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PII: S0141-8130(19)39010-5

DOI: https://doi.org/10.1016/j.ijbiomac.2020.01.098

Reference: BIOMAC 14402

To appear in: *International Journal of Biological Macromolecules*

Received date: 5 November 2019

Revised date: 8 January 2020

Accepted date: 9 January 2020

Please cite this article as: N. Gligorijević, V. Šukalović, A. Penezić, et al., Characterisation of the binding of dihydro-alpha-lipoic acid to fibrinogen and the effects on fibrinogen oxidation and fibrin formation, *International Journal of Biological Macromolecules*(2018), https://doi.org/10.1016/j.ijbiomac.2020.01.098

# Characterisation of the binding of dihydro-alpha-lipoic acid to fibrinogen and the effects on fibrinogen oxidation and fibrin formation

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**ABSTRACT**

A reduced form of the alpha-lipoic acid, dihydro-alpha-lipoic acid (DHLA) is a potent, naturally occurring antioxidant which can be consumed as food constituent or as supplement at doses up to 600 mg/day. DHLA has inhibitory effect on coagulation as it can reduce concentrations of some coagulation factors. In this study, a direct interaction between DHLA and fibrinogen, the main protein in coagulation, is described. Binding constant for DHLA/fibrinogen complex is of moderate strength ($10^4$) and interaction probably occurs in D regions of fibrinogen, as shown by docking simulations. Fibrinogen stability remains the same with only marginal structural changes in its secondary structure favouring more ordered molecular organisation upon DHLA binding. Fibrinogen with bound DHLA forms fibrin with thicker fibers, as measured by coagulation assay and is protected from oxidation to certain extent. Obtained results support beneficial effects of DHLA on fibrinogen and consequently on coagulation process, suggesting that DHLA supplementation may be indicated for persons with an increased risk of developing thrombotic complications, particularly those whose fibrin is characterised by increased oxidative modification and formation of thinner and less porous fibers. Also, DHLA in complex with fibrinogen can be located at site of injury where it may exert antioxidant effects.

**KEYWORDS**: Protein interaction, Molecular modelling, Protein function

## 1. INTRODUCTION

Fibrinogen is one of the major plasma proteins than circulates in the concentration range from 4.4 to 10.3 μM (1.5-3.5 g/L) in healthy individuals. This is a large fibrillar glyco-protein with molecular mass of 340 kDa. Fibrinogen is a homodimer with $(A\alpha B\beta\gamma)_2$ structure [1]. This protein is involved in hemostasis, both primary and secondary. Primary haemostasis involves aggregation of blood platelets as fibrinogen binds to the platelet membrane IIb/IIIa glycoprotein and secondary involves transformation to fibrin and subsequent formation of a fibrin clot [2]. Fibrinogen interacts with many molecules that alter its structure and function including proteins, ions and small molecules [3]. Also, certain pathologies are characterised by chemically modified fibrinogen, which behaves differently than the native protein [4,5]. One of these chemical modifications is oxidation and fibrinogen is the most oxidation-susceptible plasma protein [6]. Oxidised fibrinogen usually acquires thrombogenic characteristics, such as formation of fibrin clot with thinner fibrils and reduced porosity [7–9]. Even if the structure of the oxidised fibrin clot is not altered, its porosity may be reduced since protein oxidation creates more hydrophobic environment [10]. For this reason, it is relevant to investigate possible interactions of fibrinogen with small molecules that are potent antioxidants and to reveal their capacity to protect this protein from oxidation and affect the formation of fibrin clot.

Alpha-lipoic acid (ALA) is a naturally occurring compound that mammalian cells synthesise by the action of mitochondrial lipoic acid synthase. It is also commonly found in nutrients such as vegetables and meat. ALA and its reduced form dihydro-alpha-lipoic acid (DHLA) have many biological effects and act as antioxidants, metal chelators, reducing agents of the oxidised forms of other antioxidants (such as vitamins C and E), they improve endothelial dysfunction and protect against development of atherosclerosis [11]. ALA is also prescribed as a food supplement

and doses of 600 mg/day can be used. Bioavailability of the orally administrated ALA in aqueous solution can be up to 40 % and food intake reduces it. It is recommended to consume the supplement at least 30 min before the meal [12]. Beneficial effects of ALA supplementation in patients with diabetes were reported [13,14] and they include reduction of glucose and ROS levels, prevention of $\beta$ cell destruction, enhancement of glucose uptake and slowing down the development of diabetic complications. On the other hand, as reported in another study, supplementation of 600 mg/day of ALA to older patients with diabetes mellitus type 2 failed to exhibit beneficial effects regarding reduction of inflammation, oxidative stress and receptors for the advanced glycation end products [15]. The authors of that study suggested testing of higher doses of ALA.

