Journal of Non-Newtonian Fluid Mechanics 166 (2011) 932–938

Contents lists available at ScienceDirect

Journal of Non-Newtonian Fluid Mechanics

journal homepage: http://www.elsevier.com/locate/jnnfm

Rheometrical and molecular dynamics simulation studies of incipient clot formation in fibrin-thrombin gels: An activation limited aggregation approach

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ARTICLE INFO

Article history:
Available online 27 May 2011

Keywords:
Fractal clusters
Fibrin gels
Molecular dynamics
Rheometry

ABSTRACT

A rheometrical investigation of incipient clots formed in fibrin-thrombin gels is reported in which the Gel Point (GP) is characterised by frequency independence of the loss tangent in small amplitude oscillatory shear measurements over a wide range of thrombin concentration. Values of the fractal dimension (D_f) of the GP network calculated from measurements are consistent with those reported in simulations of diffusion limited cluster–cluster aggregation (DLCCA) and reaction limited cluster–cluster aggregation (RLCCA), but differ insofar as the values of D_f calculated from the present experiments increase progressively with a reduction in gel formation time. A molecular dynamics simulation (MDS) of systems of rod-like particles was designed to (i) test the hypothesis that the presence of an activation profile in a cluster aggregation model could account for the trend of D_f as a function of gel formation time observed experimentally in fibrin-thrombin gels and whole heparinised blood without recourse to the inclusion of fibrinogen-specific interactions; and (ii) to explore the effect of monomer activation kinetics on the microstructure of fractal clusters formed in systems of rigid rod-like particles. The results identify two possible mechanisms for the increase in D_f as the gel formation time decreases, both being a consequence of altering the evolution of the clustering dynamics by a process referred to herein as activation limited aggregation (ALA). This ALA-based MDS substantiates the experimental findings by confirming the trend evident in the formation of incipient clots in fibrin-thrombin gels and in whole heparinised blood. A mechanism for ALA involving the aggregation of pre-GP sub-clusters is proposed.

1. Introduction

The viscoelastic properties of blood clots are among the most sensitive measures of differences in coagulation and clot structure and can provide information relating to coagulation kinetics, clot retraction, and fibrinolysis [1,2]. A blood clot's primary microstructure consists of a network of entangled, branching fibrin fibers. Thinner fibers are associated with an increased number of network branch points, with less permeable clots having a known association with thromboembolic disease [3–6]. More permeable networks are formed from thicker fibers, the latter displaying a reduced number of branch points [7–10]. Clots with altered fibrin microstructure exhibit different susceptibility to fibrinolysis [8,10,11], clot permeability being the rate limiting factor for the activity of the fibrin network degradation enzyme plasmin. The effect of anticoagulants such as heparin in the therapeutic manipulation of fibrin clot microstructure by thrombin inhibition increases clot permeability/porosity and produces clots with thicker fibres [12,13].

The mechanical properties of clots are altered in coronary artery disease and venous thrombosis, raising the possibility that viscoelastic measurements might be used for screening, diagnosis, and monitoring therapy. Recent oscillatory shear measurements of the GP in whole blood have reported the fractal characteristics of incipient clots [1], whose fractal dimension, D_f, is a biomarker for haemostasis [2]. In whole blood and model clots (fibrin-thrombin gels), incipient clots are characterised by values of D_f which are consistent with the limiting values reported for clusters of particles formed by *diffusion limited cluster–cluster aggregation* and *reaction limited cluster–cluster aggregation* (DLCCA and RLCCA, respectively) [14–20]. Numerous studies of fractal clusters formed from spherical or rod-like colloidal particles have reported that slower aggregation results in more highly ramified structures, the latter being associated with higher values of D_f [21–24]. It is noteworthy, therefore, that rheometrical studies of heparinised blood [1] and fibrin-thrombin gels [16] report the opposite behaviour insofar as the values of D_f are found to increase progressively with a reduction in the gel formation time.

The aim of the present work was to undertake a molecular dynamics simulation (MDS) study to investigate a possible basis for this difference in behaviour. Its starting point was recognition

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doi:10.1016/j.jnnfm.2011.04.016

of the fact that in previous simulation work, all particles/rods involved are simultaneously activated to the same degree by modifying their surface charge. The present MDS of rod aggregation incorporated an activation profile in order to elucidate the effect of rod activation rates on the value of D_f. In blood coagulation, thrombin activates fibrinogen monomers which subsequently polymerise to form the incipient fibrin clot network [25,26]. The concentration of active monomers increases at a rate dictated by the time course of evolution of the thrombin concentration, this feature being represented in the present MDS by inclusion of the monomer (rod) activation profile. In addition to the present MDS studies, rheological characterisation of a range of fibrin-thrombin gels was conducted by oscillatory shear measurements in order to extend the results of previous studies.

2. Experimental

2.1. Oscillatory shear studies of fibrin-thrombin gels

Purified, plasminogen-depleted human fibrinogen (at least 95% clottable) and human thrombin (Enzyme Research Laboratories, UK) were made up to stock solutions of 45 mg/ml and 50 NIH Units/ml, respectively by gradual addition to Tris Buffered Saline, TBS, (20 mM Tris, pH 7.4, and 0.9% NaCl, Sigma Aldrich, UK) and left to fully dissolve in a waterbath at 37 °C. The stock solutions were dispersed into small vials and frozen at −80 °C until required. One part TBS (at 10 times the working concentration) was added to 9 parts 4.5% w/v human albumin (Zenalb®, Bio Products Laboratories, UK) to make a final stock solution of 4.05% human albumin in 20 mM Tris. The stock fibrinogen solutions were diluted to a concentration of 10 mg/ml with the stock albumin solution. This solution was treated with the appropriate volume of 1 M $CaCl_2$ (Sigma Aldrich, UK) to make a final $CaCl_2$ concentration of 0.005 M. Clotting was initiated by the addition of thrombin to final concentrations in the range of 0.01–0.15 NIH Units/ml to the fibrinogen. After appropriate mixing, the sample was transferred immediately to the rheometer.

