Uncovering Membrane-Bound Models of Coagulation Factors by Combined Experimental and Computational Approaches

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Abstract

In the life sciences, including hemostasis and thrombosis, methods of structural biology have become indispensable tools for shedding light on underlying mechanisms that govern complex biological processes. Advancements of the relatively young field of computational biology have matured to a point where it is increasingly recognized as trustworthy and useful, in part due to their high space–time resolution that is unparalleled by most experimental techniques to date. In concert with biochemical and biophysical approaches, computational studies have therefore proven time and again in recent years to be key assets in building or suggesting structural models for membrane-bound forms of coagulation factors and their supramolecular complexes on membrane surfaces where they are activated. Such endeavors and the proposed models arising from them are of fundamental importance in describing and understanding the molecular basis of hemostasis under both health and disease conditions. We summarize the body of work done in this important area of research to drive forward both experimental and computational studies toward new discoveries and potential future therapeutic strategies.

Keywords
► coagulation factors
► complex formation
► membrane-bound forms
► membrane lipids
► peripheral membrane proteins
► phosphatidylserine
► tissue factor

Introduction

Blood clotting is governed by a tightly regulated enzymatic cascade involving the coagulation factors in which the tiny amounts of the factors circulating, upon being triggered by trauma, amplify and control the physiological response.1–3 To elucidate the biochemical mechanisms for (in)activation of the coagulation cascade, specific or semi-specific interactions between coagulation proteins and membrane lipids need to be identified and characterized.4 The membrane surface serves as the functional platform for both colocalizing and (in)activating coagulation factors, and is as such an essential cofactor of central importance in aspects of activation, amplification, and regulation of the cascading reactions.5,6 Structural studies can provide unique insights into how exactly this is accomplished on the molecular level. Computational approaches,7–10 in particular, are apt for this purpose, providing dynamic features of the biomolecules involved, at unparalleled spatiotemporal resolution. Furthermore, computational approaches can show us the atomic details of both membrane-bound coagulants and the interacting membrane lipids, which essentially have not been achieved directly by any experimental method to date.

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The technical term “computational approach” for investigating the molecular basis of the coagulation cascade usually refers to those approaches that employ in silico methodologies, such as docking, molecular dynamics (MD), ensemble refinement, and their variations combined with bioinformatics techniques when appropriate. It is important to acknowledge up front that the computational approach relies critically on experimental observations in both requirement of initial coordinates of atoms of individual protein molecules and also restriction of the astronomical search space when setting the configuration of proteins in the systems to simulate. Suitable experimental techniques to elucidate structural and dynamic features of the coagulation cascade include site-directed mutagenesis, nanodisc, electron spin resonance, Förster resonance energy transfer (FRET), surface plasmon resonance, and cryogenic electron microscopy (cryo-EM), as well as recently developed X-ray reflectivity (XRR) and X-ray free-electron laser. The combination of both experimental and computational approaches has proven particularly powerful as they complement each other exquisitely to uncover the detailed mechanisms in the coagulation cascade.

Accurate models for membrane-bound γ-carboxyglutamic acid rich (GLA) and C2 domains, which are the two most predominant entities that anchor coagulation factors to membrane surfaces, can be the key to further model the membrane-binding mode of the whole molecules, and then the complex formation with other cofactors, dramatically reducing the search space for potential binding/docking orientations of coagulation proteins.

In this review, coagulation factors and relevant molecules are referred to as those of human’s, unless otherwise specifically noted. The basic information on individual coagulants is found elsewhere, and this review focuses on summarizing membrane-bound forms of individual coagulants and their complexes on the membrane surface where they perform their physiological functions. Some emphasis is placed on disseminating foundational principles of state-of-the-art developments in theoretical and computational methods for the broad interest of nonexperts and potential future practitioners. It is our hope that this review will support further developments aiming to uncover the structural mechanisms that underlie hemostasis in health and disease.

**Molecular Dynamics Simulations**

**Advantages and Limitations of Molecular Dynamics Simulations**

MD is a computational microscope of atomic resolution. This imaginary microscope is compact and inexpensive; in general it requires a smaller space to install and less investment when compared with the experimental equipment/approaches, while allowing for observing the behaviors of individual molecules clearly, not only detecting the statistical trends averaged over numerous molecules. Biological processes, such as membrane binding of GLA domains as described in the following sections, can be observed repeatedly and consistently to reveal the molecular mechanisms with atomic details. In addition, it is readily possible in MD methodologies to alter or gradually change the conditions of systems of interest, such as the temperature, the pressure, pH, and the molecular composition, which may be intractable or impossible in experimental approaches.

On the other hand, the wall times required by MD methodologies are far longer than the actual durations within which molecules of interest carry on certain collective motions. A few to some tens of nanoseconds-long molecular phenomena in nature may be simulated as a 1-day-long MD job, depending on the system size, the force field used, and the computer resources employed. The success of an MD simulation therefore relies both on the accessible time scales and availability of the initial coordinates for the system, which is often built as a mixture of smaller molecules (i.e., water, lipids, ligands, and/or ions) and proteins whose structures are based on specific Protein Data Bank (PDB) entries.

