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Automated, Microscopic Measurement of Fibrinaloid Microclots and Their Degradation by Nattokinase, the Main Natto Protease

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Abstract

Nattokinase, from the Japanese fermented food natto, is a protease with fibrinolytic activity that can thus degrade conventional blood clots. In some cases, however, including in Long COVID, fibrinogen can polymerise into an anomalous amyloid form to create clots that are resistant to normal fibrinolysis and that we refer to as fibrinaloid microclots. These can be detected with the fluorogenic stain thioflavin T. We describe an automated microscopic technique for the quantification of fibrinaloid microclot formation, which also allows the kinetics of their formation and aggregation to be recorded. We also here show that recombinant nattokinase is effective at degrading the fibrinaloid microclots in vitro. Flow conditions, mimicked by shaking, increase the size of the clots via aggregation. Overall, this work adds to the otherwise largely anecdotal evidence, that we review, that nattokinase might be anticipated to have value as part of therapeutic treatments for individuals with Long COVID and related disorders that involve fibrinaloid microclots.

1 Introduction

Thrombosis, the blocking of blood vessels by blood clots, along with the related thrombo-inflammation and thromboembolism, is a chief cause of cardiovascular disease [1-6]. Consequently anything that can promote safe anti-coagulation or fibrinolytic activity is likely to have therapeutic potential (e.g., [7-15]).

Nattō (usually rendered natto) is a Japanese food made via the fermentation of soy beans using the Gram-positive organism *Bacillus subtilis* var natto [16-20]. It has been widely consumed for over 2000 years, and is considered safe [21]. The proteolytic activity of natto was detected in 1906 [22] and its fibrinolytic activity in 1925 [23]. However, it was not until 1987 [21] that an enzyme exhibiting these activities was purified from natto; in spite of it being a protease it was termed nattokinase [21].

Despite having to pass through the gut wall [24-31], nattokinase is orally available (and this can be improved [32-34]), is considered a major contributor to the purported health benefits of natto [21,27,35-60], not least in cardiovascular disease [27,28,36,38,39,41,53,61-80], and is itself recognised as safe [64,81,82].

The experimental 3D structure of nattokinase, which is a serine protease related to subtilisin, is available [83,84], and it may also be produced via purification [85-88] or (as here) recombinantly [44,67,68,72,89-100]. Although not our prime focus in this paper, it is also known to cleave plasminogen activator inhibitor I [101], to have antiplatelet [102], anti-inflammatory [77], and anti-hypertensive [65,103,104] activities, and to show neuroprotective [105] and post-stroke benefits [106] as well, when dosed adequately, as having anti-lipidaemic effects [69].

Following earlier work using electron microscopy (e.g., [107-110]), we discovered that fibrinogen could polymerise or clot into an anomalous, amyloid form of fibrin (e.g., [111-118]) that exactly reflected the clots seen in both the electron microscope [119] and in bright field optical microscopy [120]. As with prions and other amyloid forms of proteins [112,121], that are often highly resistant to proteolysis (e.g., [122,123]), the existence of these 'fibrinaloid' microclots implies their comparative resistance to normal fibrinolysis [124,125], with their precise structures [126] being affected by other small and macromolecules and ions that they may have bound [111,117,127-133]. The varieties of stable macrostates into which a given amyloidogenic sequence can fold (even under the same conditions [134,135]) are referred to as different 'strains' [136-146] or 'polymorphisms' [147-158], and in some cases are sufficiently stable (i.e., kinetically isolated from other macrostates) that they are even heritable [136,159-165]. Homo- and hetero-polymerisation and their catalysis are then referred to, respectively, as (self-)'seeding' [154,166-180] and 'cross-seeding' [167,181-188]. More recently, we have established the prevalence of these fibrinaloid microclots in post-viral diseases such as Long COVID [120,189-192] (and see confirmation by others in [193,194]) and ME/CFS (myalgic encephalopathy/chronic fatigue syndrome) [195,196]. The lower amyloidogenicity of omicron versus earlier variants of SARS-CoV-2 is also reflected in its lower virulence [197], implying that these microclots are on the aetiological pathway of the disease, and they can explain many symptoms [198], including fatigue [199], post-exertional symptom exacerbation [200], autoantibody generation [121] and Postural Orthostatic Tachycardia Syndrome (POTS) [201]. Fibrin amyloid microclots also occur during sepsis [202], while amyloid deposits are also observed in the skeletal muscles of those with Long COVID [203]. Overall, this ability of fibrinaloid microclots to provide a mechanistic explanation of multiple phenomena is consistent with the 'explanatory coherence' view of science [204-207]. In common with other amyloid proteins [112], that contain a characteristic cross-β motif [187,208-224], they can be visualized using the fluorogenic stain thioflavin T [158,187,225-239] or via vibrational spectroscopy [236,240-251]. As with any other ligand or binding agent, the rotation of the bound form is more restricted than that of the free form (which is largely what makes it fluorogenic), and precise intensities of thioflavin T fluorescence depend on the location and conformation(s) to which the thioflavin T is bound [227-229,252-269] and in some cases on the presence of interferents [270].