ALA was shown to modify the coagulation process in several ways. It inhibits activation of platelets [13], prolongs the coagulation time affecting an intrinsic coagulation pathway [16] and reduces the concentration of some coagulation factors such as fibrinogen, factor VII and von Willebrand factor [17].

The aim of this study was to investigate whether ALA can interact directly with fibrinogen and if this binding can modify formation of fibrin clot and protect fibrinogen from harmful oxidation.



## 2. MATERIALS AND METHODS

### *2.1 Materials*

All chemicals used were of the analytical grade. Fibrinogen was purchased from Sigma and further purified as already reported [18]. A stock solution (5 mM) of DHLA was prepared by suspending it in 50 mM phosphate buffer, pH 7.4 and then adding few drops of 1 M NaOH until

complete clarification of the solution [19]. This solution of DHLA was used for all subsequent experiments, which were performed applying optimised ratios of reactants for each method.

*2.2 Investigation of the binding of DHLA to fibrinogen and determination of the binding constant*

The interaction between DHLA and fibrinogen and determination of the binding constant was examined by measuring the reduction of the intrinsic fluorescence of fibrinogen (20 nM) in the presence of an increasing concentrations of DHLA (0-20 μM, final concentrations), at room temperature. The spectra were recorded using RF-6000 spectrofluorometer (Shimadzu, Japan). The excitation wavelength was 280 nm and the emission spectra were recorded in the range from 290 to 450 nm, with slits width of 5 nm. The obtained spectrum for DHA/fibrinogen complex for each DHLA concentration was corrected for the background signal originating from DHLA itself. Differences in the obtained fluorescence intensities due to the inner-filter effect were corrected using the equation:

$$F_c = F_0 \text{ x } 10^{(Aex+Aem)/2} \qquad (1)$$

where $F_0$ is the measured fluorescence, $F_c$ is the corrected fluorescence, Aex and Aem are absorbances at the excitation (280 nm) and the emission (345 nm) peaks.

In order to determine the type of quenching, Stern-Volmer (SV) plot was drawn using the following equation [20]:

$$\frac{F_0}{F} = 1 + k_q \tau_o [Q] = 1 + K_{SV}[Q] \qquad (2)$$

Where $F_0$ and F are protein fluorescence intensities at 345 nm in the absence and in the presence of DHLA, $k_q$ is the bimolecular quenching rate constant, $\tau_o$ is the average lifetime of the biomolecule without quencher ($10^{-8}$ s), [Q] is the total concentration of the quencher (DHLA) and $K_{SV}$ is the SV quenching constant. The slope of the SV plot is the SV quenching constant.

The binding constant, Ka, between fibrinogen and DHLA was calculated using Stern-Volmer equation:

$$log\frac{F0-F}{F} = -nlog\frac{1}{[L]-[P]\frac{F0-F}{F0}} + nlogKa \qquad (3)$$

Where $F_0$ and F are emission signals of the fibrinogen in the absence and in the presence of DHLA, [L] and [P] are total concentrations of ligand (DHLA) and protein (fibrinogen).

*2.3 UV-VIS analysis of DHLA binding to fibrinogen*

UV-VIS analysis of DHLA binding to fibrinogen was performed using Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Sweden) in the wavelength range from 300 to 425 nm at room temperature. Spectra were recorded for DHLA alone (50 μM) and mixtures of fibrinogen and DHLA containing 5 or 10 μM fibrinogen and 50 μM DHLA. In order to detect any changes in DHLA spectra as a consequence of fibrinogen binding, the spectra obtained for mixtures were corrected for the spectra originating from fibrinogen alone.

*2.4 Determination of the thermal stability of the DHLA/fibrinogen complex*

The influence of DHLA binding on the thermal stability of fibrinogen was determined using FluoroMax®-4 spectrofluorometer (Horiba Scientific, Japan) with Peltier element. Since denaturation of fibrinogen is followed by a red shift of its emission spectrum, the ratio of fluorescence intensities at 354 and 334 nm was used to investigate its thermal stability. Thermal stability of fibrinogen alone (20 nM) or in the presence of DHLA (20 μM) was studied in the temperature range from 37 to 81 °C. The rate of the temperature increase was 2 °C/min and the equilibration time was set to 1 min. The results are presented as sigmoidal function with the inflection point representing a melting temperature (Tm) of fibrinogen.