It should be noted that the coordinates of most PDB entries are determined by either X-ray crystallography or by nuclear magnetic resonance (NMR), often under nonphysiological conditions (e.g., very high protein and/or salt concentrations, low temperature), and that the obtained structures of such proteins may not represent their physiological conformations as a whole or in part (and there exists no simple way to assess the differences). Or there may be several physiologically important conformations for a flexible protein, while a PDB structure of the protein represents just one of them. Such potential cases include 1DAN and 1PFX for GLA domain-containing coagulation factors, which share the same topology except the relative positioning of GLA and epidermal growth factor-like domain 1 (EGF1) domains, as well as 1QFK, which indicates that there could be a rearrangement of EGF1 and EGF2 upon binding to tissue factor (TF). Factor VII (FVII), factor IX (FIX), or factor X (FX) as well as prothrombin (PT) may be in either topology, in its (in)active and/or complex/free form, depending on the environment.

If a PDB entry unfortunately does not quite represent an appropriate physiological structure of the protein under the physiologic condition of choice, the systems including the protein require a long equilibration in advance of meaningful MD simulations, hoping the protein will reach its physiologic condition of choice, the systems including the protein may be intractable or impossible in experimental approaches.

**MD Force Fields**

There are three things needed to perform MD methodologies: initial atomic coordinates of the system of interest (i.e., the molecules to simulate), an MD package and a set of force field, and the computer on which the MD of the system is performed, using the force field. Several sets of MD packages with associated websites that include the manuals and tutorials have been available either for free or at
reasonable costs,\textsuperscript{42} such as AMBER,\textsuperscript{43} CHARMM,\textsuperscript{44} GROMACS,\textsuperscript{45} NAMD,\textsuperscript{46} and Tinker\textsuperscript{47} that have been commonly used for MDs of biomolecules including proteins and lipids, as well as rather recently developed OpenMM\textsuperscript{48} and GENESIS.\textsuperscript{49} Most of these packages implement their own force field (see the next section for details) of the same name as well as several other force fields including OPLS.\textsuperscript{50}

A typical potential energy function for MD at the atomic scale can be formulated as shown in Eq. 1:\textsuperscript{36,37,51,52}

\begin{equation}
V = \sum_{\text{bonds}} K_i (b - b_0)^2 + \sum_{\text{bond angles}} K_\theta (\theta - \theta_0)^2 + \sum_{\text{dihedral angles}} K_\phi \left[ 1 + \cos(n\phi - \delta) \right] + \sum_{\text{nonbonded} \ i,j} \left[ \frac{A_{ij}}{r_{ij}^{12}} - \frac{B_{ij}}{r_{ij}^6} + q_i q_j \right]
\end{equation}

which defines empirical distance-dependent, pairwise-additive interactions among atoms under the Born–Oppenheimer approximation (which results in the assumption that the average position of the electrons of an atom matches that of the nucleus—or the treatment that atomic charges are located upon the positions of the atoms). The first summation is of any pair of atoms in the system that are covalently bonded as a simple harmonic potential form, where \( b \) is the observed bond length, \( 2K_i \) is the spring constant of Hooke’s law for the bonded atom pair, and \( b_0 \) is the standard bond length of the atom pair. The second summation is a superposition of any bond angle vibration onto harmonic potentials, where \( \theta, 2K_\theta, \) and \( \theta_0 \) are the observed bond angle of any linearly bonded three atoms, the angle bending spring constant, and the bond angle at equilibrium of the atom triplet, respectively. The third sum is over any torsion angle, where \( \phi, K_\phi, n, \) and \( \delta \) are a dihedral angle, the force constant of the angle, multiplicity for the hindered rotation, and the phase to specify the equilibrium rotation angles, respectively.

The above three sums are for bonded atoms, whereas the last is for any pair of two nonbonded atoms, \( i \) and \( j \), that are separated at least four (or three in some force fields) bonds, assuming the interactions between atoms within three bonds are handled accurately enough by the first three sums. The three terms in the fourth sum represent Pauli core repulsion, van der Waals dispersion (two terms collectively, Lennard-Jones interactions), and Coulombic interactions, where \( r_{ij}, q_i, q_j, \) and \( \epsilon \) are the distance between atoms \( i \) and \( j \), the effective partial charge of atom \( i \), that of atom \( j \), and a dielectric parameter (often set at or around 2 for inside proteins), respectively. \( A_{ij} \) and \( B_{ij} \) are Lennard-Jones constants, from which the minimum of the Lennard-Jones interaction between atoms \( i \) and \( j \) is given as \(-B_{ij}^6/(4A_{ij})\) at the distance of \( \sqrt[6]{2A_{ij}/B_{ij}} \).

The formulation is rather heuristic for simplicity and therefore fast computation, especially for dihedral angles that is purely an adjustment term, while the last two terms (dispersion and Coulombic interactions) are physics-based. Yet, this type of force fields with the parameters determined from quantum-mechanical calculation or experiments of smaller molecules provides decent accuracy for exploring biological phenomena except for those that take place in the environments of very high temperature or pressure.

