Principles of Fibrinogen Fiber Assembly In Vitro

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Fibrinogen nanofibers hold great potential for applications in wound healing and personalized regenerative medicine due to their ability to mimic the native blood clot architecture. Although versatile strategies exist to induce fibrillogenesis of fibrinogen in vitro, little is known about the underlying mechanisms and the associated length scales. Therefore, in this manuscript the current state of research on fibrinogen fibrillogenesis in vitro is reviewed. For the first time, the manifold factors leading to the assembly of fibrinogen molecules into fibers are categorized considering three main groups: substrate interactions, denaturing and non-denaturing buffer conditions. Based on the meta-analysis in the review it is concluded that the assembly of fibrinogen is driven by several mechanisms across different length scales. In these processes, certain buffer conditions, in particular the presence of salts, play a predominant role during fibrinogen self-assembly compared to the surface chemistry of the substrate material. Yet, to tailor fibrous fibrinogen scaffolds with defined structure–function-relationships for future tissue engineering applications, it still needs to be understood which particular role each of these factors plays during fiber assembly. Therefore, the future combination of experimental and simulation studies is proposed to understand the inter-molecular interactions of fibrinogen, which induce the assembly of soluble fibrinogen into solid fibers.

1. Introduction

Engineering the next generation of biomaterials with novel functionalities for personalized regenerative medicine requires a fundamental understanding of biological principles from the molecular to the cellular and tissue level.[1] As a central player in the blood coagulation cascade, the adhesive glycoprotein fibrinogen has moved into the focus of tissue engineering to develop autologous scaffolds that promote wound healing and tissue repair.[2,3] Nanofibrous fibrinogen scaffolds in particular offer very promising strategies for regenerative medicine as they mimic the nanoarchitecture of native blood clots and contain important binding sites to support cell adhesion.[1,4] Hence, they can serve as a provisional extracellular matrix (ECM) during the initial wound healing phase and were even found to have immunomodulatory properties.[5–8]

Interestingly, it is known that mutations or structural changes in fibrinogen can induce thrombosis.[9] Fibrous fibrinogen aggregates were reported to support platelet binding, which may lead to thrombotic complications when hydrophobic polymer surfaces are used in the native tissue environment.[10] Moreover, conformational changes in fibrinogen can lead to the formation of amyloid fibers, which are associated with diseases like hypertension or renal failure.[11] Previous studies on the occurrence of Alzheimer’s disease have suggested that fibrinogen is infected by β-amyloids, which results in the oligomerization of fibrinogen.[12,13] It is known today that a large number of neurodegenerative diseases are associated with the aggregation of proteins into amyloid fibers, which are therefore referred to as protein misfolding disorders.[14–16] For many protein-based biomaterials it has been discussed that these amyloidogenic characteristics are related to

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conformational changes that occur during the manufacturing process.[25] Based on this discussion, it becomes extremely important to understand the fundamental mechanisms responsible for fibrinogen fibrilligenesis, which will facilitate the development of non-pathogenic fiber scaffolds.

Under in vitro conditions, fibrinogen also assembles into nanofibers on various surfaces [10,18,19] or in the presence of solvents and acidic pH values.[20,24] In particular, when functional nanomaterials from fibrinogen were developed in the presence of organic solvents, the formation of amyloid fibrils was discussed.[21,22] Such fiber assembly conditions resemble the denaturing effect of organic solvents and high electric fields, which evoke conformational changes during electrosprinning that can affect the biofunctionality of protein fibers.[23,24] Only recently, we reported that fibrinogen can self-assemble into dense nanofiber networks under physiological buffer conditions without introducing any amyloid transitions.[25,26] Despite the high relevance of fibrinogen-based biomaterials, the underlying principles of fibrinogen fibrilligenesis under in vitro conditions are still not understood at all.

Although many experimental and atomistic studies have already shed light on fibrinogen conformations under different in vitro conditions it still remains unclear which of these parameters predominate fiber assembly. Therefore, our review categorizes different surface and buffer characteristics that favor the formation of fibrillar fibrinogen superstructures in a cell- and enzyme-free environment. Based on this categorization we discuss possible key factors that may contribute to the fundamental mechanism of in vitro assembly of fibrinogen fibers.

2. Structure, Properties, and In Vivo Assembly of Fibrinogen

To understand the fundamental principles of fibrinogen assembly into fibers under in vitro conditions, it is important to gain insight into the native structure of fibrinogen and its role during blood coagulation. Therefore, in this chapter we will introduce the molecular structure of fibrinogen in Section 2.1 and summarize details on the charge distribution in fibrinogen in Section 2.2, followed by a brief presentation of native fibrin fiber assembly upon enzymatic cleavage in Section 2.3.

2.1. Molecular Structure of Fibrinogen

Fibrinogen is a 340 kDa glycoprotein with a covalent dimeric structure that is mainly synthesized in hepatocytes.[27] It is secreted into the blood plasma where it circulates at a concentration of 1.5–4 g L⁻¹.[18,28] The fibrinogen molecule is organized into three regions of independently folded structural units: two distal globular D regions, which are connected to a central globular E region via α-helical triple coiled coils.[27,29,30] The two identical subunits of the fibrinogen molecule are each composed of two sets of three non-identical Aα-, Bβ-, and γ-polypeptide chains.[29,31] In the central E region, the N-termini of all the six polypeptide chains are linked together via five disulfide bonds.[27,32] At the N-termini of the Aα- and Bβ-chains in the E region, the fibrinopeptides (Fp) A and B are located. With only 16 amino acids these peptides constitute less than 2% of the overall mass of the fibrinogen molecule.[27] They are polar and hydrophilic, which supports their cleavage by thrombin during blood coagulation.[28]

From the E region, the Aα-, Bβ-, and γ-chains symmetrically depart forming the two triple coiled-coils connectors, each held in register by two “disulfide rings” placed at their beginning and at their end.[27,33,34] At the end of the connectors, the C-terminal parts of the Bβ- and γ-chains independently fold within the outer D regions,[35] with only intrachain disulfide bridges being present.[27,33] Instead, from the second disulfide ring the C-terminal regions of the Aα-chains (aC regions) first reverse direction forming a fourth coiled helix,[35] and then are thought to be mainly disordered, with only a small structured domain.[36] It has also been proposed that two aC regions in a single fibrinogen molecule interact with each other and with the central E-region, forming a fourth node on top of it, and that they are “released” following Fp cleavage, actively contributing thereafter to the formation of fibrin (proto)fibrils.[37,38] However, a recent study has cast doubts to the effective role of the aC regions in basic fibrin polymerization.[39] In any case, these flexible and polar aC arms participate in crosslinking of fibrin during blood coagulation[29] and could lead to different arrangements of the fibrinogen molecule.