*2.5 Fourier-transform infrared spectroscopy (FTIR) analysis of the DHLA/fibrinogen complex*

The samples, 6 μM fibrinogen alone or in the presence of 60 or 600 μM (in a total volume of 50μL) were placed on the ZnSe windows (32 x 2mm, Thermo, Madison, USA) and dried under the stream of nitrogen. FTIR measurements were performed using the IRAffinity-1 FTIR spectrophotometer (Schimadzu, Japan). The measurements were acquired in a transmission mode (100 scans per sample), while the resolution was 4 $cm^{-1}$. Prior to spectral analyses, the spectra were preprocessed using Spectragryph software (Menges, 2018). The preprocessing (i.e. smoothing and baseline corrections) was done according to the described procedure [21]. Deconvolution of the amide I peak was performed using Origine Pro 8.6 software (OrigineLab, USA).

*2.6 Determination of the effect of DHLA binding on fibrinogen coagulation*

Fibrinogen coagulation assay was performed with fibrinogen at the final concentration of 0.34 μM, alone or in the presence of DHLA at final concentrations of 0.34 μM or 0.68 μM. Reaction mixtures also contained $CaCl_2$ at the final concentration of 2.2 mM and human thrombin, 1 IU/mL. Coagulation assay was carried out at room temperature in a microtiter plate. Immediately after the addition of thrombin, the microplate was placed in Victor$^3$V multilabel reader (PerkinElmer, USA) and the rise in the absorbance at 350 nm was continually followed for 8 min. The absorbances of reaction mixtures without fibrinogen were also recorded and used to correct the corresponding results obtained in the presence of fibrinogen. The results were analysed according to published procedure [22].

*2.7 Docking analysis of DHLA binding to fibrinogen*

Human fibrinogen crystal structure (PDB Code: 3GHG DOI:10.2210/pdb3GHG/pdb) was retrieved from the RCSB protein data bank (https://www.rcsb.org/ accessed Oct. 2019). The structure was prepared using Schrodinger Maestro Protein preparation module (Schrödinger Release 2018-4: Maestro, Schrödinger, LLC, New York, NY, 2018.). Ligand 2D structure was drawn in ChemDraw software (PerkinElmer Informatics, 2015). 2D structure was imported into Schrodinger Maestro software and converted to a 3D model with the LigPrep module. Prepared protein and ligand were the starting point for Induced Fit Docking protocol [23,24]. Potential binding sites were determined using Maestro Sitemap [25] module and standard Induced Fit Docking protocol was used to produce up to 20 protein-ligand complexes. The obtained complexes were evaluated and the best docking orientation was selected based on the number of protein-ligand interactions and the docking score. Results were visualised using Schrodinger Maestro software.

*2.8 Investigation of the protective potential of DHLA against oxidation of fibrinogen by AAPH*

Reduction of fibrinogen intrinsic fluorescence as a result of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced free radical oxidation was used to determine the potential of DHLA to protect fibrinogen from oxidation. The same instrument used in section 2.2 was applied for this procedure. The analysis was performed at room temperature. To fibrinogen alone (100 nM) or in the presence of DHLA (100 or 200 nM), AAPH was added at a final concentration of 5 mM. Immediately after AAPH addition, samples were placed in the spectrofluorometer and the fluorescence measured at 345 nm (5 nm slits width, 280 nm excitation). The results were corrected for the fluorescence signal originating from DHLA alone.

The protective effect (PE), expressed in arbitrary units (a.u.) of DHLA on fibrinogen oxidation was quantified using the following equation:

$$PE\ (a.u.) = AUC_{Fng+DHLA} - AUC_{Fng} \qquad (4)$$

where $AUC_{Fng+DHLA}$ is the area under the curve obtained for the oxidation of the DHLA/fibrinogen complex, while $AUC_{Fng}$ is the area under the curve for the oxidation of free fibrinogen.

*2.9 Determination of the reducing power of the DHLA/fibrinogen complex*

The reducing power (antioxidative activity) of fibrinogen (7.5 μM), DHLA (7.5 and 15 μM), and DHLA/fibrinogen complexes was measured similarly to the published procedure [26]. To 40 μL of the sample solution, 100 μL of 0.2 M phosphate buffer (pH 6.6) and 100 μL of 1% potassium hexacyanoferrate(III) were added and incubated at 50 °C for 20 min. After incubation, 20% trichloroacetic acid was added and the reaction mixture centrifuged at 10000 × g for 8 min. The supernatant (100 μL) was mixed with 100 μL of deionised water and 12 μL of 1% iron(III)chloride, incubated at room temperature for 2 h and the absorbance measured at 670 nm. The masking effect of the protein on the activity of DHLA was calculated using the following equation:

$$100\% - (100\% \times A_{Fng+DHLA} / A_{DHLA}) \qquad (5)$$

where $A_{DHLA}$ and $A_{Fng+DHLA}$ are the measured absorbances of DHLA at 670 nm in the absence and in the presence of fibrinogen.