### Computing MD Simulations

MD computation is repeated numerical integration of the classical Newton’s equation of motion. The most commonly used method is the so-called velocity Verlet algorithm.\textsuperscript{53} By differentiating \( V_i(r_i, t) \) of Eq. 1 \((r, i\), and \( t \) are added to refer to the coordinate \( r \) of any atom \( i \) in a system of interest at the current time \( t \), \( F_i(r_i, t) \), the current force on atom \( i \), can be obtained. Then using \( r_i(t), F_i(r_i, t), \) and \( F_i(r_i, t - \Delta t) \), the future position of atom \( i, r_i(t + \Delta t) \), can be obtained as follows:

\begin{equation}
v_i(t) = v_i(t - \Delta t) + \frac{\Delta t}{m_i} \left( F_i(r_i, t) - F_i(r_i, t - \Delta t) \right) \tag{Eq. 2}
\end{equation}

\begin{equation}
r_i(t + \Delta t) = r_i(t) + \Delta t v_i(t) + \frac{\Delta t^2}{2m_i} F_i(r_i, t) \tag{Eq. 3}
\end{equation}

where \( m_i \) is the mass of atom \( i \), \( \Delta t \) is a short duration, and \( v_i \) is the velocity of atom \( i \), safely assuming that \( F_i \) is constant within a very short time of \( \Delta t \). By setting the initial coordinate \( r_i \) and the initial velocity \( v_i \) (by specifying the temperature), the set of the equations (Eqs. 2 and 3) can be repeatedly updated, providing the time development of all atoms in the system. The length of \( \Delta t \), however, needs to be shorter than the cycles of the fastest motions of atoms (i.e., the hydrogen-involved bond length vibrations) for accurate integration. Therefore \( \Delta t \) is typically set at some femtoseconds, which results in millions of cycle updates of Eqs. 2 and 3 to simulate for a length of some nanoseconds.

### Running MD Software Packages

Since the potential energy function is in a pairwise form (Eq. 1), the computation time required for updating Eqs. 2 and 3 once should be proportional to the squared number of atoms in the simulation system, provided the same computer and if implemented naïvely. However, by using interaction cutoff criteria and handling long-range electrostatics with more sophisticated methods (multigrid or fast Fourier transform), the actual computation time for a unit cell (a system to simulate) with edges of several nanometers or longer is approximately proportional to the number of particles (i.e., atoms or “coarse-grained” atoms—see the next section). The typical system of coagulants and a patch of cell membrane would include over a quarter of million particles. Several tens of nanoseconds-long MD may be simulated per day for such a system by a graphics processing unit (GPU)-equipped computer, which is an acceptable computation rate for the purpose (although an even faster rate is always desirable).

One can relatively easily prepare a system to simulate\textsuperscript{54} and run MD jobs of the system by either obtaining an account at a high-performance computing center, a computing cloud, or purchasing a server equipped with multiple, fast central processing units, a few gigabytes of random-access memories, and a GPU or two. Note also that the GPU does not have to be a high-end model. A several times-inexpensive so-
called “gamers model” will perform nearly the same for MD computation.

Running an MD program for a system of interest generates the trajectory files that log the coordinates of the atoms in the system as a function of time. Visualization software such as PyMOL, UCSF Chimera, and VMD can convert the trajectories into movie files, using a wide variety of atom representations. The moving images in such files, often shown in oral presentations or available at the journal Web sites, are like slow-motion films; events within the scale of submicroseconds are presented in some tens of seconds. In such “MD films,” the fastest local vibrational motions of individual atoms, which are the majority of the whole internal degrees of freedom in case for proteins, are customarily removed (and often without mentioning so) by time-averaging the coordinates of the atoms, because such local fluctuations are usually irrelevant compared with slower, nonlocal conformation changes that can be observed clearly by cancelling local vibrations.

Models for Membranes
To accelerate the computation rate, different membrane models and associated force fields have been developed for different purposes. One obvious approach is to reduce the number of particles in a system by representing a group of atoms by a “coarse-grained” particle and employing dedicated force fields (e.g., MARTINI), sacrificing the atomic level of resolution. Furthermore, multiscale modeling and simulation methodologies combine the advantages of both atomic and coarse-grained scales, (re-)mapping a system of interest from one scale to the other.

Another dimension to explore is to represent (a part of) the membrane with smaller molecules that keep relevant characteristics, which will accelerate desired transitions in the system, sustaining the atomic resolution while having the system size basically unchanged. The highly mobile membrane-mimetic model (HMOM) is particularly suitable to simulate membrane binding of coagulants and their complex formations on the membrane. In HOMM, membrane lipids are selectively fragmented; in other words, tip parts of long acyl tails are replaced by small organic molecules of similar chemical properties. As a result, the diffusion and local fluctuations of the membrane lipids are significantly augmented, which enables membrane binding of peripheral membrane proteins within accessible computation scale by current computer processing standards.

Models for the Membrane-Binding Domains
GLA Domain
The GLA domain is one of the major membrane-binding structural motifs for coagulation factors. Since the structure of an activated FVII-TF (FVIIa-TF; “a” for activated, “-” for complex, same for other factors) complex was solved by X-ray crystallography, several models for the membrane-bound GLA domain have been proposed over the years. Due to seven linearly bound calcium ions, most of which can interact with anionic membrane lipid headgroups, the proposed models are largely in agreement on the domain’s orientation with respect to the membrane. The membrane insertion depth of GLA domains, however, remains in disagreement. The difference seems to originate from the consideration of the balance of electrostatic and hydrophobic interactions between the GLA domain and the membrane.

