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Review

A physical organic mechanistic approach to understanding the complex reaction network of hemostasis (blood clotting)

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ABSTRACT: This review focuses on how the mechanistic approach of physical organic chemistry can be used to elucidate the mechanisms behind complex biochemical networks. The dynamics of biochemical reaction networks is difficult to describe by considering their individual reactions, just as the dynamics of organic reactions is difficult to describe by considering individual electrons and atomic nuclei. Physical organic chemists have developed a useful set of tools to predict the outcome of organic reactions by separating the interacting molecules into modules (functional groups), and defining general rules for how these modules interact (mechanisms). This review shows how these tools of physical organic chemistry may be used to describe reaction networks. In addition, it describes the application of these tools to develop a mechanistic understanding of the dynamics of the complex network of hemostasis, which regulates blood clotting. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: coagulation; microfluidics; spatiotemporal dynamics; complexity; nonlinear; enzyme models; networks; modules

INTRODUCTION

This review describes a mechanistic approach to understanding the spatiotemporal dynamics of complex reaction networks. Biochemical reaction networks perform a wide range of functions indispensable for the existence of living organisms, from energy conversion, self-regulation, and computation, to signal detection and amplification. Understanding the mechanisms by which biochemical reaction networks perform their function is currently the focus of a major research effort and a primary goal of the field of systems biology.[1] However, the inherent complexity of these networks makes theoretical and experimental characterizations of their underlying mechanisms challenging for two reasons: these networks comprise a large number of reactions, not all of which are typically known, and the dynamics in these systems is spatially heterogeneous and time-dependent. Therefore, we have developed a two-fold strategy to study complex systems. We begin by reducing the complexity of the network by using a physical organic approach to develop a simple chemical model of the complex network. Then, we utilize microfluidics[2,3] and patterned surfaces[4,5] to experimentally probe the chemical model and the biochemical network, which allows control and manipulations of reactions on the length and timescales relevant for biochemical reaction networks. This review focuses on the first component of our strategy—the mechanistic approach to understanding the spatiotemporal dynamics of a complex reaction network.

APPLYING PHYSICAL ORGANIC APPROACHES TO COMPLEX SYSTEMS

The success of physical organic chemistry provides motivation to apply its tools to other fields, including systems biology. At first glance, the connection between physical organic chemistry and the study of reaction networks may not be obvious, but these two fields are conceptually parallel. The mechanisms of organic reactions are a result of the complex interactions among a set of components—the atomic nuclei and electrons of the organic molecules. One may use the Schrödinger equation to describe these interactions, but solving this equation to describe realistic reactions in polar solvents is not easy, even with today's computer power. Similarly,

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the function of networks is also a result of the complicated interactions among a set of components—biochemical reactions. Differential equations may be written to describe these systems, but solving these equations is also difficult because of kinetics with time delays, highly nonlinear interactions among many components, and compartmentalization. In addition, for many biological and chemical networks, new players are constantly being discovered.[6,7] Therefore, we wished to capitalize on this parallelism to help uncover the mechanisms by which reaction networks operate.

Physical organic chemists have developed an approach that has been surprisingly successful for elucidating and predicting the mechanisms of organic reactions. Rather than treating molecules as an interacting set of electrons and nuclei and then solving the Schrödinger equation, this approach first separates molecules into modules—functional groups, such as thiols and carbonyls. The functional groups are further categorized into classes, such as nucleophiles and electrophiles. Then, general descriptions of the interactions of the functional groups in a reaction are developed—reaction mechanisms, such as the cycloaddition and the S_N2 substitution mechanisms. This approach is powerful because the knowledge gained by studying functional groups and mechanisms in one set of molecules and reactions is transferable to other molecules and reactions.

We applied this approach to elucidate the mechanisms governing complex networks. In networks, it is likely that groups of interacting reactions are actually functioning together as modules, as has been proposed.[8–10] It is also likely that the interaction of these modules constitutes the basic mechanism for the network's function. In addition, these modules and mechanisms may be generally applicable to other networks, as many networks share similar dynamics, such as threshold responses, amplification, oscillations, and hysteresis.