## 3. RESULTS AND DISCUSSION

DHLA binds to and reduces the intrinsic fluorescence of fibrinogen upon its excitation at 280 nm in a concentration-dependent manner, Figure 1A. The SV quenching constant was calculated to be 1.15 x $10^4$ $M^{-1}$, Figure 1B and, from this value, the bimolecular quenching rate constant was calculated to be 1.15 x $10^{12}$ $M^{-1}s^{-1}$. This last constant is two orders of magnitude higher than the diffusion rates of biomolecules ($10^{10}$ $M^{-1}s^{-1}$) which suggests that static quenching of fibrinogen fluorescence occurs in the presence of DHLA [26]. The binding constant for the DHLA/fibrinogen complex was calculated to be 1.01 ± 0.15 x $10^4$, Figure 1C. There was no shift in the maximum of the emission spectra due to DHLA binding, implicating that amino acids Tyr and Trp in fibrinogen remain in the same or very similar environment in the native protein and DHLA/fibrinogen complex.

Another evidence that the formation of DHLA/fibrinogen complex occurs was acquired from the UV-VIS spectral analysis. The absorption maximum of DHLA was at 340 nm. In the presence of fibrinogen, there was no change in the intensity of absorbance of DHLA, but a blue shift of the maximum occurred. This shift was as large as 22 nm in the presence of 10 μM fibrinogen, Figure 2.

The analysis of thermal denaturation revealed that binding of DHLA to fibrinogen doesn't affect protein stability. Fibrinogen alone and DHLA/fibrinogen complex, Figure 3, both had the same melting temperature of 53 °C which corresponds to the melting point of the fibrinogen D region [27]. These results confirmed spectrofluorometric data, i. e. that there is no significant structural alteration of protein upon DHLA binding. To investigate whether any change in the secondary structure of fibrinogen occurs upon its interaction with DHLA, FTIR analysis was performed. Again, no significant change was detected, Figure 4, even in the presence of high concentration of DHLA. A slight increase in the content of α-helix (for 2 %) and β-sheets (for 5 %) at the

expense of reduction of random structures (for 3 %) and turns (for 4 %) was seen, suggesting a small contribution of DHLA to the structural order of fibrinogen.

To investigate possible functional consequences of the interaction between fibrinogen and DHLA, two sets of experiments were performed, a coagulation assay (i.e. fibrin formation) and an assay to test antioxidative protection of fibrinogen by DHLA. Both experiments were carried out at molar ratios of DHLA and fibrinogen which can be obtained under physiological conditions. In the coagulation assay, when the molar ratio DHLA:fibrinogen was 2, an increase in the maximal absorbance at 350 nm was recorded, without affecting the coagulation time and speed, Figure 5. Since this result indicates that thicker fibrin fibers form in the presence of DHLA [28], we hypothesised that DHLA may bind to fibrinogen in protein regions involved in fibrin formation.

In order to verify this hypothesis, the Schrodinger Maestro Sitemap module was used to identify possible binding sites for docking simulations. Sitemap suggested two most probable binding sites and the results of the Induced Fit Docking simulation are presented in Figure 6. The key interaction for complex formation is hydrogen bonding between fibrinogen and ALA/DHLA carboxyl group, while DHLA can form additional hydrogen bond via a thiol group. In the case of the binding site 1, interacting amino acids are Asp 364, Arg 375, Lys 356 and His 340. Similar interactions occur at the binding site 2, involving Trp 385, Arg 406 and Asp 432. The significance of both binding sites is that they are located in terminal D domains of the fibrinogen molecule which are directly involved in the initial association of fibrin fibrils and the latter lateral association of growing fibrils [29]. Thus, the proposed model supports our hypothesis and shows that DHLA binding may affect the formation of fibrin fibers.

As predicted by docking analysis, DHLA needs to be in certain conformation(s) in order to bind to fibrinogen at binding sites 1 and 2, and such structural change(s) may be the reason for the detection of a blue shift of DHLA in UV-VIS spectral analysis in the presence of fibrinogen. This hypothesis, however, needs further experimental confirmation.

Thicker fibers usually give rise to a fibrin clot with greater porosity, which may be beneficial in pathologies accompanied by thrombotic complications, as this implies that the clot can be more easily penetrated and lysed by plasmin [30]. This is, however, not always straightforward as in some conditions thrombotic fibrin with thicker, but also more rigid fibers forms [31]. One should keep in mind that the coagulation assay in our study was performed with the isolated fibrinogen and further *in vivo* investigations using entire blood samples from patients with or at high risk of developing thrombotic complications is needed. It must be considered that many factors determine the final structure of fibrin clot, such as the concentration of fibrinogen, its structural modification, interaction with other proteins, ionic strength of the medium, activity of thrombin etc [3,32]. Also, fibrin susceptibility to lysis by plasmin depends on other determinants besides fibrin structure and its porosity, such as the protein mass fraction of the clot, the extent of cross-linking and the presence of some pathology-associated components in plasma, including immunoglobulins and cellular microvesicles [33–36].