The first pilot model for the FVII GLA domain (tagged as “St. Paul-1999” in Table 1) was proposed as having the ω-loop and the K32 residue being located on the surface of the membrane and the line intersecting bound calcium ions being slanted to the membrane surface (Fig. 1). The model is based on the observations that bound calcium ions and GLA residues are mostly not in contact with the membrane and that the protein–membrane interactions are primarily hydrophobic. This model and none other employs a GLA domain orientation with an oblique calcium line. Later studies reported such positioning was observed during MD and it was suggested that the model might be of a binding intermediate rather than the final equilibrium orientation.

The membrane-bound form of the FX GLA domain was proposed (“Kiyose-2001” in Table 1), based on the crystal structure of the FX GLA domain bound by an anticoagulant, hundred-pacer snake’s venom. In this model, protruded hydrophobic residues at the tip of the ω-loop, namely F4, L5, and V8, penetrate the membrane and the calcium line is about level above the membrane. This arrangement is essentially identical to another model for the PT GLA domain mutant as F4W (“Boston-2001”). An equivalent model was also suggested for the bovine PT (bPT) GLA domain (“Boston-2003”), using the NMR-solved structure of the bPT GLA domain bound by a lysophosphatidylserine (lysoPS). Yet another equivalent model was proposed for the FIX GLA domain, relying upon NMR study of an octapeptide that mimicked the FiX ω-loop.

A later MD study (“Chicago-2008”), however, reported that the outer four of the bound calcium ions can interact with membrane lipid phosphatidylserine (PS) headgroups and that the membrane penetration depth of the GLA domain is approximately 1.2 nm deeper than other models while the orientations of the domains are equivalent (Fig. 1). This model was further supported by a repeatedly observed membrane-binding process, using a mobility-augmented membrane model named HOMM. It was also reported that the GLA domain positioning with a slanted calcium line on the membrane surface was often observed during MD as if we were a possible binding intermediate. The Urbana-2008 model served as the basis of a novel explanation for the membrane–GLA domain interaction as “single PS-specific interaction and multiple phosphate-specific interactions,” or in short, the “Anything But Choline” (ABC) hypothesis.

Monitoring the membrane binding of GLA domains to anionic membranes is possible by solid-state NMR (SSNMR) or XRR to compare the results with the models proposed; the bound divalent calcium ions may be problematic for SSNMR, but not for XRR. Experts’ trials are highly anticipated, because finding the actual positioning of the
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\(^a\) Tag: Indicated crystallographic structure.
\(^b\) Factors: Membrane-bound coagulation factors.
\(^c\) Methods: Techniques used for model building.
\(^d\) Coordinates: Crystallographic coordinates for membrane-bound complexes.
\(^e\) Membranes: Membrane type or lipid composition.

(Continued)
Table 1 (Continued)

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<td>c/w Urbana-2008 + Milano-2006/Seattle-1999</td>
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<tr>
<td>Cambridge-2013</td>
<td>FXa-FVa</td>
<td>X-ray, modeling</td>
<td>4BXS</td>
<td>+</td>
<td>Upright FVIIIa</td>
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<td>Albany-2002</td>
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<td>Cryo-EM</td>
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Abbreviations: The symbol “~” immediately followed by a tag indicates the model is similar to the tagged model; AFM, atomic force microscopy; b/w, between; bFVai, APC-activated FV from bovine; CG, coarse-grained; c/w, consistent with; DOPS, 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]; DVPS, 1,2-dioleyl-sn-glycero-3-[phospho-L-serine]; FCM, flow cytometry; GLA, γ-carboxyglutamic acid rich; h/p, human/porcine chimeric; i/a, interactions; lysoPS, lysophosphatidylserine; MD, molecular dynamics; 1PFX-like or 1DAN-like, the topology of the GLA-containing factors based on relative orientation of GLA and EGF1; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]; SDM, site-directed mutagenesis; var., variations in (a domain).

bRelevant models in solution are also included.

cTags to individual models in the format of “town-year,” where town is the location of the institution that hosts the main group in the reference and year indicates the year in which the reference was published. The models named by the authors themselves, if available, are shown in parenthesis.

dThe symbol “+” indicates that the coordinates of the system are available upon request; “++”, “available upon request” written in the reference. PDB IDs for FVII, FVIIa, TF, and TF pathway inhibitor (TFPI) are also listed elsewhere.145

eBlank indicates the membrane is not included in the system; +, the membrane represented as a line or sheet; ++, the membrane represented at atomic resolution.

fRenamed in Stoilova-McPhie22 and Dalm et al117; originally “X-ray,” “EM-2D” and “EM-LNT,” respectively.
GLA domain with respect to the membrane, as well as that of the catalytic triad (CT) and substrate (Table 2), reduces sterically possible arrangement of neighboring domains, and therefore leads to further modeling the whole molecules and complexes on the surface of the membrane, excluding the otherwise possible vast majority of the potential arrangement candidates.

