hottest point after addition to water.

blood or plasma. TEG measures the torsion of clotting blood as a function of time, providing a precise determination of time until initial clot initiation (denoted R), the rate of clot formation (α), and the strength of the clot (Ma) (schematic in Figure 2a). Previous research has shown that in vitro clotting times, specifically clotting times equal to or shorter than those induced by fully dehydrated QC, correlate with increased survival rates in a large animal model of a fatal femoral injury.[15] Therefore, we focused specifically on clotting times in this comparison between QC and layered clays.

The clotting times corresponding to the layered clays and QC are shown in Figure 1. The layered clays displayed a wide range of blood clotting activities in vitro, sometimes with only modest variations in structure and composition. For example, sodium-exchanged montmorillonite conferred significantly faster clotting than the natural calcium form, presumably because of the higher swelling capacity and therefore greater exposed surface area of Na-montmorillonite.[16] Dehydrated clays (for example, dehydrated pillared montmorillonite, which has a hydration capacity similar to that of QC) did not confer faster clotting than the hydrated forms, suggesting that water absorption by clays does not dominate their hemostatic activity. In contrast, QC is significantly more active in the dehydrated form (shown in red). Hydrotalcite and fully hydrated QC (which does not release heat) were the least effective clotting agents tested, showing no enhanced rate of clotting over untreated porcine whole blood. All of the hydrated smectite clays and kaolin were more effective than the fully hydrated (heat-free) form of QC, underscoring the potential of layered clays for hemorrhage control. Furthermore, kaolin elicited a rapid clotting response that has been matched only by dehydrated QC among the dozens of metal-oxide-based materials screened for clotting activity in our laboratory.

Kaolin was selected from the clays that we studied for a more detailed comparison with QC because of its exceptionally fast clotting response. A typical TEG showing the response of porcine whole blood to fully dehydrated QC and to kaolin is shown in Figure 2a. The traces nearly overlap, indicating that the materials have very similar clotting characteristics, including R, α, and Ma. A dose−response comparison (see the Supporting Information, Figure S1) verifies the similarity in clotting activities between the two agents at different concentrations. Because of the widespread use of anticoagulant drugs, which can make patients susceptible to hemorrhage, we compared the clotting properties of kaolin and QC in human plasma taken from patients receiving the anticoagulant Coumadin, and found that kaolin and QC had comparable efficacies in this medium (see the Supporting Information, Table S1).

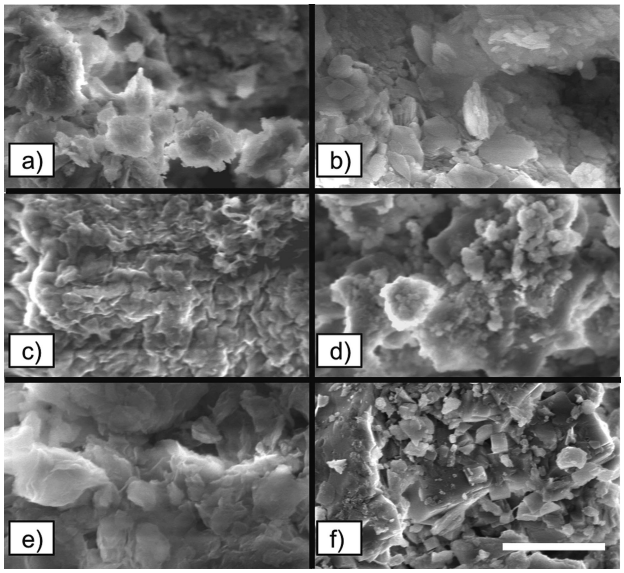

Figure 3. Scanning electron microscope images of clays used this study: (a) pillared montmorillonite, (b) kaolin, (c) saponite, (d) hydrotalcite, (e) montmorillonite, and (f) QuikClot. Scale bar, 2 μm.

Given the motivation to use clays as an alternative to QC to reduce the risk of thermal injury, we used infrared imaging to map the heat released by QC and kaolin upon application to water, which served as a model for blood. The images show that the QC formulation used in these studies locally heats water to 100 °C, whereas the kaolin layered clay, which has similar efficacy in vitro, does not release measurable heat upon addition to water.

Because the dramatic effectiveness of the QC material has been attributed to local dehydration and/or release of heat upon contact with blood, it was surprising that a layered clay such as kaolin could share comparable blood-clotting activity with QC, despite the observation that dehydration and release of heat do not appear to play a role in the clotting activity of the clays. Therefore, we examined the materials properties of the clays that might influence their effectiveness.