From hydrodynamic studies, as a function of the pH value and the ionic strength of the surrounding buffer system, fibrinogen was hypothesized to exist in different shapes (see Figure 1). At neutral pH and physiological ionic strength, a compact shape with collapsed side chains compatible with that of the human fibrinogen crystal structure[40] is predicted, where the molecules have been reported to have an “effective length” $L_e$ of 53–55 nm with the maximum extension length from cryotomography being in the order of 47 nm. On the other hand, at more basic pH and low ionic strength individual fibrinogen molecules have been reported to adapt a more extended shape predicted from the $L_e$ of 63–68 nm.[41,42]

Fibrinogen molecules dried under in vitro conditions were also characterized by electron microscopy, which revealed an approximate length of 45 nm and a diameter of 2–5 nm.[37,43] Thus, dried fibrinogen also exhibited an elongated shape, which is known to be related to its particular biological function during blood coagulation.[44] When the intermolecular interaction of fibrinogen in aqueous solution was studied with dynamic light scattering (DLS) the hydrodynamic radius $R_H$ was found to change depending on the ionic strength and pH of the buffer system.[42,44,45] Both, human and bovine fibrinogens, have $R_H$ values of 10.5–10.7 nm in close to physiological solutions.[42,46] In other solutions, for instance pure water or buffers with ionic strengths below $3 \times 10^{-5}$ M, fibrinogen molecules are reported to be quite unstable forming large aggregates, which can reach dimensions up to 100 nm.[47,48] However, aggregation of fibrinogen molecules can be reversed, for example by adding salts to increase the ionic strength to 0.15 M.[47]

2.2. Charge Distribution within the Fibrinogen Molecule

The D and E regions of fibrinogen are considered less hydrophilic than the aC regions.[39] Amino acid sequence analysis
Figure 1. Possible molecular shapes of a fibrinogen molecule in electrolyte solutions derived from hydrodynamic measurements. A) Fibrinogen can assume a compact shape under physiological conditions having an ionic strength of 0.15 m and pH 7.4 or B) an extended shape in a solution with an ionic strength of 0.01–0.1 m and pH > 9 or pH < 4. The effective length of the molecule (Lef) under these conditions was calculated using experimentally determined intrinsic viscosity values. Adapted with permission.© 2009, American Chemical Society.

revealed that the part of the αA chains emerging from the end of the coiled-coils is highly polar and hydrophilic, whereas the C-terminal residues of the αA chain, i.e., the αC regions, consist of unequally distributed polar and nonpolar amino acid residues. Therefore, the unstructured αA chain extending out from the main body (see Figure 1) contains both, hydrophobic and hydrophilic, regions, while the D, E, and αC regions have a greater hydrophobic content. However, the amino acid sequence analysis does not provide any information about surface properties on globular domains of the 3D molecule. At physiological pH, the distal D regions and the central E region of fibrinogen are negatively charged while the extending αC regions exhibit a positive charge. The fibrinopeptides A and B in the E region are short polar hydrophilic peptides. Theoretical predictions of molecular shapes of fibrinogen in electrolyte solutions (as shown in Figure 1) were used to determine the charge distribution within the fibrinogen molecule. Thus, an anisotropic charge distribution over the whole molecule was observed in the pH range of 5.8–9.5. Consequently, positive and negative charges within the fibrinogen molecule are separated by a considerable distance in this pH range.

Using the hydrodynamic radius R_H value of around 12 nm determined by DLS the number of uncompensated, electrokinetic charges N_c per individual fibrinogen molecules was calculated from electrophoretic mobility data (see Figure 2). However, the absolute values reported from the calculations in ref. should be considered with caution, since in the derivation of R_H from the diffusion coefficient, the dynamic viscosity η of the solution appears to have been substantially underestimated and seems to have a value close to that of water.

At low ionic strength of 10^{-4} m and an acidic pH 3.5, a positive charge of N_c = +26 was reported. These results nearly exclude the possibility of aggregation of fibrinogen at these particular pH conditions, if the charges are uniformly distributed over the molecule. At an ionic strength of 10^{-4} m and a basic pH of 9.5 or 9.7, fibrinogen molecules were found to have a high negative charge of N_c = -26 respectively. On the other hand, for an acidic pH of 3.5 with an ionic strength of 0.1 m a positive charge of N_c = +22 to +26 was reported. At the same time, when the ionic strength was increased to 0.15 m at an acidic pH of 3.5, the total N_c was reduced to +13. This reduced total charge indicates a screening effect due to the reduced electrostatic repulsion between individual molecules. When the pH was increased beyond the isoelectric point (IEP) of 5.8 to the physiological value of 7.4 at an ionic strength of 0.15 m the overall net charge decreased to N_c = -13 as it was confirmed by electrophoretic mobility measurements. The reported N_c varies considerably likely as a result of the different techniques used and models applied to calculate the uncompensated charges on fibrinogen as well as the fibrinogen concentration adopted in the different studies.

Overall, a clear correlation between increasing ionic strength in the solution and a change in the number of uncompensated charges N_c per fibrinogen molecule toward an equilibrium value of zero charges can be observed as indicated by the dashed lines in Figure 2. This gradual change of N_c toward zero uncompensated charges can be associated with an increased charge shielding effect of fibrinogen molecules due to an increase in salt ion concentration in the dispersant. That encompasses changes of the thickness and composition of the electrical double layer.

Sequence analysis revealed that at physiological ionic strength of 0.15 m fibrinogen is 41% polar, 26% charged, and 33% hydrophobic. When a coarse grain model of fibrinogen was used, which was based on its crystal structure, the far distal ends of the molecule were found to exhibit a more hydrophilic character than the rest of the protein and an overall net charge of -8 at pH 7.4 and a low ionic strength of 0.025 m was found. By including the αC regions in the same study a total negative charge of -12 at the same pH and low ionic strength was reported for the whole molecule. This implies an overall negative net charge of the αC region contrary to the work of Adamczyk et al., for which a positive net charge of +3e is reported at pH 7.4 for the αC-terminal domain.

For both, experimental and theoretical studies, the reported charge values vary significantly. Most of the theoretical studies are based on the 3ghg model only while Zuev et al. used an extended version of this structure. However, the different authors seem to assign their charges based on varying protocols. On the other hand, experimentally observed charge variations can be explained with varying buffer conditions and different fibrinogen sources or purity grades. To clarify the
question of the internal charge distribution within the fibrinogen molecule, a visualization of the isopotential surfaces can be helpful. With this visualization, the presence of asymmetrically distributed charged areas on the fibrinogen molecule at an ionic strength of 0.15 \( \text{m} \) was revealed\(^{[61]} \) as it is illustrated in Figure 3. Molecular dynamics (MD) studies confirmed the presence of a large negatively charged area per dimeric fibrinogen protomer, which was located on one side of the surface of the \( \text{D} \) region\(^{[61]} \) (see Figure 3).

Moreover, MD simulations showed that the concentration of fibrinogen in solution also affects the overall molecular conformation.\(^{[45]} \) Fibrinogen changes from a compact shape with \( \approx 35 \text{ nm} \) diameter in dilute solutions of \( 1 \times 10^{-6} \text{ m} \) fibrinogen to an extended shape with an approximate length of 70 nm in solutions with \( 100 \times 10^{-6} \text{ m} \) fibrinogen.\(^{[48]} \) Atomistic studies revealed that this change in molecular shape originated from a high internal molecular flexibility, which influences the overall hydrodynamic properties of the fibrinogen molecule.\(^{[45,61]} \) Due to this bending, owing to a molecular flexibility observed in MD trajectories, the effective length of full-length fibrinogen in solution was reported to be only about 35–37 nm for the intramolecular \( \text{D–D} \) domain distance,\(^{[43]} \) which is much smaller than the effective length reported in previous experimental studies.\(^{[41,54]} \)

In addition, Zuev et al. found an approximate distance of 70 nm for the \( \alpha \text{C–} \alpha \text{C} \) domain distance, showing a much higher range, since the \( \alpha \text{C} \) domains are connected via highly flexible connection regions.\(^{[45]} \) Hence, this intrinsic flexibility is assumed to be responsible for the conformational variability of fibrinogen in solution/colloidal dispersion as well as upon adsorption to different inorganic surfaces.\(^{[61]} \)

**Figure 3.** Typical bent conformation of the symmetric fibrinogen dimer. The molecular structure as published in the pdb entry 3ghg is displayed after 40 ns of MD simulations with \( \alpha, \beta, \) and \( \gamma \)-chains colored in gray, green, and blue, respectively. For one symmetrical unit, the isopotential surfaces\(^{[83,64]} \) with values of \( \pm 2 \text{ K}_\text{B}T \)\(^{[65]} \) show a non-uniform charge distribution along the molecule. Red indicates negative and blue displays positive potential values. Especially at the outer \( \text{D} \gamma \) domain, a higher negative charge density is visible as published in ref. \([61]\). Via rotation around 180°, the backside of the molecule is visible in the lower image of the fibrinogen molecule.
2.3. Fibrin Fiber Assembly during Blood Coagulation