C1/C2 Domains

C2 and C2-like domains are another major family of membrane-binding sites of coagulation factors. In this review “C2-like domains” refers only to the extracellular phospholipid-binding discoidin domains of certain coagulation factors. We note that there are other domains also referred to as “C2-like” such as lactadherin and PKCα-class C2 domains. Factor V (FV) and FVIII include homologous C1 and C2 domains that individually have membrane-binding properties and facilitate cofactor binding. Curiously, there is nontrivial interplay and cooperation between the domains in determining the precise binding kinetics and lipid component specificity for the full-length cofactors. The structures of FV and FVIII C2 domains were solved by X-ray crystallography as an eight-stranded Greek-key topology barrel with moderately long hairpins, or spikes (or “fatty feet”) at the bottom (the opposite side of N- and C-terminal ends). These spikes include hydrophobic residues toward the tips, and it is suggested that C2 domains bind to membrane surfaces facilitated by these spikes, while a few other strategically placed basic residues interact with anionic headgroups of the membrane lipids, yielding Ca\(^{2+}\)-independent stereospecific recognition toward PS lipids. Based on these considerations, and the domain surface hydrophobicity distribution, a slightly tilted β-barrel, where the domain leans toward one side on the membrane, was suggested as the membrane-bound form of the FVIII C2 domain (Martinsried-1999, Seattle-1999 in Table 1; Fig. 2). A similar form would be expected for the FV C2 domain. Alanine-scanning mutagenesis on the FV C2 domain also indicated the spikes as the membrane-binding sites. Since then, several models for membrane-bound forms of C2 (and/or C1) domains have been suggested. They are however at variance in the insertion depth and/or the orientation of the domains with respect to the membrane (Fig. 2).

Before the structures of C2 domains became available, the first models for membrane-bound FV and FVIII C2 domains were proposed, based on the results of threading their sequences through the known structure of a fungal galactose oxidase-binding domain (the barrel is upright with respect to the membrane; “La Jolla-1998” in Table 1). Later, an MD study showed that the FVa C2 domain autonomously bound neutral 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) membrane with a part of the β-barrel buried into the membrane and the axis of the barrel nearly aligned with the membrane normal similarly to La Jolla-1998 (the barrel is upright with respect to the
membrane; “Milano-2006” in 

table_1). Furthermore, it was reported that the FVa C2 domain exhibited conformational changes upon membrane binding, including significant orientational changes of the side chains of W26 and W27 residing in the first spike. This conformational change between what has been called the “open” and the “closed” forms of FV C2 domain was investigated in another study by means of free-energy calculations. It was found that while

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<tr>
<td>FVIIa-dcTF</td>
<td>PC/PS/PE (56:6:40) vesicles</td>
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<td>7.7 (0.2)</td>
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<tr>
<td>FVIIa</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>8.31 (0.33)</td>
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<tr>
<td>FVIIa-dcTF</td>
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<td>FVIIa</td>
<td>In aqua</td>
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<td>FVIIa-dcTF</td>
<td>PC/PS (4:1) vesicles</td>
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<td>7.6 (0.3)</td>
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<tr>
<td>FVIIa</td>
<td>PS bilayer patch</td>
<td>-</td>
<td>MD</td>
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<td>99</td>
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<td>FVIIa-sTF</td>
<td>PS bilayer patch</td>
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<td>8.81 (0.14)c</td>
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<td>FVIIa-dcTF</td>
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<td>MD (AMBER)</td>
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<tr>
<td>FVIIa-dcTF</td>
<td>PC/PS (4:1) bilayer patch</td>
<td>-</td>
<td>MD (NAMD)</td>
<td>7.686 (0.222)d</td>
<td>101</td>
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<tr>
<td>FVIIa</td>
<td>PC/PS (4:1) bilayer patch</td>
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<td>MD</td>
<td>7.704 (0.257)</td>
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<tr>
<td>FVIIa-TF</td>
<td>PC/PS (4:1) bilayer patch</td>
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<td>MD</td>
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<tr>
<td>FIXa–FVIIa</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-A-FPR</td>
<td>FRET</td>
<td>8.9 (0.3)</td>
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<tr>
<td>FIXa</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-A-FPR</td>
<td>FRET</td>
<td>8.9 (0.3)</td>
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<tr>
<td>FIXa–FVIIa</td>
<td>PC/PS (4:1) vesicles</td>
<td>DEGR</td>
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<td>FXa</td>
<td>PC/PS vesicles</td>
<td>DEGR</td>
<td>FRET</td>
<td>6.1 (0.2)</td>
<td>149</td>
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<tr>
<td>FXa–FVa</td>
<td>PC/PS vesicles</td>
<td>DEGR</td>
<td>FRET</td>
<td>6.9 (0.5)</td>
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<td>FXa</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-FPR</td>
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<td>8.4 (0.3)</td>
<td>148</td>
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<tr>
<td>FXa–FVa</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>7.2 (0.2)</td>
<td>150</td>
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<tr>
<td>FXa (desEGF1)</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>7.5 (0.1)</td>
<td>150</td>
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<tr>
<td>FXa–FVa (desEGF1)</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>5.6 (0.1)</td>
<td>150</td>
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<tr>
<td>FXa (S195C in SP)</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-C195</td>
<td>FRET</td>
<td>9.5 (0.6)</td>
<td>150</td>
</tr>
<tr>
<td>FX (S195C in SP)</td>
<td>PC/PS (4:1) vesicles</td>
<td>Fl-C195</td>
<td>FRET</td>
<td>9.7 (0.2)</td>
<td>150</td>
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<tr>
<td>APC</td>
<td>PC/PS (4:1) vesicles w/o PrS</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>9.4 (0.4)</td>
<td>119</td>
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<tr>
<td>APC</td>
<td>PC/PS (4:1) vesicles w/ PrS</td>
<td>Fl-FPR</td>
<td>FRET</td>
<td>8.4 (0.4)</td>
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<tr>
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<td>MT–FVa</td>
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<tr>
<td>MT</td>
<td>PC/PS (4:1) vesicles</td>
<td>DEGR</td>
<td>FRET</td>
<td>6.7 (0.3)</td>
<td>123</td>
</tr>
</tbody>
</table>