DEVELOPING A MODULAR MECHANISM FOR HEMOSTASIS

We used this mechanistic approach as a basis for our investigations of the complex network of hemostasis, which regulates blood clotting (also called blood coagulation). Specifically, we focused on initiation of blood clotting and on the threshold dynamics of this system. Like many complex networks, key molecular players in hemostasis are known, but many overall properties of the network are not understood. Elucidating the general mechanism by which the network operates may shed light on how clotting is initiated on areas of significant vascular damage and not on small areas of damage, which are believed to be present throughout the vascular system (Figure 1a). A general mechanism may also explain how clotting, once initiated, remains localized to regions of damage without spreading throughout the vascular system.

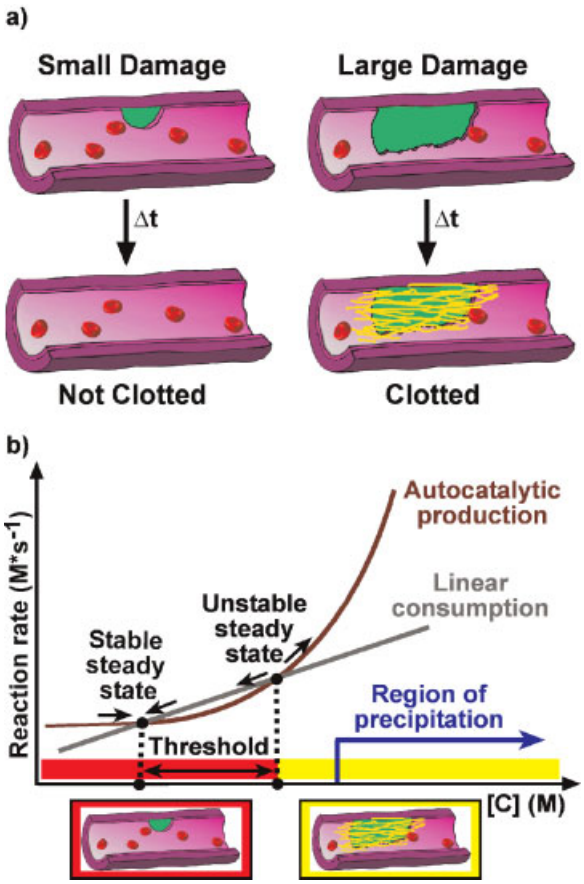

Figure 1. A modular mechanism for the threshold response in blood clotting may account for the dynamics at sites of vascular damage. (a) A threshold response to clotting based on the size of a patch of vascular damage (green) has been postulated, in which small patches of damage do not initiate clotting and large patches do initiate clotting (yellow). (b) A rate plot for the proposed modular mechanism for the threshold response.[11] Three rate equations, one for each module, are graphically represented (brown, gray, and blue curves). The reaction rate for each equation is plotted against the concentration of activators of clotting, [C]. The interaction between two modules, autocatalytic production of C (brown line) and linear consumption of C (gray line), creates two steady states in the system, which is the basis for the threshold response. Reprinted with permission from reference 11 with minor modifications. Copyright 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. This figure is available in colour online at www.interscience.wiley.com/journal/poc

Utilizing the physical organic ideas described above, we simplified the complex network of hemostasis by reducing the ~80 reactions of clotting into three interacting modules.[11] Each of the three modules was assigned a corresponding rate equation, which are graphically represented in a rate plot (Figure 1b), and, together, describe the threshold response of initiation of clotting. In this mechanism, one module autocatalytically produces an activator of clotting, C; a second module linearly consumes C; and a third module forms a solid clot at high [C]. Competition between production and consumption of activators creates three steady states in the system

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(two are shown), which leads to a threshold response to [C]. For small areas of vascular damage, [C] does not exceed the threshold [C], $[C]_{thresh}$, and clotting does not occur. For large areas of vascular damage, [C] exceeds $[C]_{thresh}$, and clotting occurs.

TESTING THE MODULAR MECHANISM

To experimentally test this modular mechanism, we utilized an additional physical organic approach—we built a chemical model. Chemical models have been used to advance our understanding on a broad scale of complexity, from the reactivity of organic molecules[12] and the functions of enzymes[13–17] to spatiotemporal dynamics of catalysis,[18] oscillations in chemistry[19] and biology,[20] oocyte development,[21] and fibrillation in myocardium.[22] Importantly, the ability of the chemical model to reproduce and predict the properties of blood clotting would strongly support the proposed modular mechanism for hemostasis.