Blood coagulation in vivo induces the formation of nanofibrous fibrin clots upon enzymatic cleavage of fibrinogen during the wound healing process.[12] The formation of an insoluble fibrin network, the final step in blood coagulation, is achieved by a cascade of reactions and changes in the molecular shape of fibrinogen (see Figure 4).[27,38,53] Initially, the enzyme thrombin cleaves off Fp A from the N-terminal portion of the Aα chain in the E region of fibrinogen, generating the so-called “desAA fibrin monomer,”[28] where the Aα chains have acquired new N-terminal sequences termed as “knobs” “a”.[28,66] These knobs bind to holes “a” present in the α subunit of the D regions of fibrin units, thus leading to “A-a” interactions.[28,35] In the classic scheme of fibrin formation, two desAA-fibrin monomers are held together by knob–hole interactions to form a double-stranded network of insoluble fibrin fibrils.[61,73] Finally, lateral aggregation of fibrin protofibrils into a 3D, multistranded network of insoluble fibrin fibrils takes place, aided by the cleavage of Fp B and the subsequent “B-b” interactions.[28,62] A somewhat alternative mechanism involving delayed double-strand formation and early branching taking place at the protofibril formation step has also been proposed recently.[70]

Interestingly, the formation of knobs “A” and the existence of holes “a,” in particular the amino acid residues therein, are necessary and already sufficient to initiate fibrin polymerization.[28,39] However, there has been evidence of both, an increase in the binding affinity between “a” holes and “A” knobs and a domain rearrangement in the D regions of fibrinogen, that favor the aggregation of protofibrils due to the “B-b” interactions.[71,72] MD simulations have provided evidence of the allosteric regulation of “a” and “b” holes in the D-region of fibrinogen, which ultimately help to create lateral non-covalent connections between neighboring fibrin molecules via dynamic association.[61,73]

Overall, the enzymatic conversion of fibrinogen to fibrin is accompanied by changes in the quaternary structure.[12] Although the secondary structure of fibrinogen remains largely unchanged during thrombin-mediated conversion to fibrin,[74] minor conformational changes from α-helices to β-sheets have previously been observed under in vitro conditions.[75] The cleavage of Fp A and Fp B causes small conformational changes, which coupled to the Fp loss could weaken the intramolecular interactions between the αC arms and the central E region within individual fibrin molecules. Consequently, these regions could become available for intermolecular interactions between neighboring fibrin monomers,[37,51] which are important in determining the structure and mechanical properties of the resulting fibrin network.[76] Nevertheless, a direct evidence of this mechanism in solution is still lacking.

Fibrin aggregation into a nanofibrous network reinforces the initial plug formed by thrombocyte aggregation, leading to hemostasis and wound closure.[77,78] The 3D plug consisting of the insoluble fibrin network and platelets is called a blood clot.[78] As a consequence of blood coagulation in response to vascular damage, this fibrin-rich nanofiber network emerges immediately following an injury and enables tissue repair in vivo.[79] This nanofibrous network, supported by platelets, serves as a provisional ECM, which ultimately undergoes fibroblast-mediated remodeling to facilitate wound healing.[79] Since fibrinogen and fibrin both have a high affinity for vascular endothelial growth factor and fibroblast growth factor, they play a critical role in the adhesion of endothelial cells and fibroblasts.[80,81] Integrins facilitate the adhesion of cells to both, fibrinogen and fibrin, via Arg-Gly-Asp (RGD) binding sites present on the α-chains so that different cell types can migrate into a blood clot to promote tissue repair and regeneration.[12,77,78]

3. Fiber Assembly of Fibrinogen In Vitro

Due to its variability of molecular conformations, fibrinogen is also able to assemble into fibers in vitro, without the enzymatic cleavage by thrombin so that no crosslinking mechanism is involved in the formation of fibrinogen fibers.
Although fiber formation of fibrinogen is very important to avoid thrombogenic reactions to fibrinogen-based biomaterials, the available literature on in vitro fiber assembly that we review here is very scattered. Nevertheless, various conditions were already found to induce intermolecular interactions of individual fibrinogen molecules in an enzyme- and cell-free in vitro environment, thus favoring the assembly into nanofibers. Different surface properties were associated with substrate-driven fiber formation of fibrinogen, which will be presented in Section 3.1. On the other hand, varying buffer conditions were reported to induce fiber assembly, which are summarized in Section 3.2.

### 3.1. Substrate-Driven Fibrillogenesis

Varying surface characteristics were found to favor fiber formation of fibrinogen in vitro. In Table 1 we have categorized these properties into the main groups of “hydrophobic surfaces” and “hydrophilic surfaces” to compare the manifold environmental conditions, during which fiber assembly was reported. When comparing different environmental conditions, it needs to be taken into account that fibrinogen concentrations reported in Table 1 refer to the starting concentrations used in the respective studies. While some samples were completely dried prior to analysis (named “in air”), others were analyzed in aqueous environment (named “in liquid”). Based on these different treatments it needs to be assumed that the protein concentration as well as the ionic strength will have increased over the total incubation time, which makes it difficult to compare particular concentrations among different studies. With all surface characteristics summarized in Table 1 different hierarchical levels of fibrous superstructures were obtained, which ranged from small protofibrils, single nanofibers and nanofiber bundles to dense nanofiber networks. These different categories are based on the fiber dimensions and morphologies reported in the respective studies. Exemplary images of these different hierarchical fiber arrangements observed by substrate-driven fibrillogenesis are shown in Figure 5.

| Surface          | Surface properties | Buffer         | Metal ions | Ionic strength (molar) | pH   | Fg concentration [µg mL⁻¹] | Temperature | In air/in liquid | Fiber morphology                          | Ref.   |
|------------------|--------------------|----------------|------------|------------------------|------|---------------------------|-------------|-----------------|--------------------------------------------|--------|
| HOPG with nanosteps | Hydrophobic         | PBS            | Na⁺, K⁺    | 0.15                   | 7.4  | 3–200                     | 37 °C       | In air          | Single nanofibers, nanofiber bundles, and nanofiber networks | [18]   |
| Carbonate        | Hydrophobic         | Na⁺            | 0.35                   | 8.4                  |      |                           |             |                 |                                            |        |
| Nanostructured polyethylene single crystal surfaces | Hydrophobic | PBS | Na⁺, K⁺ | 0.15 | 7.4 | 0.5 – 5                  | 37 °C       | In air          | Single nanofibers and nanofiber networks | [83]   |
| TOMA clay        | Hydrophobic         | TBS-EDTA       | Na⁺        | 0.15                   | 7.4  | 4 × 10¹                   | 21 °C       | In air/liquid    | Nanofiber bundles                          | [19]   |
| TOMA clay        | Hydrophobic         | PBS            | Na⁺, K⁺    | 0.15                   | 7.2  | 4 × 10¹                   | 21 °C       | In air          | Nanofiber bundles and nanofiber networks |        |
| Polystyrene      | Hydrophobic         | PBS            | Na⁺, K⁺    | 0.15                   | 7.4  | 4 × 10¹                   | RT          | In air          | Nanofiber networks                          | [10]   |
| Poly(methyl methacrylate) | Hydrophobic | PBS | Na⁺, K⁺ | 0.15 | 7.2 | 4 × 10¹                   | RT          | In air          | Nanofiber networks                          |        |
| Polybutadiene    | Hydrophobic         | PBS            | Na⁺, K⁺    | 0.15                   | 7.4  | 4 × 10¹                   | RT          | In air          | Nanofiber networks                          |        |
| Poly(methyl-4-vinyl-pyridine) as reference | Hydrophobic | PBS | Na⁺, K⁺ | 0.15 | 7.4 | 4 × 10¹                   | RT          | In air          | Nanofiber networks                          |        |
| GM-HOPG          | Hydrophilic         | Phosphate      | Na⁺        | 0.02                   | 7.2  | 2.3–23                    | RT, 65 °C and 90 °C | In air          | Protofibrils                                | [82]   |
| CM-HOPG          | Hydrophilic         | Phosphate      | Na⁺        | 0.02                   | 7.2  | 2                        | RT          | In air          | Protofibrils                                | [85]   |
| PBS              | Na⁺, K⁺, Ca²⁺      | 0.007          | 7.4 | 50–250                 | In liquid | Single nanofibers          |             |                 |                                            | [86]   |
| Au (111)         | Hydrophilic         | Tris-HCl       | Na⁺, Mg²⁺  | 0.33                   | 7.6  | 4                        | In liquid   | Single nanofiber bundles                      |        |
| Aluminum oxide membrane | Hydrophilic | PBS | Na⁺, K⁺ | 0.15 | 7.4 | 10                        | RT          | In air          | Nanofiber bundles                          | [87]   |