Although nattokinase preparations are widely available commercially, and as noted above they are considered to have significant therapeutic value, including in Long COVID [271,272], their exact contents are uncertain, and so we decided that it was best to create and use purified, recombinant material.

While the proteolytic specificity of nattokinase, as an alkaline serine protease [44,73,273], is surprisingly underexplored, beyond a broad similarity to that of plasmin [44,274] (and nattokinase can even degrade spike protein [275] and certain 'classical' amyloids [276-278]), the question arises as to whether or not nattokinase can degrade the amyloid 'fibrinaloid' form of microclots. The purposes of this paper are (i) to describe an efficient, quantitative, automated microscopic method that can be used to determine the size and number of amyloid microclots and any time-dependent changes therein, and thus (ii) to assess any such nattokinase-induced degradation of the microclots, concluding that nattokinase can indeed degrade fibrinaloid microclots effectively. The therapeutic implications of this are discussed.

2 Materials and methods

2.1 Assay method

In vitro microclots were made by mixing 45 μL commercially obtained fibrinogen (Sigma catalogue number 9001-32-5, at a final concentration of 2 mg/mL) with 5 μL bacterial LPS (Sigma product code L2630-10MG) and used at a final concentration of 1 ng/mL were incubated at 37 ºC for 15 min. 25 μL were removed and replaced with 25 μL thrombin (Sigma, final amount 14U) and incubated at 37 ºC for a further 15 min. 3 μL were removed and replaced with 3 μL of the fluorogenic amyloid dye, Thioflavin T (ThT) (final concentration: 0.03 mM) and incubated for 20 min (protected from light) at room temperature. Following incubation, 10 μL of the recombinantly produced nattokinase at different concentrations / PBS (control) were added. This was then followed by immediately pipetting 15 μL of assay sample onto a 15-well slide 'angiogenesis' glass bottom plate used without a lid (Ibidi: https://ibidi.com/chambered-coverslips/245--slide-15-well-3d-glass-bottom.html), reproduced in Figure 1, and without shaking (cf. [156,279-291]). The excitation wavelength band for ThT was set at 450 nm to 499 nm and the emission at 499 nm to 529 nm. Samples were viewed using Gen5 software on an Agilent BioTek Cytation 1 Cell Imaging Multimode Reader, essentially following the protocol developed and described by Dalton and colleagues [193]. The Cytation instrument is an automated fluorescence microscope with 8-bit intensity resolution in which an entire, large field of view can be constructed at high magnification by taking serial images and moving the stage automatically. With the 4× objective used, each final image (as in Figure 1) was composed of 1,296 individual images. The typical file size of a final, stitched .tif image was 19 Mb. Each experiment was run multiple times, each time being in triplicate (three separate wells). Other relevant settings that we optimized for this assay were as follows: the Cytation 1 temperature was set at 37 ºC, and images were taken every 41 min for 6 hours. The colour channel used was GFP 469,525. A fixed focal height setting, with a bottom elevation of 549 μm and

0 μm offset was selected. A Z-Stack montage of the entire well was applied, with a step size of 86.9 μm, and 12 slices. Samples were analysed using the Gen5 Image Prime 3.13.15 software supplied with the instrument, and the thresholds for minimum and maximum object (clots) size that could be detected were set at 5 and 500 μm, respectively.

Figure 1 Ibidi 15-well 3D glass-bottomed microslide as used herein (the incubation system used herein, allowing imaging from below).