Fibrin formation and its structure were shown to be altered in many pathologies characterised by thrombotic complications [37–39]. The formation of thrombotic fibrin clot may result as a consequence of the oxidation of fibrinogen [10]. This chemical modification is detrimental for its function. One possible way to protect fibrinogen from oxidation may be via its interaction with a small ligand with potent antioxidant activity. DHLA is one such ligand [40] and in this study we have demonstrated its capacity to bind to fibrinogen without altering protein structure and

stability, and exerting a beneficial effect on its coagulation properties at the same time. Free radical oxidation assay using AAPH as an oxidising agent enabled verification of the protective role of DHLA against oxidation of fibrinogen, Figure 7A. Surface areas under the curves defining progress of fibrinogen oxidation in the absence and in the presence of DHLA served as a measure of protein modification. DHLA was able to reduce fibrinogen oxidation by 23 % when the molar ratio DHLA:fibrinogen was 2.

In the last experiment, a reducing activity of the DHLA/fibrinogen complex was investigated using hexacyanoferrate (III)/$Fe^{3+}$ assay, Figure 7B. A reducing power of DHLA and fibrinogen alone was greater than that of the DHLA/fibrinogen complexes. This finding can be explained by the masking effect, which is a well-known consequence of the protein-ligand complex formation [41]. A masking effect on the reducing activity in the case of DHLA/fibrinogen complexes was calculated to be 31 % and 37 % for the molar ratios of DHLA:fibrinogen 1 and 2, respectively. Although masking effect was present, this may not necessarily be a definitive negative consequence of the DHLA/fibrinogen interaction, as DHLA may be released from fibrinogen during fibrin formation and/or lysis by plasmin. In either case, a released DHLA could exert antioxidative effect at the site of injury.



## 4. CONCLUSION

Fibrinogen can bind DHLA without significant effect on its structure and stability. The binding constant is of the moderate strength and the interaction most probably occurs in D regions of fibrinogen. Under conditions which correspond to the possible physiological ratio of the protein and the ligand, DHLA can protect fibrinogen from oxidation and stimulate formation of thicker fibrin fibers. The obtained results support beneficial effects of DHLA on fibrinogen and

consequently on the coagulation process, suggesting that DHLA supplementation may be indicated for persons with an increased risk of developing thrombotic complications, particularly those whose fibrin is characterised by an increased oxidative modification and formation of thinner and less porous fibers.

**FUNDING**: This work was supported by the Ministry of Education, Science, and Technological Development of the Republic of Serbia [grant number 173042].

**ACKNOWLEDGEMENTS**: We are grateful to Professor Steva Lević from the Faculty of Agriculture, for obtaining FTIR results. The authors declare no potential conflict of interest regarding this paper.

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**FIGURE CAPTIONS**

**Figure 1.** Spectrofluorometric determination of the binding constant between fibrinogen and DHLA: **A.** Reduction in the fluorescence emission intensity of fibrinogen upon DHLA binding (fibrinogen concentration was 20 nM and the concentration of DHLA varied in the range 2 - 20 μM). **B.** Stern-Volmer (SV) plot for the DHLA/fibrinogen interaction, the slope of the plot is SV quenching constant ($R^2$ = 0.989). **C.** Double log plot used for calculation of a binding constant between DHLA and fibrinogen ($R^2$ = 0.992).

**Figure 2.** UV-VIS spectra of DHLA alone (black line), and in the presence of 5 μM (red line) and 10 μM fibrinogen (blue line).

**Figure 3.** Melting curves for fibrinogen alone (black line), and DHLA/fibrinogen complex (red line). Tm is the melting point.

**Figure 4**. Investigation of the secondary structure of fibrinogen upon DHLA binding by FTIR. Deconvoluted FTIR spectra of the amide I signal were obtained for: **A.** Fibrinogen (6 μM); **B.** DHLA/fibrinogen complex (6 μM and 60 μM, respectively) and **C.** DHLA/fibrinogen complex (6 μM and 600 μM). Individual secondary structures are represented by color-coding.

**Figure 5.** The effect of DHLA bound to fibrinogen on fibrin formation. A coagulation curve for: fibrinogen alone (0.34 μM, black squares) and in the presence of DHLA (0.34 μM, red triangles or 0.68 μM, blue circles).