**Table 1.** Substrate-driven assembly of fibrinogen (Fg) into fibers. Properties of pristine substrate surfaces and associated buffer conditions, which were found to drive the assembly of fibrinogen into fibrous arrangements at different hierarchical levels.
3.1.1. Fiber Formation on Hydrophobic Surfaces

Globular proteins are well known to unfold on hydrophobic surfaces. Interestingly, the fibrillar protein fibrinogen, which contains three globular regions, also experiences unfolding on hydrophobic surfaces, such as graphite. Therefore, it is likely that hydrophobic surfaces induce conformational changes in the fibrinogen molecule that lead to fiber assembly.

The influence of the chemical surface composition on fibrinogen conformation and fiber assembly was systematically studied by Koo and co-workers using a variety of hydrophobic surfaces. In a first study, synthetic fluoromica clay with a quaternary ammonium cation, trioctylmethylammonium (TOMA) clay with a water contact angle (WCA) of 69° was used as a model hydrophobic surface to study the role of the αC region during fiber formation. Short nano fiber bundles with a height of 3.2 ± 1.2 nm were observed when 4 mg mL\(^{-1}\) fibrinogen in Tris-buffered saline (TBS) solution were incubated with TOMA surfaces for 8 min. When the adsorption time was increased to 20 min, networks with long overlapping fibers were found, which exhibited a mean height of 19.2 ± 6.9 nm. After 18 h incubation the fiber dimensions increased even further, and networks with straight fibers and an average height of 30.7 ± 18.0 nm were observed (see Figure 5D). However, at concentrations below 1 mg mL\(^{-1}\) no fibers formed on TOMA. To study the particular role of the extending αC region in fiber formation on hydrophobic substrates a fibrinogen construct lacking the intact αC region was prepared by plasmin digestion. When these des-αC fibrinogen molecules were incubated with TOMA surfaces in either TBS or Ethylenediaminetetraacetic acid (EDTA) or phosphate-buffered saline (PBS) for up to 24 h no fiber assembly was reported.

Following up on these results, the influence of different polymer surfaces on fibrinogen fibrillogenesis was studied using hydrophobic TOMA clay with a WCA of 69°. Moreover, hydrophilic mica was used as a reference substrate, which is known to have a WCA of 2.7°. On hydrophobic polymer surfaces such as polystyrene (PS), polybutadiene (PB), and poly(methyl methacrylate) with WCAs between 60° and 90° fibrinogen was found to assemble into fibers when the surfaces were incubated with 4 mg mL\(^{-1}\) fibrinogen solution in either TBS or PBS (see Figure 5C). On hydrophobic TOMA clay fibrinogen formed extended trinodular structures while hydrophilic references from polar mica clay (WCA = 46°) only yielded globular fibrinogen aggregates, but no fibers were formed. Likewise, no fibers were formed on poly(4-vinyl pyridine) (PVP), which only exhibited contact angles of 37° and 55°. When 2.5% of hydrophilic PVP were added to hydrophobic PS to achieve a WCA of 71°, fibrinogen still assembled into fibers. A higher content of PVP in PS, which reduced the WCA below 60°, however, prevented fiber formation.

Interestingly, when hydrophobic PS with a WCA of 82° was functionalized with increasing amounts of hydroxyl groups, fiber formation was suppressed completely despite the relatively high WCA. This finding indicates that changes in the surface chemistry can alter the interaction with fibrinogen in addition to the WCA, and it was suggested that hydroxyl groups might interact with the αC regions, which are rich in carboxyl and amine groups. This interaction could possibly lead to a similar molecular conformation as otherwise postulated for fibrinogen on hydrophilic surfaces (see Section 3.1.2). Later on, hydrophobic PS surfaces were also exposed to UV-ozone to reduce the contact angle to 62° by increasing the surface charges. This treatment completely suppressed fiber formation, yet without changing the surface chemistry as it was previously achieved with the introduction of hydroxyl groups.

In summary, for hydrophobic surfaces with WCAs above 60° fiber formation was observed when 4 mg mL\(^{-1}\) fibrinogen were used, and with increasing incubation time the resulting fiber dimensions increased. It is known that fibrinogen strongly adsorbs to hydrophobic surfaces via the relatively more hydrophobic E and D regions. This adsorption to hydrophobic surfaces distorts the molecule into an extended shape and releases the outer αC regions, which are less hydrophobic (see Figure 6A). Such an extended fibrinogen conformation with lateral spreading has previously been reported for a wide range of hydrophobic surfaces including graphite, TOMA clay, and modified gold. Consequently, the molecule is extended and the αC regions are available to bind to adjacent fibrinogen molecules to induce fiber assembly.

Interestingly, no fiber assembly was observed when des-αC fibrinogen interacted with hydrophobic surfaces, or when low fibrinogen concentrations of 1 mg mL\(^{-1}\) were used. Hence, an involvement of accessible αC regions in fiber formation seems plausible. On the other hand, the plasmin digestion of fibrinogen that was used to prepare des-αC fibrinogen...
may have attacked the N-terminal ends of the Bβ-chains.[100] Based on this assumption it may also be possible that potentially missing portions of the Bβ-chains play a role in fiber assembly when fibrinogen adsorbs to hydrophobic surfaces. In the future, studies with recombinant fibrinogens having intact N-terminals and constitutively deleted αC regions could also shed more light on this issue.

At the same time, conformational changes in the D region were found to occur upon fibrinogen adsorption on relatively hydrophobic but not on hydrophilic surfaces.[100] which may mimic the effect of thrombin-induced fiber assembly.[10] Moreover, fibrinogen molecules were reported to stay immobilized on some hydrophobic polymer surfaces to form fibers that were insoluble in aqueous solutions.[19] This finding suggests that fibrinogen molecules may even have been irreversibly unfolded during adsorption onto hydrophobic polymer surfaces.

A particular example of hydrophobic surfaces leading to fiber assembly of fibrinogen are the nanostructured surfaces previously introduced by Jandt and co-workers.[18,83] When fibrinogen in PBS or carbonate buffer (CB) was exposed to highly oriented pyrolytic graphite (HOPG) surfaces with 8 nm high steps, single nanofibers were formed after incubation at 37 °C for 30 min (see Figure 5B).[18] After drying with nitrogen (N2), fibrinogen preferentially adsorbed at the hydrophobic HOPG nanosteps and formed single parallel nanofibers with widths around 15 nm for low fibrinogen concentrations of 3–5 µg mL−1, while 5–10 µg mL−1 resulted in nanofiber bundles with diameters around 40 nm. With much higher concentrations of 200 µg mL−1, dense porous fibrinogen layers or here called “nanofiber networks” were observed. However, when the adsorption of fibrinogen solutions to HOPG nanosteps was monitored directly in buffer using atomic force microscopy (AFM) fibrinogen did not assemble into fibers.