2.2 Recombinant nattokinase

Recombinant nattokinase was produced within the Liverpool Gene Mill. The nucleotide sequence for *Bacillus subtilis* nattokinase (Uniprot Q93L66, GenBank: AER52006.1) was synthesised by Twist Bioscience and supplied in the pET28a(+) plasmid. The sequence was modified to include a C-terminal poly-Histidine tag for purification, as well as an N-terminal PelB leader sequence in which the terminal QPAMA residues are replaced by APOIA, and with a penta-aspartate linker for targeting to the periplasmic space [292] plus a ENLYFQ TEV cleavage site and a further SGS linker prior to the nattokinase sequence (beginning AQSVPY). The vector was used to transform chemically competent cells of the Rosetta™ strain of *Escherichia coli* (Novagen) according to the method described by Inoue et al. [293]. Transformed cells were plated on plates of LB-agar (0.5% w/v yeast extract, 1% w/v NaCl, 1% w/v tryptone and 2% agar) supplemented with 50 μg/mL kanamycin and 25 μg/mL chloramphenicol. A single colony from the agar plate was used to inoculate 5 mL of LB broth (0.5% w/v yeast extract, 1% w/v NaCl, 1% w/v tryptone) supplemented with kanamycin and chloramphenicol as described above, for overnight culturing at 37 °C with shaking. The culture was diluted to an OD_{600} of 0.05 in 500 mL of LB broth supplemented with kanamycin and chloramphenicol as described above, and incubated with shaking at 37 °C. When an OD_{600} of 0.6 was reached, recombinant protein expression was induced by addition of 0.75 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and the culture was incubated overnight at 18 °C with shaking. Cell pellets were harvested by centrifugation at 4,000× for 10 min, and the pellets were resuspended in 50 mL of a solution of Tris-HCl (pH8) and 10 mM EDTA, and incubated at 60 °C for 2 hours [294]. The suspension was centrifuged at 4 °C at 16,000× for 10 min, and the supernatant was passed through 1 mL of HisPur™ Ni-NTA resin (Thermo Scientific) to purify poly-Histidine-tagged proteins. Bound proteins were eluted using 500 mM imidazole, followed by desalting and concentration using a Pierce™ Protein Concentrator PES (Thermo Scientific) with 30 kDa cut-off. Protein yield was quantified using the Pierce^{TM} Bradford Protein Assay Kit (Thermo Scientific), and samples were frozen with 10% v/v glycerol until further use. Inclusion body formation [295] was not here a significant issue. Figure 2 shows a gel illustrating the final preparation.

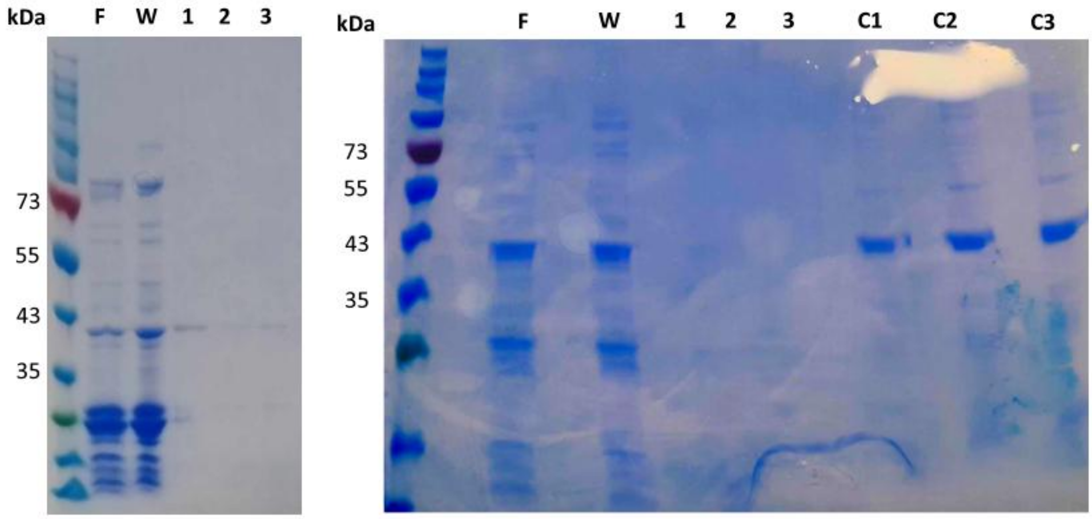


Figure 2 SDS-PAGE of recombinant Nattokinase. F: sample flow-through (unbound proteins); W: fraction of wash buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 50 mM imidazole); 1-3: purifed fractionsusing elution buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 500 mM imidazole); C1 & C2: purified samples concentrated through 30 kD cut-off protein concentrator unit; C3: C1 & C2 samples pooled and further concentrated through 3 kD cut-off protein concentrator unit.

A kinetic experiment was set up on the Cytation 1 and the effect of nattokinase on microclots was studied at final concentrations of 28 ng/μL and 14 ng/μL, using ThT at a final concentration of 0.005 mM, as the fluorogenic dye. Measurements were taken every 40 min.

3 Results

3.1 Basic phenomenon, and effect of concentration of NK and incubation time

To give an indication of the kinds of data obtained in this study, Figure 3 (left panels) shows three Cytation images representing clots as stained with thioflavin T following incubation of fibrinogen plus thrombin plus LPS (as in [111]) for 6 hours, either with no further additions (Figure 3A, top), with PBS (Figure 3B, middle), or after simultaneous exposure to 28 ng/μL nattokinase.