To build this model, we substituted one reaction for each module, for a total of three reactions.[11] Each reaction had the same overall kinetics as the proposed module. In the chemical model, the autocatalytic production and linear consumption modules were represented by the chlorite–thiosulfate reactions,[23] which autocatalytically produced and also consumed H_3O^+. The precipitation module was represented by the precipitation of alginic acid from alginate under acidic conditions, which is representative of ''clotting.''

To test if this chemical model of the network of hemostasis operated by the same mechanism as the one we proposed for the threshold response in blood clotting, we exposed the chemical model to surface patches of clotting stimulus of various sizes.[24] As was postulated for clotting of human blood, ''clotting'' in the chemical model showed a threshold response to patch size, where ''clotting'' occurred on large patches, but not on small patches (Figure 2). This dynamics of the chemical model was rationalized by considering reaction-diffusion equations and competition between production of activators from the patch and diffusion of activators away from the patch. This competition between production and diffusion is described by the Damköhler number,[25] $Da = t_D/t_r$, where t_D [s] is the timescale for diffusion of activators from the center to off of the patch, and t_r [s] is the time scale for the production of activator at the patch. For small patches, *Da* is small and diffusion out-competes production. Diffusion of activators off the patch is fast, and the concentration of activators necessary to initiate clotting ($[C]_{thresh}$) is not produced. For large patches, *Da* is large and production out-competes diffusion. Diffusion of activators from the center of the patch to outside the patch is slower than the time it takes for the concentration of activators to reach the threshold. Based on this analysis, the threshold patch size, p_{tr} [m], can be predicted by the equation, $p_{tr} \approx (D \times t_r)^{1/2}$, where D [$m^2 s^{-1}$] is the diffusion coefficient of the activator. Using this equation, the experimental threshold patch size could be predicted within a factor of ~2 for the chemical model.

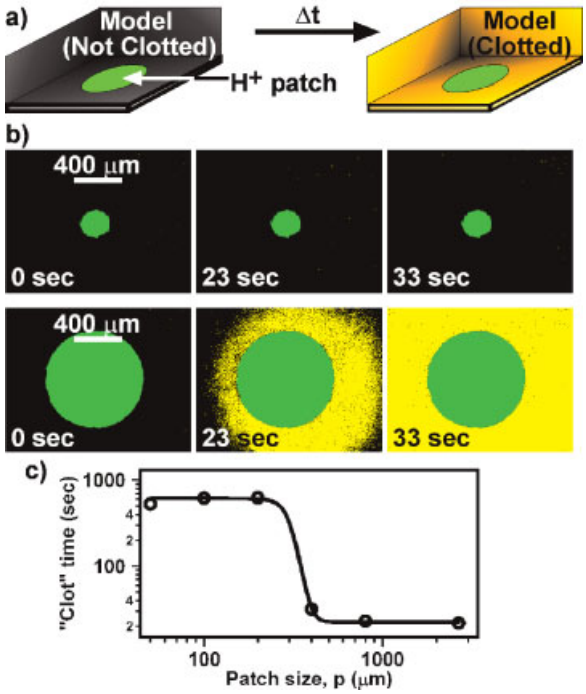

Figure 2. ''Clotting'' in the chemical model shows a threshold to stimulus patch size.[24] (a) Schematic showing that over time the chemical model initiates ''clotting'' (yellow) on large patches (green) that produce H^+, the stimulus of ''clotting'' in the model. (b) Time-lapse fluorescence micrographs showing clotting on large patches, but not small patches. (c) Graph quantifying the threshold response of initiation of ''clotting'' to stimulus patch size in the chemical model. Reprinted with permission from reference 24. Copyright 2006 National Academy of Sciences, USA. This figure is available in colour online at www.interscience.wiley.com/journal/poc

To test whether the chemical model described the dynamics of the real biological system, we tested the clotting of human blood plasma on patches of stimulus.[24,26] Clotting of human blood plasma showed a threshold response to patches of stimulus of different sizes, where the stimulus was either a lipid bilayer[27] containing a reconstituted enzyme (tissue factor[28]) or hydrophilic glass (Figure 3). Surprisingly, the experimental threshold patch size for human blood plasma could also be predicted within a factor of ~2 by using the same simple scaling equation developed for the chemical model.