Abbreviations: APC, activated protein C; DEGR, dansyl-Glu-Gly-Arg; EGF1, epidermal growth factor-like domain 1; Fl-A-FPR, Nα-(2-mercaptoacetyl)-FPR; Fl-FPR, fluorescein-(D-Phe)-Pro-Arg; FRET, Förster resonance energy transfer; GD-FVIIa, GLA domainless FVIIa; GLA, γ-carboxyglutamic acid rich; MD, molecular dynamics; MT, meizothrombin; PC, phosphatidylincholine; PE, phosphatidylethanolamine; PrC, protein C; PrS, protein S; PS, phosphatidylserine; sTF, soluble tissue factor; TF, tissue factor.

*Recalculation of the data in McCallum et al.136

bDistance between Cα of S195 in SP and N of L5 in GLA.

cHeight of Cα so fC Tf r o mc a r boxyO' si nP Sh e a g r o u p s.

dDistance between Cα of S195 in SP and the nearest P of PC or PS.

eHeight of CT from GLA-bound Ca²⁺ ions.
rearranging W26 and W27 from the “open” to the “closed” form required the concerted motions of several spike residues, the stability of each state was comparable and only a low transition barrier (≈1.5 kcal/mol) separated the two states. A cryo-EM study of the nanotube-bound FVa C2 domain suggested a model for the membrane-bound FVa C2 domain, which is different from the above two in the insertion depth of the β-barrel (►Fig. 2). The model proposes upright, more-than-half buried β-barrels of juxtaposed C1 and C2 domains (“Coventry-2008”).

Solving the FVIII structure by X-ray crystallography and by docking it with porcine FIXa (pFIXa), the binary complex of FVIIIa and FIXa was built ("Woods Hole-2008"), in conjunction with a membrane-bound GLA domain model to position FIXa. In this model, juxtaposed C1 and C2 domains lie on the membrane surface without inserting their spikes into the membrane. A model based on FRET measurement is in accordance with that by FIXa–FVIIIa docking. The angle between the membrane surface and that spanned by the centers of the individual domains of FVIIIa ranges between 30° and 50° with the A3 domain being in contact with the membrane surface. In a recent MD study ("Måløv-2015"), multiple spontaneous binding events of FVIII to a PS HMMM membrane by the C1 and C2 domains were reported. The observed membrane binding modes of C1 and C2 domains were consistent, keeping their β-barrels close to juxtaposed and upright (or slightly tilted) toward the membrane, with more fluctuations in domain tilt for the C1 domain. Based on individual C1/C2 domain membrane orientations (and assuming negligible domain rearrangement in FVIIIa), putative models of the FIXa–FVIIIa tenase complex were proposed. From those results, it was concluded that likely the C1 (and not C2) domain is primarily responsible for directing the membrane-bound orientation of the putative FIXa–FVIIIa tenase complex, since those models where generally consistent with the requirement that the FIXa GLA domain is known to be membrane-bound as well. Deep insertion of either C1/C2 domain or tilted whole FVIIIa was not observed.

**Models for the Whole Factors and Complexes**

**TF, TF-Interacting Factors, and Their Complexes**

sTF, dCTF, and TF

Soluble TF (sTF) is the extracellular domain of TF, which can be used in most cases when building a model for coagulation complexes that include TF, because the FVIIa–sTF complex is known to be enzymatically active. Provided that sTF is an active coagulant, it can be safely assumed that the membrane-bound form of sTF should be nearly identical to that of the whole TF. An MD study reported the membrane-bound modes of free and FVIIa-bound sTF ("Urbana-2010"). In its binary complex with FVIIa, sTF leans in a way in which the tip of the N-terminal fibronectin type III domain is lowered when compared with its free form (►Fig. 3). As a result, TF–membrane lipid interaction patterns differ in the two forms.
For instance, some residues in Ser-loop of the C-terminal fibronectin type III (TFC) domain, such as K165 and K166, are significantly less interacting with membrane lipids in the FVIIa–TF complex, implying a mechanism for the preparation of the FX-binding exosite. The model for the whole TF including the transmembrane and cytoplasmic regions is required when studying crosstalk between coagulation and cancer-signaling cascades, in which the cytoplasmic domain of TF is involved. The model for des-cytoplasmic TF (dcTF; “Chapel Hill-2012”) in the binary FVIIa–dcTF complex, as well as that for the whole TF (“Kolkata-2018”), was proposed by other groups (see the next section). The mechanistic details of FVIIa–TF binary complex formation, however, have not been reported yet.