While we sought to avoid any 'cherry picking' in the past, the great attraction of the present approach is that the entire sample is imaged (serially) so this issue is completely avoided. Although not necessarily obvious to the naked eye, there are variations in pixel intensity that allow a thresholding to determine what counts as a clot boundary. Figure 3 also shows the pixel intensity variation for the images displayed on its left side; the logarithmic plot in particular makes clear how much the pixels of larger intensity differed following the addition of the nattokinase.

The time evolution of these data (Figure 4) shows that in the absence of nattokinase the clot numbers increase for an hour or so then decrease slightly before stabilizing (Figure 4A). When nattokinase is present the clot numbers decrease after the first time point and by 2 hours have attained their lowest level, this being approximately half that of the 14 ng/ μ L nattokinase (in which the nattokinase level is thus halved), possibly implying a loss of activity over time. In Figure 4B we see the dynamics of the clot intensity (total number of pixels), this being substantially lower in the presence of NK, especially at the higher level of enzyme. Figure 4C shows the time evolution of the median clot size.

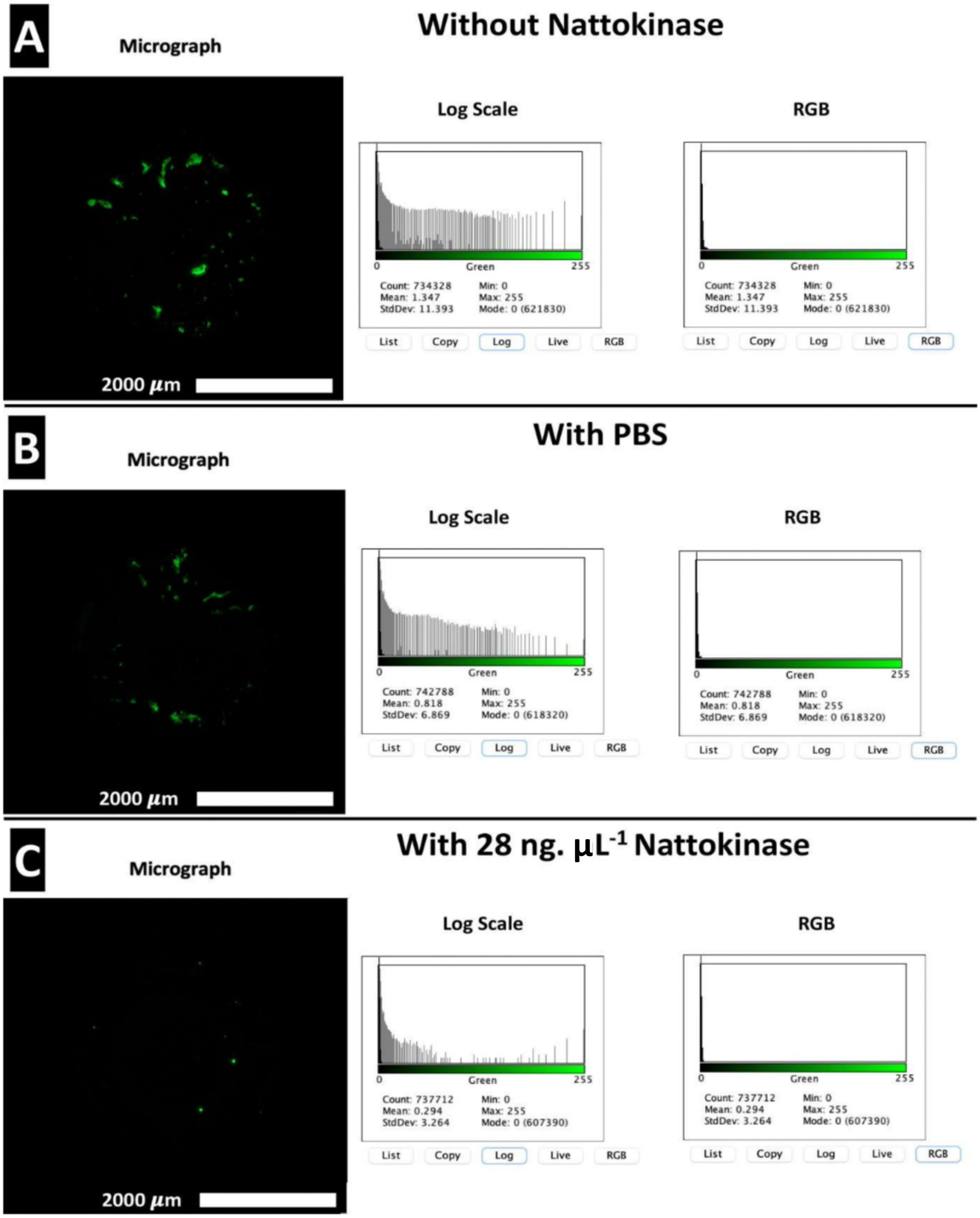


Figure 3 Images of fibrinaloid microclot formation and their removal via nattokinase. Thrombin and fibrinogen were incubated together with thioflavin T and LPS, and imaged after 6 hours in a Cytation 1, as described in Methods. Further additions were (A) none, (B) PBS, (C) recombinant nattokinase 28 ng/µL. Bar = 2 mm (2,000 µm).

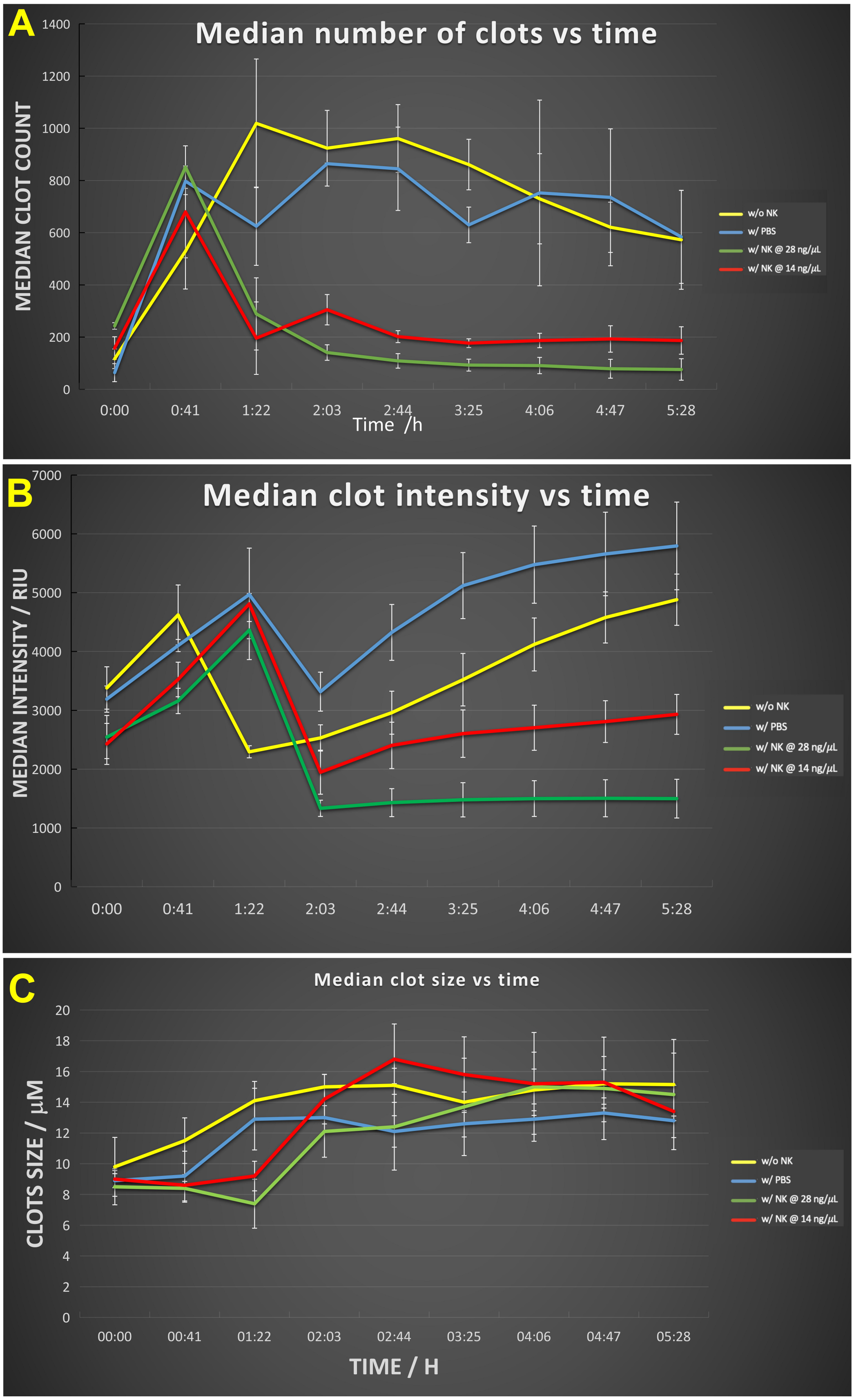


Figure 4 Time evolution of (A) clot number, (B) intensity and (C) median clot size during the development of fibrinaloid microclots and their incubation with nattokinase. Thrombin and fibrinogen were incubated together with thioflavin T, and imaged in a Cytation 1, as described in Methods. Further additions were none (yellow), PBS (blue), recombinant nattokinase 28 ng/μL (green), or recombinant nattokinase 14 ng/μL (red). Videos of the

incubation with PBS and with nattokinase are given in Supplementary Information. Error bars represent standard deviations of triplicates within a single experiment (and the experiment was repeated on two separate days). The *p*-values (paired, one-tailed *t*-test) at 2 hours were as Table 1:

Table 1 *p*-values (paired, one-tailed *t*-test) at 2 hours.

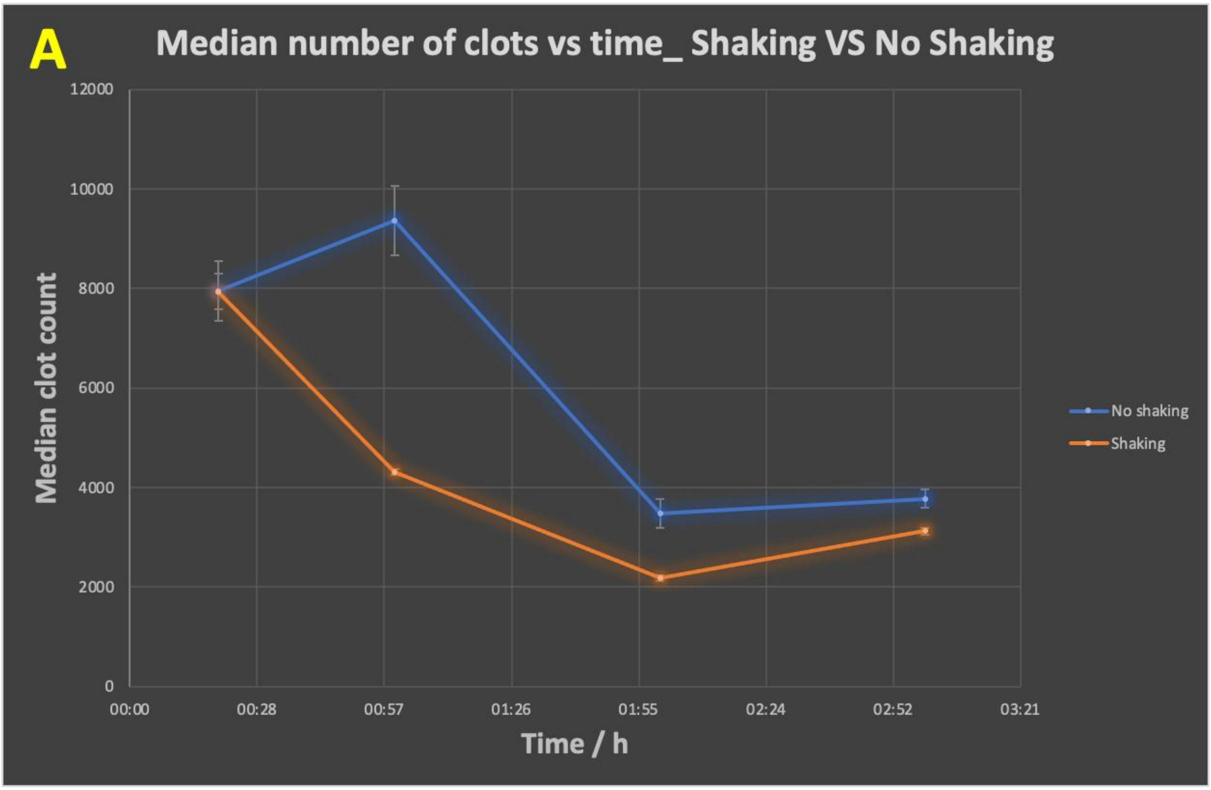
	p value			
	w/o NK and w/ PBS	**w/o NK and w/ NK at 28 ng/μL**	**w/o NK and w/ NK at 14 ng/μL**	**NK at 14 ng/μL vs. 28 ng/μL**
For median cell count	0.35	0.0003	0.0014	0.055
For median clot intensity	0.004	0.0008	0.015	0.023
For median clot size	0.019	0.008	0.126	0.25

3.2 Effect of flowing conditions, as implemented by shaking

The above analyses were done online within the Cytation 1 throughout, and under static conditions. However, it is known that flow conditions—as would be the case in blood *in vivo*—can themselves stimulate amyloidogenic fibre formations [288-290,296-301]. It was thus of interest to compare (with individual time samples added to the Cytation 1), the effect of flow. To mimic this we used simple shaking (Figure 5). As indicated (Figure 5), shaking had a significant influence in decreasing the clot number (Figure 5A) while increasing the clot size (Figure 5B).