**Figure 6.** Docking simulation results. **A.** Partial 3D model of human fibrinogen (PDB: 3GHG) with two potential DHLA binding sites in D-domain: 1 (red rectangle) and 2 (blue rectangle).

Trp residues are displayed as CPK model and secondary structure is depicted by ribbons. **B.** DHLA interaction at two binding sites 1 (left) and 2 (right). Both 3D model and 2D interaction schemes are given. Magenta lines represent hydrogen bonds. Only amino acid residues in close contact with the ligand are shown for clarity.

**Figure 7.** Investigation of antioxidant and reducing power of DHLA. **A.** Oxidation of fibrinogen (100 nM) by AAPH in the absence (black line) and in the presence of DHLA (100 nM, red line or 200 nM, blue line); a reduction in protein fluorescence at 345 nm upon AAPH addition was recorded for 15 min. **B.** Determination of the reducing power of fibrinogen (7.5 μM), DHLA (7.5 and 15 μM) and their complexes by using potassium hexacyanoferrate (III)/$Fe^{3+}$ assay; the absorbance at 670 nm was recorded.

**Author statement**

**Nikola Gligorijević:** Conceptualization, Investigation, Writing - Original Draft, Visualization. **Vladimir Šukalović:** Investigation, Methodology, Investigation. **Ana Penezić:** Investigation, Visualization. **Olgica Nedić:** Supervision, Funding acquisition, Writing - Review & Editing

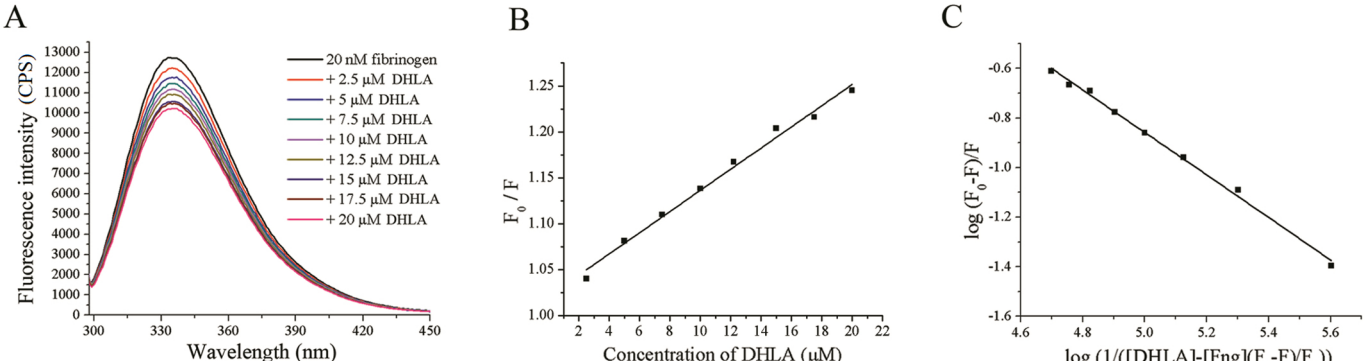
A
Fluorescence intensity (CPS)
Wavelength (nm)
20 nM fibrinogen
+ 2.5 μM DHLA
+ 5 μM DHLA
+ 7.5 μM DHLA
+ 10 μM DHLA
+ 12.5 μM DHLA
+ 15 μM DHLA
+ 17.5 μM DHLA
+ 20 μM DHLA
B
F0 / F
Concentration of DHLA (μM)
C
log (F0-F)/F
log (1/([DHLA]-[Fng](F0-F)/F0))