In a similar study, the assembly of fibrous fibrinogen structures on nanostructured, hydrophobic polyethylene single crystals (PE-SC) was reported.[83] Hydrophobic PE-SC nanocrystals were fully covered with fibrinogen solution and stored at 37 °C for 2 h prior to rinsing with PBS or CB and drying with compressed air. Single nanofibers as well as networks of fibril-like structures were found on the hydrophobic PE-SC crystals.[83] Interestingly, the fibrous networks resembled the fibrinogen structures previously observed on HOPG nanosteps[18] although much lower protein concentrations were used. Overall, increasing protein concentrations yielded fibers with larger dimensions and a higher network density: 0.5 µg mL−1 of fibrinogen yielded single nanofibers with an average width of 30 ± 5 nm and 2 µg mL−1 resulted in a nanofiber network with nanofiber widths of 45 ± 10 nm.[83] With a concentration of 5 µg mL−1 fibrinogen sponge-like structures with interconnected fibers were observed.[83]

In conclusion, fiber formation on hydrophobic PE-SC nanocrystals and HOPG nanosteps was observed with much lower fibrinogen concentrations than on smooth hydrophobic polymer or TOMA surfaces. For these hydrophobic nanostructures, fiber assembly was explained by the preferential orientation of fibrinogen molecules along the crystallographic PE-SC directions or the parallel nanosteps, which favor intermolecular interactions and fiber assembly at the surface-buffer interface.[18,83] This molecular orientation may originate from the intrinsic flexibility of fibrinogen,[61] which encourages surface mobility on nanostructured surfaces.[102] For the non-polar, hydrophobic PE-SC nanocrystals it was also assumed that fibrinogen adsorbs via its nonpolar D regions and the central E region, like it was suggested for varying smooth hydrophobic surfaces. Consequently, the polar αC-regions may also be involved in fiber assembly on PE-SC surfaces[83] and it may be concluded that the particular nanotopographies were responsible for the fiber formation rather than the hydrophobic polymer itself. More experiments with similar nanotopographies from other materials will be required to fully understand their involvement in fibrinogen fibrillogenesis.

When comparing all studies of fibrinogen fibrillogenesis on the hydrophobic substrates in Table 1, it becomes clear that one important aspect also needs to be considered: the drying of fibrinogen samples. Jandt and co-workers even explicitly mentioned the influence of sample drying in their work on HOPG nanosteps where they did not observe any fiber formation during AFM monitoring in liquid.[18] When fibrinogen solutions are dried during incubation, an increase in protein concentration and ionic strength takes place, which may actually be a major cause for fiber formation. However, this aspect has not been explicitly addressed among the studies available to date. Likewise, information on incubation times of fibrinogen with different surfaces has not been reported throughout all studies, either. Therefore, it is not yet possible to conclude whether fibrinogen fibers have already formed in solution or only after drying.

3.1.2. Fiber Formation on Hydrophilic Surfaces

In contrast to hydrophobic surfaces, for hydrophilic materials it has been suggested that fibrinogen adsorption can be caused by
electrostatic attraction, hydrogen bonding, coordinative interaction, or hydrophilic interactions. Yet, many of the studies focusing on hydrophobic substrates did not observe fibrillogenesis of fibrinogen on hydrophilic reference surfaces although fibrinogen was found to adsorb to them. In contrast to these studies fibrinogen was later found to assemble into fibers on a variety of hydrophilic materials ranging from modified graphite, gold, mica, and aluminum oxide to hydrophilic and silanized glass.

Klinov and co-workers reported the formation of fibrillar fibroinogen structures on HOPG surfaces with oligoglycine-hydrocarbon graphite modifier (GM-HOPG, WCA = 59°). Fibrinogen was exposed to the GM-HOPG surfaces for 1 s at low concentrations of 2–23 μg mL\(^{-1}\), before the surfaces were incubated in water or PBS and subsequently dried with \(N_2\). AFM analysis revealed that fibrinogen on GM-HOPG underwent a transition from tri-nodular, native-like molecules into fibrillar structures (i.e., protofibrils, see Figure 5A). This transition was promoted when the incubation time was increased to 35 min. Moreover, by increasing the incubation temperature from room temperature (RT) to 65 or 90 °C, pre-denatured fibrinogen molecules were more likely to assemble into protofibrils. For AFM analysis in wet environment GM-HOPG was placed in PBS with additional CaCl\(_2\) at RT before fibrinogen solutions between 50 and 230 μg mL\(^{-1}\) were added. These in situ studies also revealed the formation of protofibrils on GM-HOPG in aqueous environment, which is in contrast to previous studies in aqueous environment. For fibrinogen on GM-HOPG it was concluded that protofibril formation was driven by the protein-surface interaction under both, dry and wet, conditions although no control experiment was performed where fibrinogen was dried in the presence of CaCl\(_2\)-containing PBS.

Hydrophilic gold surfaces in (111) orientation were found to induce fibrinogen assembly into protofibrils in wet environment when 4 μg mL\(^{-1}\) fibrinogen in Tris buffer were incubated on Au (111) for 50 min at pH 7.6. It has been postulated that the interaction of gold with sulfur atoms in the cysteines of the fibrinogen molecules leads to stable Au–S bonds that break the intramolecular disulfide bonds in the E domain, releasing the outer αC regions to interact with other fibrinogen molecules to assemble into nanofibers.

In contact with hydrophilic aluminum oxide nanopores, which are known to have a WCA of 25 to 30°, we recently observed fibrinogen to assemble into bundles of aligned nanofibers when a solution of 10 μg mL\(^{-1}\) of fibrinogen in PBS was extruded through the pores. Scanning electron microscopy (SEM) analysis of extruded and dried bundles, which had formed in the shear flow of the protein solution through the nanopores, revealed that individual nanofibers exhibited diameters around 34 ± 3 nm. When the same process was applied to other proteins it was observed that the fiber dimensions depended on the protein concentration and the nanopore diameter. For extruded fibronectin nanofibers conformational changes were observed, which also correlated with pore size and protein concentration. Hence, it can be assumed that extrusion of fibrinogen nanofibers was actually driven by shear forces in the confined alumina nanopores rather than the surface properties.

Overall, when fibrinogen assembled into fibers on hydrophilic surfaces much lower fibrinogen concentrations were present than on smooth hydrophobic surfaces and the protein concentrations were more in the range of previous studies on fibrinogen fiber assembly on hydrophobic nanostructures. In general, the adsorption energy of hydrophobic surfaces is higher (more negative) than for hydrophilic surfaces, which facilitates irreversible adsorption of fibrinogen to hydrophobic surfaces as compared to hydrophilic surfaces. However, on hydrophilic surfaces the αC regions have a higher affinity to directly adsorb to the surface than on hydrophobic surfaces (see Figure 6B). Hence, on hydrophilic surfaces the αC regions are less likely able to recruit other fibrinogen molecules, thus inhibiting the assembly of individual molecules into fibers.