3.3 Using Amytracker dyes instead of ThT

Because it is valuable to have other dyes should one wish to use multiple wavelengths (as in [117]), we also assessed the red oligothiophene-class Amytracker^{TM} dyes (Ebba Biotech) (see e.g., [115,117,119,302-309]). However, these gave highly anomalous traces in this system, and given that they did previously stain the fibrinaloid microclots as mentioned in those references we suspect may have inhibited the nattokinase, so were not further pursued.



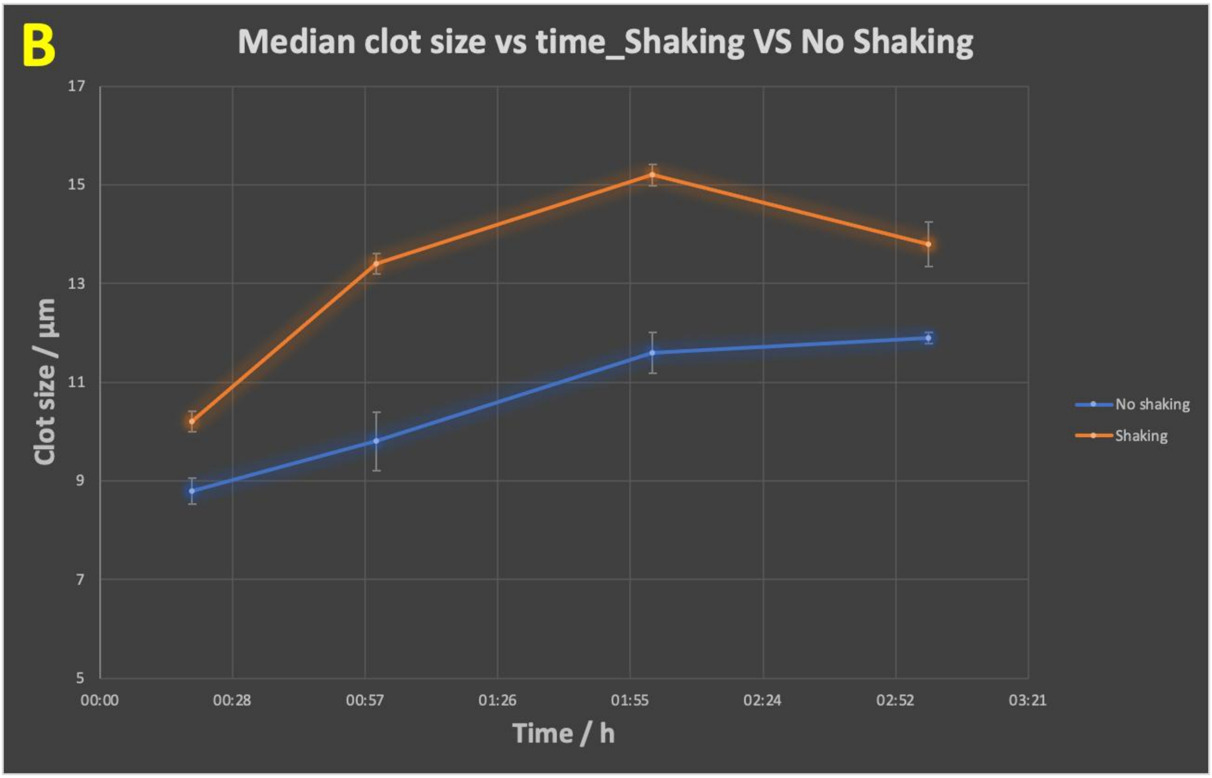


Figure 5 Time evolution of (A) clot number, (B) median clot size during the development of fibrinaloid microclots whilst shaking versus no shaking of the slides. Fibrinogen, thrombin and LPS were incubated together with thioflavin T, followed by immediate pipetting of 15 µL of assay sample onto a 15-well slide 'angiogenesis' glass bottom plate (as per Figure 1), which was left incubating for 20 mins, 1 hour, 2 hours and 3 hours, in a dark room, at 37 ºC, in a shaking incubator at 170 rpm. The no-shaking control samples were incubated under the same conditions but under stationary conditions. Slides were then imaged on the Cytation 1, using the GFP filter block, and the appropriate exposure settings. The *p*-value (paired, one-tailed *t*-test) for the difference in median clot size is 0.01. The experiment was duplicated, with each experiment having three technical replicates.