Figure 1

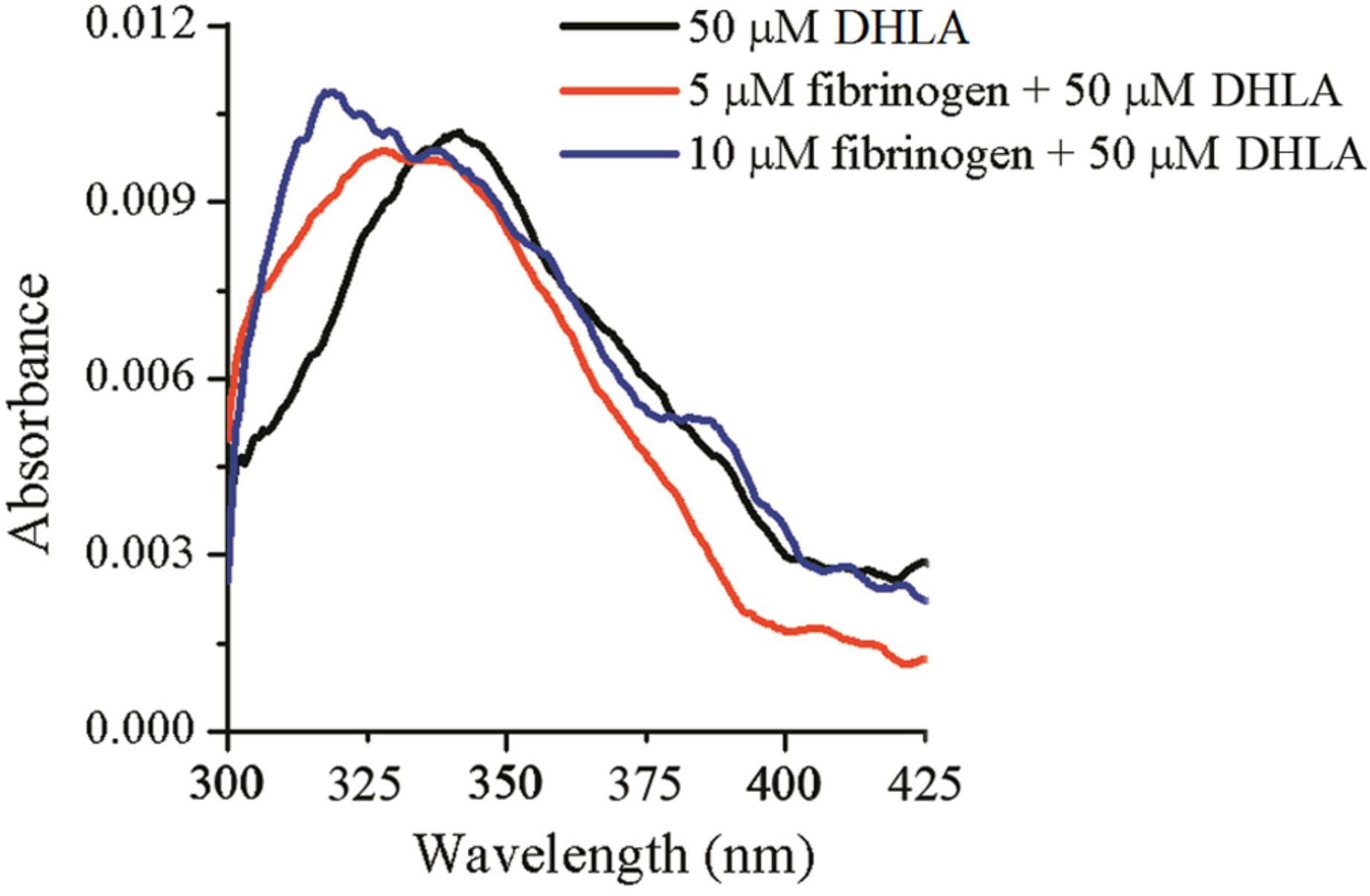
50 μM DHLA
5 μM fibrinogen + 50 μM DHLA
10 μM fibrinogen + 50 μM DHLA
Absorbance
0.012
0.009
0.006
0.003
0.000
300
325
350
375
400
425
Wavelength (nm)