CONCLUDING REMARKS

This mechanistic approach of reducing the complex network into a modular mechanism appears to work, at least for blood clotting *in vitro*. Reducing the complex network of hemostasis into modules allowed us to

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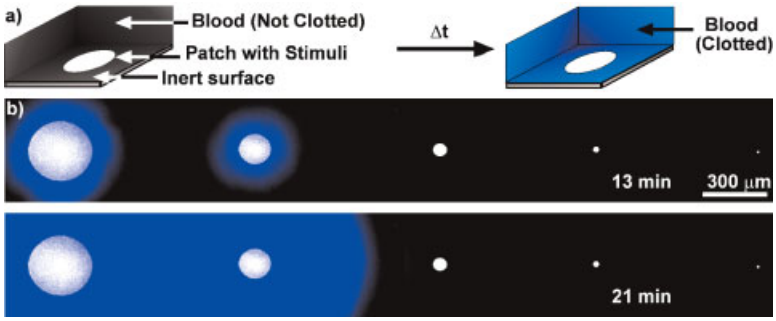

Figure 3. Clotting of human blood plasma displays a threshold response to the size of patches containing a stimulus.[24] (a) Schematic showing that over time blood will clot (blue) on large patches (white) of stimulus. In this experiment, the stimulus was hydrophilic glass. (b) Time-lapse fluorescence micrographs showing clotting on large patches, but not small patches. Reprinted with permission from reference 24. Copyright 2006 National Academy of Sciences, USA. This figure is available in colour online at www.interscience.wiley.com/journal/poc

identify a possible modular mechanism for the threshold response of initiation of blood clotting. By building a simple, experimental chemical model based on this modular mechanism, we were able to reproduce and predict properties of the hemostasis network, suggesting that the modular mechanism is reasonable. In this review, we have argued that the mechanisms of complex networks can be elucidated by using a physical organic approach. There are two main criteria that must be satisfied to conclusively validate this approach. First, it must be shown that mechanisms for one network can be determined using this method. Here, we satisfied part of this criterion by showing that predictions based on the proposed mechanism for the complex network of hemostasis could be made and verified in a simple *in vitro* system. However, in order to conclusively show that this mechanism is physiologically relevant, more experiments must be done, especially experiments involving flow and experiments *in vivo*.[29] A future *in vivo* experiment demonstrating the existence of a threshold response to patch size for patches of a stimulus, either tissue factor, collagen, or some other stimulus, would strongly support the proposed mechanism. Second, modules and mechanisms of one reaction network must prove to be transferable to other networks, just as the functional group properties and reaction mechanisms of organic molecules can be transferred from one set of molecules to another. Several other reaction networks have groups of autocatalytic reactions leading to threshold responses, such as networks of apoptosis and quorum sensing. These networks may be described by a modular mechanism similar to the one we proposed for hemostasis. Additional experiments must be conducted to test this hypothesis.

In addition to investigating the dynamics of specific biological networks, the techniques described in this review have the potential for investigating other aspects of reaction networks as well. The combination of microfluidics and nonlinear chemical systems may be used to engineer artificial systems that perform the functions of complex biological networks. For example, we previously used microfluidics to build a synthetic reaction network that performed chemical 5000-fold amplification of autocatalysts with a threshold response.[30] This synthetic reaction network has similarities to the chemical model for hemostasis in that competition between production of autocatalytic species and the removal of these species by transport determines whether or not the system produces a response. We also used these principles to design systems that can detect low quantities of specific chemicals[30] and systems that can control nucleation and growth of protein crystals.[31] These types of synthetic networks have also been used previously to understand molecular connectivity and mechanisms within complex reaction networks.[32,33] Combining engineered reaction networks with micro-scale technologies has broad potential applicability to both understanding complex networks and to the engineering of artificial systems.

We envision that this general approach of reducing the complexity of networks with a modular mechanism and model may be useful for understanding the mechanisms in complex networks performing other functions as well. Currently, we are applying these ideas to the study of embryonic development.[34] This approach for elucidating a mechanism in blood clotting was based on principles of physical organic chemistry. We find it exciting that these approaches developed for physical organic chemistry may be general and used for elucidating the mechanisms of reactions as well as the mechanisms of other types of chemical processes.

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