4 Discussion

Fibrinogen, especially its α-chain, is known to be amyloidogenic [112,310-316], and certain alleles are especially prone to causing fibrinogen-driven amyloidoses (e.g., [317-326]). Indeed, our own studies [111,113,115-118,120,191,197,199,327-333], and those of others [193,194,202], have demonstrated via staining with thioflavin T the authentic amyloidogenesis of fibrinogen to form fibrinaloid microclots. In the present work, we describe a medium throughput method for their quantitative estimation.

Three features stand out from the data in Figure 4. First, especially in the absence of NK (yellow trace), the clots increase in both number and size over time (Figure 4A,4B), illustrating how microclots may aggregate to form macroclots, as part of the normal amyloidogenic process (e.g., [187,227,230,254,312,334-344]), and such aggregation was increased under flow conditions (Figure 5). This kind of aggregation may be highly significant in stroke and myocardial infarctions, where clots may be far larger (e.g., [345-349]) than the simple sloughing off of atherosclerotic plaques might reasonably create. Secondly, the enzyme effectively decreases the rate and extent of microclot formation, in rough proportion to the amount of enzyme (compare e.g., red and green traces at 5 hours). The lowest intensity point was observed in the interval 2-4 hours, implying a die-off in activity or instability of the enzyme over time. This is good, in that untrammelled fibrinolytic activity may not be of the greatest therapeutic benefit. Lastly, the median clot size (Figure 4C) increases briefly then stabilizes. This reflects the fact that smaller clots will tend to be degraded preferentially as their surface area per unit mass is significantly greater than that of larger clots. (It is not commonly recognised, but if one imagines two solid spheres, of which one is twice the diameter of the other, the degradation of a given (i.e., the same) mass in the two spheres leads to a loss in mass of just 12.5% of the larger sphere when the smaller one is completely degraded, and a loss in radius of the larger sphere that is less than 5% of its starting value. Consequently, although possibly at first glance surprising, this is, given the traces in Figures 4A and 4B, in fact the result expected for Figure 4C.

The ability to assess the rate of fibrin amyloid formation and degradation noninvasively is highly desirable, as it precisely permits studies of the present type that can then be automated. While still not a high-throughput approach in the usual sense (flow clotometry [191,333], albeit using more expensive instrumentation, is certainly quicker), this does provide a substantial advance in scoring fibrinaloid microclot formation that is both fully quantitative and without undue operator fatigue. This has allowed us, for the first time, to conclude at least three important features: (i) the formation kinetics of fibrin amyloid microclots in whole samples may be imaged noninvasively in an automated manner, (ii) such microclots can aggregate over time, and (iii) the fibrinaloid microclots may be degraded by nattokinase. This latter has significant therapeutic implications for those suffering from Long COVID and related disorders, as NK preparations are widely available commercially. Our approach also thus allows for the comparison of different preparations of NK. Future work could usefully include recombinant serrapeptase (NK/SP), lumbrokinase (NK/LK) and/or sequence variants of NK/SP made using the methods of synthetic biology [350], since both serrapeptase and lumbrokinase, and even papain [351], also have fibrinolytic and amyloid-degrading properties [60,352-363].

Of course these results might also be recognized as having clinical potential. Given the evidence for the relevance of fibrinaloid microclots in the aetiology of Long COVID and other post-infection diseases as rehearsed above, and the potential shown here of such enzmes to remove them, a randomized controlled trial of these kinds of fibrinolytic enzymes (vs. placebo) seems more than warranted.

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Conflicts of Interest

E.P. is a named inventor on a patent application covering the use of fluorescence methods for microclot detection in Long COVID. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Author Contributions

Conceptualization, D.B.K., E.P.; methodology, J.M.G., J.E.S.-S., C.W.T.; resources, D.B.K., E.P.; writing—original draft preparation, D.B.K.; writing—review and editing, all authors; project administration, D.B.K., E.P.; funding acquisition, D.B.K., E.P. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The manuscript didn't involve any human or animal subjects, therefore no ethical approval was required for this article.

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Availability of Data and Materials

All data are available either in Supplementary Information or on request from the first author.

Supplementary Materials

The following supporting information can be downloaded at: https://ojs.exploverpub.com/index.php/jecacm/article/view/201/sup. Movies showing a kinetic series of images for samples treated with either PBS (Supplementary Figure 1) or 28 ng/ μ L nattokinase (Supplementary Figure 2). Frames start from read 1 at time zero and end at read 9 at time 5 h 28 min. Movies play at 0.5 frames per second. Time zero is the start of the reaction when PBS/NK was added to the sample containing fibrinogen, LPS, Thrombin, and ThT as described in the text. Green annuli are an artefact that may be ignored.

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