Nevertheless, many studies reported fibrillogenesis of fibrinogen on hydrophilic surfaces as we have summarized in Table I. In these works, it is very unlikely that the adsorption of more hydrophobic fibrinogen regions has occurred on the hydrophilic surfaces. Experimental studies on hydrophilic surfaces mainly reported two different conformations of fibrinogen molecules upon adsorption. A globular shape has been identified together with a more compact, bent conformation, where the αC regions are located between the D and E regions and the respective surface. This conformation is referred to as the “αC-hidden” conformation (see Figure 6B). Under these conditions, no intramolecular αC-interactions between neighboring fibrinogen molecules are possible and an absence of molecular aggregation or fiber formation has been reported. On the other hand, fibrinogen molecules have been described to adapt a “flat-on” conformation on hydrophilic surfaces with visible αC regions, which is known as “αC-exposed” conformation (see Figure 6B).

Regardless of the αC-hidden or the αC-exposed conformation of fibrinogen, its binding to hydrophilic surfaces has been reported to occur mainly through electrostatic interactions between negatively charged surfaces and positively charged αC regions at a lower pH range of 3.5–7.4. Under these low pH conditions, fibrinogen molecules can adsorb irreversibly to negatively charged surfaces because of a large (negative) binding energy. The αC-hidden conformation of fibrinogen on hydrophilic surfaces seems to prevail at physiological pH of 7.4, however this quickly changes to the “αC-exposed” conformation on hydrophilic surfaces when the pH is changed to 3.5 and back to 7.4. A similar molecular reorientation has been observed on hydrophilic polyurethane, where fibrinogen first adsorbed loosely via its αC regions, followed by more favorable interactions via the D regions, which successively left the αC regions accessible for intermolecular interactions with surrounding fibrinogen molecules.

Although many studies have mentioned αC-αC interactions as a possible factor for fibrillogenesis of fibrinogen on hydrophilic surfaces, it is still not clear whether this is the major cause for in vitro fiber formation. In particular the role of the αC extensions was recently questioned in fibrin formation.

Although Litvinov and co-workers recently provided direct evidence for specific interactions of αC regions with the central E region and other αC regions, these interactions were reported to be rather weak. Since the conformation of the αC regions within individual fibrinogen molecules is critically
dependent on pH, the properties of the buffer system will also be extremely important in understanding whether fibrinogen assembles into fibers on hydrophilic surfaces.

3.2. Buffer-Induced Fibrillogenesis

MD simulations of fibrinogen have previously underlined the interdependence of the surface properties and the molecular orientation and conformation of adsorbed fibrinogen molecules,[59,61,108] which consequently influences whether molecular aggregation into fibrillar structures under in vitro conditions will take place or is prevented.[10,19,86] Besides surface properties, buffer conditions like pH and ionic strength are known to influence the molecular orientation and conformation of fibrinogen. Although hydrophobic surfaces have previously been mentioned as the main driving force for fibrillogenesis of fibrinogen, a lot of studies also reported fiber assembly on hydrophilic surfaces.[20–22,25,26,86] In these works the influence of different buffer conditions, such as pH, ionic strength, protein concentration, and the presence of metal ions, on the conformation and orientation of adsorbed fibrinogen molecules was studied, although these parameters were not considered as the main driving force for fibrillogenesis.[54,95,109]

Only few studies have explicitly considered varying buffer conditions as being responsible for the formation of fibrinogen fibers when no enzymes or cells are present. Like for substrate-driven fibrillogenesis, they reported the assembly of fibrous superstructures with different hierarchical levels that ranged from protofibrils to single nanofibers and nanofiber bundles as well as to dense nanofiber networks that even resemble the morphology of native fibrin clots.[20,21,25,26,48,110] Exemplary images of the different hierarchical fiber assemblies observed by buffer-induced fibrillogenesis are shown in Figure 7. In Table 2 we have categorized the different buffer conditions leading to fiber assembly into the groups of denaturing and non-denaturing buffer conditions. Again, the protein concentrations and ionic strengths refer to initial values used in the respective studies, which often lack detailed information on the incubation time. Nevertheless, an increase in fibrinogen and salt concentration over time needs to be assumed for all studies on buffer-induced fiber assembly upon drying.

3.2.1. Denaturing Buffer Conditions

Organic Solvents: Wei and co-workers observed the formation of nanofibers on hydrophilic mica surfaces when fibrinogen was mixed with ethanol (EtOH) at varying ratios and incubated at 37 °C for 1 h.[21] AFM analysis in the dried state revealed thin, straight nanofibers with 4–5 µm length at 5 µg mL⁻¹ fibrinogen concentration. Interestingly, shorter fibers of ~ 2 µm length were obtained with higher concentrations of 20 µg mL⁻¹ fibrinogen.[21] At 50 µg mL⁻¹ branched nanofiber bundles were described whereas no fibers were formed with 200 µg mL⁻¹. At this concentration only spherical aggregates were observed on the mica surfaces.[21] Variation of the ethanol content in a 20 µg mL⁻¹ fibrinogen solution revealed that thin protofibrils formed with a minimum of 33% of ethanol.[22] The assembled fibers increased in thickness and length and became more branched when the ethanol content was increased to 80%.[21] The same denaturing condition was later used to assemble fibrinogen nanofibers on silanized silicon wafers.[22] In this study branched fibrinogen fibers were found to promote the nucleation and growth of hydroxyapatite crystals[22] while they were previously used to aggregate gold nanoparticles along the fibers.[21] Both studies claimed the preparation of amyloid-like fibers by ethanol denaturation since the fibers were found to be insoluble. Nevertheless, no detection of amyloid-specific characteristics by thioflavin T (ThT) staining[112] or structural analysis by X-ray diffraction[113] was presented to support these findings.

Organic solvents like dimethylformamide are also a major component of the well-established technique of electrospinning, which is commonly used to fabricate nanofibers from a variety of different (bio-)polymers.[114–116] In 2003 electrospinning has been introduced for the first time to prepare nanofibrous mats from fibrinogen dissolved in a 9:1 mixture of 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and minimum essential medium (MEM).[117] However, HFP was found to increase the α-helical content of fibrinogen by ≈ 25% as compared to fibrinogen in physiological PBS solution.[118] Since such conformational changes are often associated with protein denaturation and can lead to toxic reactions, a mixture of formic and acetic acid was recently introduced as an alternative solvent for electrospinning of fibrinogen.[119] Even though organic solvents in combination with the high electric fields can induce denaturation during
electrospinning, many studies demonstrated that electrospun fibrinogen nanofibers have great potential for tissue engineering. Good biocompatibility of electrospun fibrinogen nanofibers have great potential for tissue engineering. [3,120] Likewise, Barinov and co-workers reported thermal denaturation of fibrinogen aggregate into thicker bundles as it was previously observed without the presence of metal ions. [20] At high ionic strength with fibrinogen concentrations of 50 and 200 μg mL⁻¹ fibrillar networks were formed, which were less compact than without metal ions. [20]

Recently, an acidic pH of 3.6 in combination with incubation at 37 °C for 1 h was found to induce gelation of highly concentrated fibrinogen solutions of 40 mg mL⁻¹. Analysis by transmission electron microscopy (TEM) revealed very thin, protofibril-like structures on the surface of acidic fibrinogen gels. The gels were found to be stable upon neutralization or increase in ionic strength and temperature. Likewise, Barinov and co-workers reported thermal denaturation of fibrinogen on GM-HOPG substrates upon heating to 65 or 90 °C, which resulted in the formation of fibrillar or globular aggregates. Only recently, the same group observed unfolding of fibrinogen molecules and aggregation into protofibrils on GM-HOPG substrates in the presence of the enzyme myeloperoxidase.