Measurements of the dynamic rigidity (G′) and loss modulus (G″) were made over a range of frequencies [16] using an AR-G2 controlled stress rheometer (TA Instruments, UK) fitted with either a low inertia cone-plate (60 mm diameter, 1° cone) or a double-gap measuring system. The system was maintained at the test temperature by means of a peltier temperature control system fitted to a recirculating water bath. In addition, a vapour hood was fitted to minimise any effects due to sample evaporation. Prior to each test, the sample was transferred to the rheometer's measuring geometry, the sample being cooled to the test temperature of 10 °C (this procedure typically taking approximately 10 s). The test was initiated on attainment of the test temperature STARD guidelines being followed throughout [27].

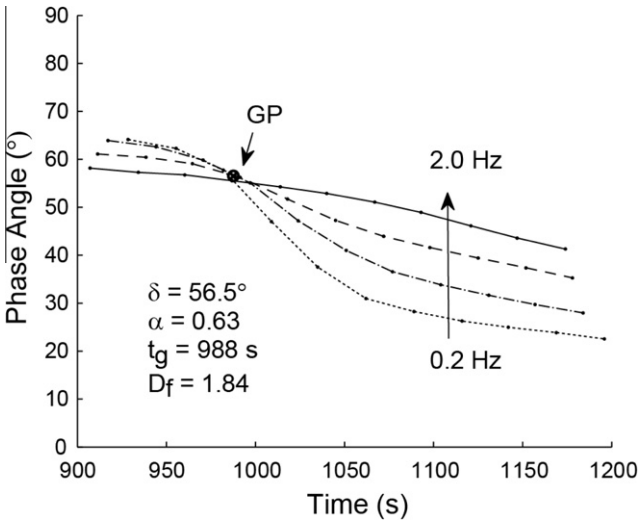

Fig. 1. Gel Point data for a low thrombin concentration (0.03 NIH/ml) fibrin-thrombin gel.

At the GP, G' and G'' scale as power-laws in frequency, ω as $G'(\omega) \sim G''(\omega) \sim \omega^{\alpha}$, the Gel Point being identified by the corresponding *frequency independence* of the loss tangent, tanδ [28] (see Fig. 1). The value of α was used to calculate D_f using the established relationship $D_f = (D + 2)(2\alpha - D)/2(\alpha - D)$ where D is the space dimension [29]. The higher the value of D_f the more compact is the network, whereas low values of D_f correspond to more open networks. An automated numerical method for GP location was employed to analyse the raw data and the resulting value of α (hence D_f) and the time, t_{GP}, taken to reach the GP were recorded [17].

2.2. Results of oscillatory shear measurements

An example of the results obtained on a fibrin-thrombin gel are shown in Fig. 1. The results illustrate the initial, pre-incipient clot response which is characteristic of an elasticoviscous fluid, with increasing frequency of oscillation causing δ to decrease. The frequency dependence of δ decreases progressively as gelation proceeds, becoming frequency *independent* as the incipient clot is established at the GP. Thereafter the frequency dependence is characteristic of a viscoelastic solid. Tests conducted on a range of fibrin-thrombin systems, in which the concentration of thrombin was gradually increased, revealed that the effect of increasing the thrombin concentration was to form fibrin-thrombin gels with (i) a decrease in t_{GP} and (ii) a decrease in α, corresponding to an increase in D_f in the range of values $1.78 < D_f < 2.00$. The results obtained (t_{GP} and D_f) for gels formed over the range of thrombin concentration are shown in Fig. 8.

3. MDS Studies

In its simplest form MDS involves the numerical solution of Newton's laws of motion. If the position and velocity of each particle involved in the simulation is known at time $t = 0$, and the inter-particle interactions (and particle–wall interactions if necessary) are also known then the position and velocity of each particle can be calculated at any later time. MDS differs from other formalisms used to model aggregation processes in that the particles follow deterministic trajectories defined by the laws of motion. Conversely, Brownian dynamics utilises probability density functions or Monte-Carlo (MC) methods to determine particle trajectories. Typically, DLCCA/RLCCA models utilise such a lattice-based MC approach, in which pseudo-random number generators are used to randomly select the nearest neighbour lattice site to which a particle moves during a time step, thereby simulating the diffusion of particles according to Brownian trajectories. MDS offers the advantage of off-lattice techniques which are preferable to lattice-based methods since particle movement is unconstrained within the simulation volume.

Relatively few MDS studies of *sol–gel* transitions have been reported. In a study of collagen, Parkinson et. al. [30] represented procollagen monomers (the precursor to collagen fibril formation) using rod-like particles, whose aggregation was simulated on-lattice in a manner similar to that of the simplest DLA routines. Inclusion of details of the collagen-specific binding processes in the simulation did not influence the results, leading to the conclusion that specific collagen-collagen interactions were unimportant in