Scanning electron microscope images indicate that the clay platelets, with the exception of those of saponite, have similar morphologies and diameters (Figure 3). Therefore, we do not expect that, for the samples studied, clay platelet morphology or size greatly influenced the clotting performance. Increased external clay surface area, as determined by nitrogen sorption isotherms (see the Supporting Information, Table S2), did not appear to be a determining factor on clotting times. A plot showing the relationship between clotting time and external surface area is shown in Figure 4c. This result was unexpected, given that the extent of clotting activation has been shown to scale with procoagulant surface area for other materials.[17]

Calcium and magnesium ions are required for certain enzymatic reactions in the coagulation cascade, and therefore

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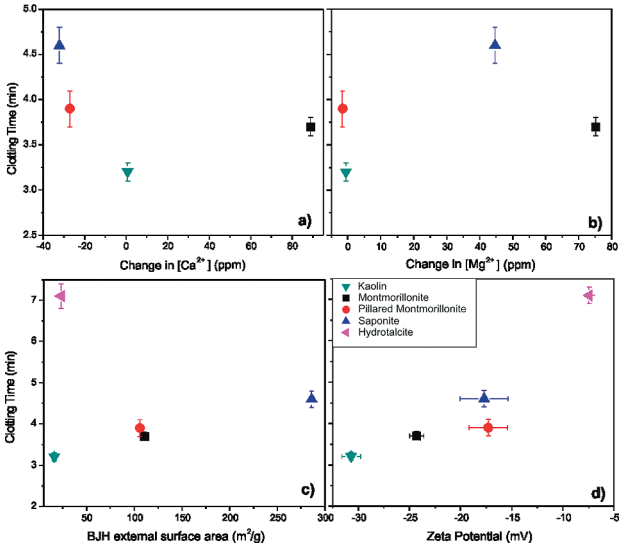

Figure 4. Porcine plasma clotting time plotted versus: (a, b) change in concentrations of Ca and Mg ions, respectively, in SBF after 3 min exposure to clays, (c) external surface area, (d) zeta potential of clay platelets as measured in SBF. The zeta potentials of the clays were measured in SBF in order to mimic the effects of absorption and shielding by the ions in blood.

their delivery by materials might influence coagulation kinetics. In particular, delivery of Mg^{2+} ions intravenously has been explored as a candidate therapy for treating patients with prolonged clotting.[18] We found that the addition of clays (with the exception of kaolin) to simulated body fluid[19] (SBF) greatly altered the concentrations of Ca^{2+} and Mg^{2+} ions in the fluid (panels a and b in Figure 4). Despite the critical role of these ions, their apparent increase in blood did not appear to influence clotting time response (see the Supporting Information, Figure S2). However, because a threshold level of Ca^{2+} is required for propagation of clotting,[20] we speculate that the removal of Ca^{2+} by pillared montmorillonite and saponite adversely affects the clotting activity of these materials (Figure 4a).

We found a striking correlation between the zeta potential of clay platelets measured in SBF and clotting times. Figure 4d shows that decreasing clotting times correspond with decreasing (more negative) clay platelet surface charge. Our studies show that the quantitation of surface charge of layered clays in SBF provides a useful metric for clotting agent design. For example, we found that hydrotalcite exhibited the least negative surface charge in SBF and did not stimulate clotting. Kaolin, however, displayed the most negative surface charge in SBF and was the most active clotting initiator. Dehydration and the release of heat and calcium ions, which are characteristics of the interaction between dehydrated QC and blood, are not essential for activation of blood coagulation by clays.

The correlation between clay surface charge and clotting time that we observed is likely due to preferential absorption and activation of blood coagulation factor XII on the more negatively charged clay surfaces in the presence of plasma proteins.[21] It has been shown that certain "negatively charged" materials (e.g., kaolin, glass, and celite) activate factor XII, which initiates contact activation proteins and triggers the intrinsic pathway of blood coagulation.[13] Although it has been assumed that factor XII activation correlates with negative surface charge density,[22] quantitation of material surface charge density under physiologically relevant conditions (which was done in the present study) has not been reported. Furthermore, it has been shown that factor XII does not autoactivate in the presence of all negatively charged moieties, implying that the interaction between factor XII and negatively charged surfaces may be more complex than simple electrostatics.[22] The binding of the protein fibrinogen to clay surfaces, which can activate blood platelets,[23] may also play a role in the high clotting activity of the smectite and kaolin clays. However, in what is termed the "Vromen effect," fibrinogen is rapidly displaced by other plasma proteins from negatively charged surfaces,[24] so may play a lesser role in clot initiation by negatively charged surfaces than factor XII activation.

Although charge appears to be an important variable in the clotting activity of layered clays, charge does not appear to be a controlling parameter in the clotting activity of QC. The zeta potential of QC in SBF was determined to be -14.6 ± 0.4 mV, placing it among the less effective clotting agents in terms of zeta potential on the plot in Figure 3d, which reinforces the hypothesis that the properties of QC and clays that determine their effectiveness as hemostatic agents are distinct from one another. Studies are underway to further elucidate these differences.

We have shown that layered clays are a class of materials with a wide range of clotting properties that appear to be greatly influenced by surface charge. In contrast to what has been proposed for the zeolite (QC) material, we found that clay dehydration, surface area, and ion delivery did not appear to play a major role in blood clot initiation by layered clays. The most active clay clotting agent in our studies, kaolin, is as effective as dehydrated QC, but does not release heat, making it a promising alternative to dehydrated QC. The layered clays are lightweight, inexpensive, stable, and can be nontoxic. Therefore clays may represent a new class of clotting agents for use in hemorrhage control both in military and in civilian trauma.

Acknowledgment. This work was funded by the Office of Naval Research, ONR Award N00014-06-1-0145. We thank Prof. Thomas Pinnavaia for helpful discussion and Z-Medica for providing QuikClot.

Supporting Information Available: Supplementary tables and figures mentioned in the text, synthesis of clays, experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

CM071457B

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