FVIIa–TF
A pure PS membrane-bound form of FVIIa was built, aligning the FVIIa part of PDB ID: 1DAN (with missing portions being modeled) onto the membrane-bound GLA domain. A membrane-bound model for the FVIIa–sTF complex on the PS membrane was also built similarly, including sTF in 1DAN (“Urbana-2010”). The membrane-bound, isolated FVIIa exhibited significant flexibility during MD simulation, fraying its serine protease (SP) domain. FVIIa in the membrane-bound FVIIa–sTF, on the other hand, was stabilized by sTF, sustaining the CT in its SP domain at a certain orientation at 8.81 ± 0.14 nm from the surface of the membrane. Other groups reported the CT height above phosphatidylycerol/PS 4:1 membrane by MD as 7.686 ± 0.222 nm for FVIIa–dcTF and 8.497 ± 0.069 nm for FVIIa–TF, respectively. These results are not in good agreement, and it is not quite clear what the origins of the differences are: the form of TF (sTF, dcTF, or TF), the lipid compositions in the membranes, or both. More specifically, do both TF form and membrane lipid composition have little influence on the membrane-binding mode of the binary complexes, or do they have offsetting effects instead? At least in these three works, the membrane-bound form of the GLA domain seems to be equivalent to one another (Table 1); if 1DAN is used as a template for the FVIIa–TF complex, assuming that TF interacts with the membrane via the tip of the TFC domain, the calcium line of the GLA domain would need to be about level near the surface of the membrane (► Table 1). The reported CT heights measured by FRET also vary, ranging from 7.5 to 8.3 nm (► Table 2). One noticeable difference between the FRET-measured heights and the MD-measured ones is that the CT height tends to be shorter for FVIIa–TF by FRET, while MD measurements show the opposite trend. The larger CT height for isolated FVIIa indicates that FVIIa is more extended linearly in the absence of TF, presumably as close as possible, with little conformational variety, which seems to imply that FVIIa does not exist as isolated molecules (but as homodimers instead, for instance) on the membrane.

FVIIa–sTF–FIX and FVIIa–sTF–FXa
The models for the TF-involved ternary complexes have not been developed very much, presumably due to the fact that the membrane-bound form of GLA domains is not quite established yet. There are only a few computational trials, and all of them primarily employed protein-docking methodologies between the FVIIa–sTF complex and FIX (“Grenoble-2002”) and FIXa (“Chapel Hill-2003” and “La Jolla-2003”). As a result, the membrane is not explicitly included in these models, and one of these works indicated the putative membrane surface by a line in a side view of
the ternary complex model they obtained. In these works, the relative orientations of the FVIIa and FXa GLA domains vary (→ Table 1, “Significance” column), which indicates the importance of establishing the actual membrane-bound mode of GLA domains. Again, trials for determining the configurations of membrane-bound GLA domains by the experts of SSNMR, XRR, etc. are immensely expected.

In all these models, it seems to be assumed that both GLA domains bind to the membrane, and that the domains are close to each other. As for the FVIIa-TF-FXa ternary complex, upon its engagement in TF-dependent cell signaling, however, it is proposed that the FXa GLA does not bind to the membrane but to endothelial cell protein C receptor, which complicates matters further.

**FV, FVIII, and Their Complexes**

In the first models for the whole cofactors FVa or FVIIa, the C2 domain binds to the membrane with C1 located on top of it, such as FVa (PDB ID: 1FV4) by modeling (→ “La Jolla-2000”) and FVIIa by cryoEM (→ “Albany-2002”). Recent experiments in various conditions are, however, in accord with the juxtaposed C1/C2 domains, while the membrane insertion depth of the domains remains unsettled (→ “Burlington-2004,” “Coventry-2008”).

Activated protein C (APC)-inactivated bovine FVa (bFVa), which is missing the A2 domain (i.e., A1 and A3-C1-C2), was crystallographically determined (PDB ID: 1SDD). In this work, A domains (A1, A2, and A3) were modeled from those of human FV and then combined with the crystal structure of bovine C1/C2 domains. The A domains, therefore, have the same topology as those of FVIIIa, while both C1 and C2 domains bind to the membrane (→ Fig. 4). Based on this FVa model and TEM studies of FVa on PS-containing lipid tubes, another model of membrane-bound FVa, in which upright, juxtaposed C1/C2 domains are half-buried into the membrane, was proposed (→ “Burlington-2004,” “Coventry-2008”).

A model for the FXa–FVa binary complex was then obtained by docking (→ “Paris-2006”), which is consistent with a site-directed mutagenesis study. Another group built a whole structure of FVa from the above-mentioned A2 domainless FVa model and homologous human ceruloplasmin (PDB ID: 1KCI), equilibrated the whole FVa molecule in solution, and then performed rigid-body docking of the equilibrated FVa and FXa (→ “Chapel Hill-2008”: → Fig. 4). Based on the docking results, they also...
suggested potential binding modes for the FXa-FVa-PT ternary complex.

Afterwards, the structure of FVIIIa was solved by X-ray crystallography (PDB ID: 3CDZ) with C1/C2 domains juxtaposed, as proposed by the homologous FVa. However, a slanted orientation of C1/C2 domains (and therefore the whole FVIIIa as well) against the membrane was proposed, considering the interaction with FXa,94 as opposed to the upright C1/C2 domains in the membrane-bound FVas. The FRET measurement supported this slanted FVIIIa as its membrane-bound model96 (“Rochester-2013”; – Fig. 2).