Table 2. Buffer-driven assembly of fibrinogen (Fg) into fibers. Surface properties and associated buffer conditions, which were found to drive the assembly of fibrinogen into fibrous arrangements at different hierarchical levels.

| Surface | Surface properties | Buffer | Metal ions | Ionic strength (molar) | pH  | Fg concentration (μg mL⁻¹) | Temperature | In air/in liquid | Fiber morphology | Ref. |
|---------|--------------------|--------|------------|------------------------|-----|---------------------------|-------------|-----------------|-----------------|-----|
| Mica    | Hydrophilic        | 32–76% EtOH | –          | –                      | 5–50 | 37 °C                     | In air      | Single nanofibers, nanofiber bundles, and nanofiber networks | [21] |
| APTES-Graphene oxide | Hydrophilic | 76% EtOH | –          | –                      | 20  | 37 °C                     | In air      | Nanofiber bundles | [22] |
| Mica    | Hydrophilic        | H₂O/HCl | Na⁺, K⁺    | 0.17                   | 2   | 1–200                     | 37 °C       | In air          | Single nanofibers, nanofiber bundles, and nanofiber networks | [20] |
| Carbon coated TEM grid | Hydrophobic | H₂O/HCl | –          | –                      | 3.6 | 4                         | 37 °C       | In air          | Protofibrils     | [110] |
| HOPG    | Hydrophilic        | H₂O/NaCl | –/Na⁺      | 0–0.17                 | 5.5 | 1–150                     | 21 °C       | In air          | Nanofiber networks | [111] |
| Glass   | Hydrophilic        | PBS    | Na⁺, K⁺    | 0.07–0.75              | 7–9 | 2–5×10⁻¹                   | RT          | In air          | Nanofiber networks | [26] |
| APTES-coated glass | Hydrophilic | PBS | Na⁺, K⁺ | 0.41 | 7.4 | 5×10⁻¹ | RT | In air | Nanofiber networks | [26] |
| Gold    | Hydrophilic        | PBS    | Na⁺, K⁺    | 0.37–0.42              | 7–9 | 2–5×10⁻¹                   | RT          | In air          | Nanofiber networks | [25] |
| Polystyrene | Hydrophilic | PBS | Na⁺, K⁺ | 0.01–0.15 | 0.38 | 7.4 | 5×10⁻¹ | RT | In air | Nanofiber networks | [58] |
| Quartz glass | Hydrophilic | PBS | Na⁺, K⁺ | 0.07 | 7.4 | 5×10⁻¹ | RT | In air | Nanofiber networks | [58] |

Acidic pH Conditions, Heating, and Protein Binding: Wei and co-workers also observed fiber assembly of fibrinogen on hydrophilic mica under acidic pH conditions. At neutral pH of 7.0 and a temperature of 37 °C varying fibrinogen concentrations were incubated with mica for 1 h, followed by air drying for 24 h. At low concentrations of 1 and 5 μg mL⁻¹ molecular fibrinogen was found to adsorb to the mica surfaces while aggregates were formed when the concentration was increased to 50 and 200 μg mL⁻¹, respectively. As soon as the pH was lowered to 2.0 the 1 and 5 μg mL⁻¹ fibrinogen solutions yielded single nanofibers and nanofiber bundles with several micrometers in length (see Figure 7A,B). Higher concentrations of 50 and 200 μg mL⁻¹ resulted in the formation of uniform nanofiber networks. Interestingly, when the ionic strength in the acidic solution was increased to 0.17 m by adding 10×10⁻³ m phosphate and 150×10⁻³ m NaCl the assembled fibers differed in morphology. With 1 and 5 μg mL⁻¹ fibrinogen, long thin nanofibers and branched bundles were observed, which did not aggregate into thicker bundles as it was previously observed without the presence of metal ions. At high ionic strength with fibrinogen concentrations of 50 and 200 μg mL⁻¹ fibrillar networks were formed, which were less compact than without metal ions.
in deionized water with a pH of 5.5. On the other hand, SEM analysis of dried fibrinogen-myeloperoxidase samples on HOPG revealed dense nanofiber networks, which resembled the dimensions and architecture of fibrinogen nanofibers prepared by our process of salt-induced self-assembly (see Section 3.2.2) although concentrations below 150 µg mL\(^{-1}\) were used in this study. Barinov et al. suggested that the denaturation upon protein binding is driven by electrostatic interactions since myeloperoxidase is highly positively charged while fibrinogen is neutral at pH 5.5. Interestingly, when 171 × 10\(^{-3}\) m NaCl were added to the aqueous solution, absorbance spectrophotometry revealed a decline in fibrinogen aggregation. This reduced aggregation was interpreted by an increased Debye screening of charges in the electrolyte, which successively reduced the electrostatic attraction between fibrinogen and myeloperoxidase.

Overall, denaturing buffer conditions mainly induced fibrillogenesis for fibrinogen concentrations in the lower µg mL\(^{-1}\) range whereas higher concentrations did not yield any fibers. When comparing the different buffers used during substrate- and buffer-driven fiber assembly in Tables 1 and 2 it can be seen that acidic pH values of 2 and 3.6 triggered fibrillogenesis of fibrinogen on both, hydrophobic and hydrophilic, surfaces. In the acidic pH regime, the fibrinogen molecule has a positive \(N_c\) (see Figure 2), which results in an extended conformation and thus promotes molecular aggregation. Although it was postulated that fibrinogen fibers, which assembled at pH 2, exhibit amyloid-like properties, no further analysis, for instance with a ThT staining, was presented to support this hypothesis. Nevertheless, it can be assumed that fiber formation at these low pH values is accompanied by fibrinogen denaturation, as it is known to occur for many other proteins.

With organic solvents, high temperatures or the presence of other enzymes than thrombin as alternative denaturing buffer conditions, fibrinogen fibers were also observed on hydrophilic substrates such as mica, (3-Aminopropyl)triethoxysilane (APTES)-coated graphene oxide, or GM-HOPG. Like acidic pH values, these buffer conditions have a tendency to completely unfold the fibrinogen molecule, thus inducing irreversible conformational changes. From other proteins it is already known that such denaturing factors lead to the formation of insoluble fibers. Since insoluble fibers are often associated with amyloid fibers it is very likely that denaturing buffer conditions can lead to pathogenic amyloid transitions, as postulated by Wei and co-workers.

3.2.2. Non-denaturing Buffer Conditions

In contrast to the works, which reported denaturing acidic pH to induce nanofiber assembly, we recently showed that fibrillogenesis of fibrinogen is also induced at neutral and basic pH conditions in the absence of thrombin. For hydrophilic substrates such as glass, quartz glass, gold, and glass with APTES modification as well as for hydrophobic PS we observed the formation of dense nanofiber networks in a pH range of 7–9 when metal ions were present during drying. SEM analysis revealed nanofibers with diameters between 100 and 300 nm, which were assembled into dense, porous networks and thus resembled the nanoarchitecture of native fibrin clots (see Figure 7C). However, planar fibrinogen films with a very smooth surface topography were formed in the same pH range when NH\(_4\)HCO\(_3\) buffer without any metal ions was used to dry fibrinogen at RT (see Figure 7C inset). Moreover, no fibers were observed on glass or gold at slightly acidic pH values of 5 or 6. We found that a minimum fibrinogen concentration of 2 mg mL\(^{-1}\) was required to induce fibrillogenesis and fiber networks became more dense when the concentration was raised to 5 mg mL\(^{-1}\).

Apart from PBS other salt solutions containing bare sodium or potassium phosphate as well as NaCl and KCl also induced fiber formation upon salt-induced self-assembly. Interestingly, the ionic strength, which was associated with fiber formation, differed widely for the respective salts. With 5 mg mL\(^{-1}\) fibrinogen and pH 7.4, a minimum ionic strength of 0.07 m in PBS buffer induced fibrillogenesis, whereas a minimum ionic strength of 0.01 and 0.05 m, respectively, was required in bare sodium and potassium phosphate buffer to form fibers. However, in NaCl and KCl buffer, with 5 mg mL\(^{-1}\) fibrinogen at pH 7.4, a minimum ionic strength of 0.38 m was required to induce fiber assembly upon drying. Moreover, increasing ionic strength was found to yield higher fiber densities and coverage on hydrophilic glass substrates while the diameter of individual fibers remained unchanged.

To elucidate the role of secondary structure changes in the presence of salt buffers during drying we combined Fourier transform infrared and circular dichroism spectroscopy with morphological SEM analysis. These studies revealed that salt-induced self-assembly of fibrinogen nanofibers was accompanied by partial transitions from \(\alpha\)-helices to \(\beta\)-sheets in the protein conformation. We could correlate these conformational trends with a morphological transition from planar to nanofibrous fibrinogen scaffolds, which were both found to depend on protein concentration and pH. Nevertheless, towards future applications in tissue engineering we could show with a ThT staining that the observed conformational changes were not accompanied by any pathogenic amyloid formation, which was supported by the observation that self-assembled fibrinogen networks dissolved again in aqueous environment. Hence, a crosslinking step in formaldehyde vapor was introduced to stabilize the fibrinogen nanofibers in aqueous buffers (see Figure 7D), which did not affect the resulting secondary structure.

Interestingly, most studies that observed surface-driven fibrillogenesis of fibrinogen on hydrophobic and hydrophilic surfaces also used neutral to slightly basic pH without explicitly discussing the contribution of this parameter to fibrillogenesis (see Table 1). In this pH range fibrinogen has an overall negative \(N_c\) (see Figure 2) and is present in a compact shape, which in itself does not favor the aggregation into fibers. Accordingly, we showed that fibrinogen did not assemble into fibers at neutral to basic pH when no metal ions were present in the buffer. However, we reported fiber formation when the initial ionic strength in buffers of neutral pH was increased to a range of 0.01 to 0.85 m followed by a drying step. Moreover, our studies required a drying step to assemble fibrinogen nanofibers, which yielded a further increase in protein concentration and ionic strength. This observation underlines the
importance of increasing fibrinogen concentration and ionic strength for fiber assembly, as previously suggested by Jandt and co-workers. In another study, which focused on acidic buffer conditions, the Jandt group hypothesized that the presence of positively charged metal ions, such as K⁺ and Na⁺, can mediate the self-assembly of fibrinogen molecules into fibers.\(^{[20]}\)

The particular influence of the ionic strength on fibrinogen aggregation in solution has been studied in more detail by Hämisch et al., who found fibrinogen to be very stable at physiological ionic strength while aggregation increased at lower ionic strength.\(^{[48]}\) They correlated the reduced aggregation probability of fibrinogen at high ionic strength with an increase in electrostatic screening that is mediated by the metal ions.\(^{[48]}\) This effect is also clearly visible in Figure 2 as a reduction of the total number of uncompensated charges \(N_c\) (indicated by dashed lines). Interestingly, on hydrophilic surfaces fibrinogen assembled into protofibrils at 0.007 M ionic strength\(^{[85]}\) or into single nanofibers at 0.33 M ionic strength\(^{[86]}\) when Ca\(^{2+}\) or Mg\(^{2+}\) ions were present in solution and no drying was involved. When Ca\(^{2+}\) and Zn\(^{2+}\) ions were present during fibrinogen adsorption to hydrophilic glass surfaces these divalent ions favored the formation of nodular rod-like fibrinogen assemblies while monovalent K⁺ or Na⁺ ions yielded branched aggregates.\(^{[134]}\) Although we could already show that our novel self-assembly method to prepare fibrinogen fibers provides good control of several relevant parameters governing the fibrinogen assembly,\(^{[25,26]}\) more fundamental insight into parameters like ion charge and size, pH or fibrinogen concentration is required to understand the in vitro mechanisms that lead to fiber formation of fibrinogen.

4. Conclusion and Future Perspectives

Based on our findings in Tables 1 and 2 we have categorized the manifold factors leading to fibrillogenesis of fibrinogen in vitro into different groups and summarized them graphically in Figure 8. The categorization we introduced in our review shows that there are many experimental conditions and parameters that trigger the self-assembly of fibrinogen, which can either

Figure 8. Schematic representation of in vitro conditions that favor fiber assembly of fibrinogen. Denaturing buffer conditions (red field) induce irreversible structural changes in the fibrinogen molecule leading to complete unfolding and denaturation, often including the central coiled-coil structures, which favors the assembly of insoluble fibrinogen fibers. Substrate interactions (yellow field) together with non-denaturing buffers (blue field) are mostly associated with a reversible extension of the fibrinogen molecule and mainly lead to the formation of soluble fibrinogen fibers.
maintain the native conformation of fibrinogen or denature the molecule. Accordingly, we have divided the main process-related driving forces for in vitro fibrillogenesis of fibrinogen into three major categories:

- denaturing buffers, leading to complete unfolding and irreversible changes in the conformation of fibrinogen molecules, which often result in the formation of insoluble fibrinogen fibers,
- substrate interactions comprising hydrophobic and hydrophilic surfaces, both interacting with fibrinogen molecules to induce fiber formation while maintaining a more native conformation,
- non-denaturing buffers, which favor the self-assembly of fibrinogen molecules into soluble fibrinogen fibers without inducing irreversible conformational changes.

Nevertheless, since all studies we discussed in this review were conducted involving different surfaces, it cannot be concluded unambiguously whether the mentioned buffer conditions are the sole driving force of fibrinogen fiber assembly. A detailed comparison of Tables 1 and 2 and in particular of our recent studies\cite{25,26} strengthens the hypothesis that monovalent metal ions play a major role when fibrinogen fibers assemble upon drying. Nevertheless, it will be important to also investigate the particular role of divalent ions during drying of fibrinogen solutions at varying pH values in comparison to the influence of monovalent ions. These studies will provide fundamental insight into the respective role of ion charge and size to understand possible steric constraints during the assembly of fibrinogen fibers. For future studies on fibrinogen fibrillogenesis, it will therefore be important to understand whether the presence of metal ions plays a predominant role compared to different surface properties, which will also require more extensive control experiments.

Based on our meta-analysis of the multiple factors that induce fibrillogenesis of fibrinogen under in vitro conditions, it becomes evident that a more thorough understanding of the underlying mechanisms is needed to develop fibrous, fibrinogen-based biomaterials with tailored morphology, biological activity, and defined degradation profiles. These characteristics will be important to use fibrinogen nanofibers, for instance, as novel wound dressing materials, co-culture systems, for blood vessel replacement or to support stem cell differentiation. Although the available literature on fibrinogen fiber assembly is very scattered, we could identify increased fibrinogen concentration and ionic strength as key parameters to favor fiber assembly. On the other hand, it remains elusive whether specific surface characteristics play a major role in fiber assembly since fibers were formed on both, hydrophilic and hydrophobic surfaces. Hence, in future studies, it will be important to understand whether individual parameters trigger the assembly of soluble fibrinogen into solid fibers or whether several conditions might actually be interrelated and jointly responsible for the fibrillogenesis of fibrinogen in vitro.

To understand the respective influence of individual in vitro conditions on fibrillogenesis in detail, further experimental studies on the intermolecular interaction of fibrinogen are required. These studies would benefit from recombinant constructs that lack specific regions of the fibrinogen molecule to study their particular involvement in fibrillogenesis. Moreover, MD simulations will offer a powerful addition to these experimental studies to unravel the atomistic principles of fibrinogen assembly into fibers. Understanding the fundamental principles of these multiscale mechanisms during fibrinogen fibrillogenesis will be crucial to design functional tissue engineering scaffolds and biomaterial surfaces for personalized medicine that do not evoke any pathogenic or thrombogenic reactions.

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Conflict of Interest
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