Unlike GLA domains that are not in contact with the remaining part of the whole molecule, C1/C2 domains are close to each other as well as to the A1 and A3 domains. It is not clear whether there is rearrangement of C1/C2 domains upon membrane binding, or whether there is significant difference in C1/C2 (re)arrangement between FVas and FVIIIa. For FVIII, however, a heterodimeric mode was suggested for FXIII binding to platelet-resembling LNTs (lipid nano tubes), in which FVIII binds to the membrane only by the C2 domain22,116,117 (“Galveston-2013”). To address this flexibility issue, additional investigations should be performed.

Other Relevant Targets

Molecules whose membrane-bound modes have not been explored to a great extent include (activated) protein C,108,118–120 protein S,119 protein Z,121,122 and meizothrombin.123 The structures of these GLA domain-containing proteins are partly available, and a few models have been proposed,121,122 often together with protein Z inhibitor.121,122,124 The CT heights of APC118–120 and meizothrombin123 have been measured by FRET. As for TF pathway inhibitor125,126 and polyphosphate,127,128 the modeling of the complexes of these molecules and other membrane-bound coagulants or cofactors is still to be performed. Protease activated receptor 2 (PAR2) is found to be proteolytically activated by the FVIIIa-TF and FVIIIa-TF-FXa complexes.129 Recently, the structures of PAR2,130 as well as of the homologous protease activated receptor 1,131 in a complex form were solved by X-ray crystallography, making computational modeling and simulations feasible.

Summary of Experimental and Computational Models

As discussed in the previous sections, experimental and computational approaches to membrane-bound forms of coagulation factors are mutually complementary and evocative. For GLA domains, the experimental71–73 and computational5,67,132 models seem to match relatively well, assuming that the bound form would be equivalent among different factors as a whole or isolated GLA domains only, due to the presumably flexible connection between the GLA and EGF1 domains.41,133 Different insertion depths of the ω-loop, however, have been suggested (– Fig. 1), and direct experimental validation is needed.

The flexible GLA–EGF1 connection makes modeling of whole GLA-containing factors, especially PrC,108 FXa,94,97,134 and Fxa,112,115,135 difficult when they form complexes (– Fig. 4). 1PFX40-based structures are used in their computational models, but it is not quite clear that the factors are really in (the neighborhood of) the 1PFX conformation. Simple MD or adaptive docking methods to seek for potentially more feasible structures are time-consuming, and any other effectual attempts have not been made either experimentally or computationally to our knowledge. As for FVIIa, there are ample experimental and computational data on the CT height from the membrane surface (– Table 2), which exhibit the opposite trends on the height change upon TF binding, hypothesizing potential modes of membrane-bound FVIIa as discussed above.

As for C1/C2 domains, the experimental models85,86,91,94,96 do not match well with regard to both the insertion depth and the tilt angle, while a few computational models suggest the upright β-barrel orientation with different insertion depths (– Fig. 2). The interactions between the domains and membrane lipids therefore do not match as well. Besides, it is not clear whether C1/C2 domains are reoriented upon membrane binding, with respect to the membrane, as well as to the whole factors, which is not explored computationally yet due to the large sizes of FV and FVIII. TF, dcTF, and sTF are assumed to hold the upright orientation in the computational models99,101,102,104 as experimental data indicate136,137 in conjunction with the 1DAN39 structure. There may be reorientation of TF upon binding to FVIIa so that TF becomes available for FX binding at its exosite (– Fig. 3), but this remains largely unclear.

For the binary and tertiary complexes, data with explicit information about the membrane is mostly lacking, because the models for the complexes require in general the model for individual factors, to which solid models for membrane-bound GLA or C1/C2 domains are prerequisite. Alternatively, the opposite approach may be taken, in which the interactions between CT and the substrate (and the vicinity of them) are first sought (computationally)14,175,135,138 as the restricting factors to possible membrane-bound forms of GLA and C1/C2 domains.

Future Directions and Concluding Remarks

Since models for the membrane-bound C2 and GLA domains were first suggested in 1998 and 1999, respectively, several membrane-bound models of individual coagulation proteins and complexes have been proposed based on experimental observation. The models provide unique insights into the spatiotemporal activation, amplification, and regulation of the enzymatic cascade governing blood coagulation that ensures a proper physiological response to tissue trauma. The central balance between hemorrhage and thrombosis is determined by the precise regulation of the clotting processes under healthy conditions. In addition, lessons learnt here have immediate consequences for several hereditary diseases (notably bleeding disorders such as hemophilia) as they facilitate the rational design of coagulation-factor-based
therapeutic strategies. This review has outlined the current state of knowledge on structural features on how exactly the activated coagulation factors interact with their primary cofactor—the membrane surface itself.

Recent development of membrane models with high-mobility lipids and the application of GPUs to MD computation enable computational approaches to provide clearer views on the membrane-bound coagulation factors with atomic details and dynamics which, in turn, illustrate the molecular basis of hemostasis. Reliable membrane-bound models will help to locate key residue interactions in or around the active sites or exosites upon complex formation on the surface of the membrane that can serve as candidates for mutagenesis and conceptual design of replacement therapies. On a fundamental level, we have seen how the theoretical models for GLA domains and those for TF-involved complexes match relatively well their experimental counterparts, and further validation by the experiments is desired. The models for FV- or FVIII-involved complexes, on the other hand, exhibit a large (but intriguing!) variation to one another, and more computational trials for different environmental conditions are expected to assist the experimental ones in fully elucidating the structural and dynamic determinants of the essential molecules that help provide proper hemostasis.

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Conflict of Interest
None declared.

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