Figure 2

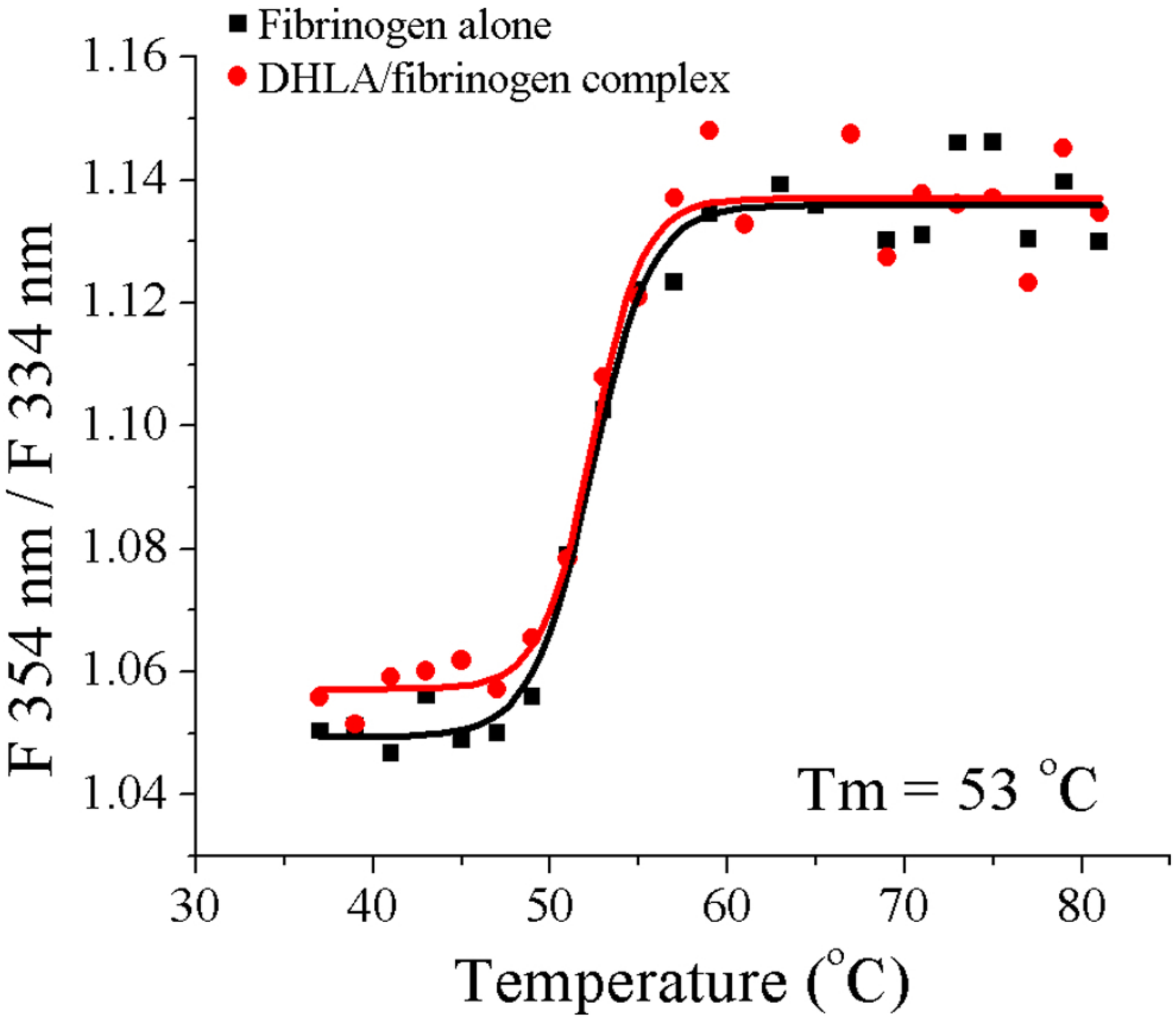
Fibrinogen alone
DHLA/fibrinogen complex
F 354 nm / F 334 nm
1.16
1.14
1.12
1.10
1.08
1.06
1.04
30
40
50
60
70
80
Temperature (°C)
Tm = 53 °C

Figure 3

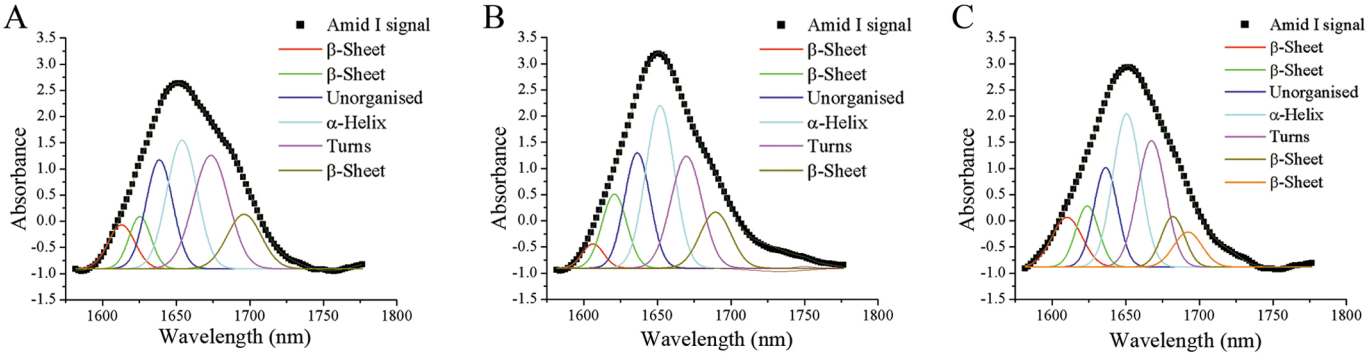
A
Amid I signal
β-Sheet
β-Sheet
Unorganised
α-Helix
Turns
β-Sheet
Absorbance
Wavelength (nm)
B
Amid I signal
β-Sheet
β-Sheet
Unorganised
α-Helix
Turns
β-Sheet
Absorbance
Wavelength (nm)
C
Amid I signal
β-Sheet
β-Sheet
Unorganised
α-Helix
Turns
β-Sheet
β-Sheet
Absorbance
Wavelength (nm)

Figure 4

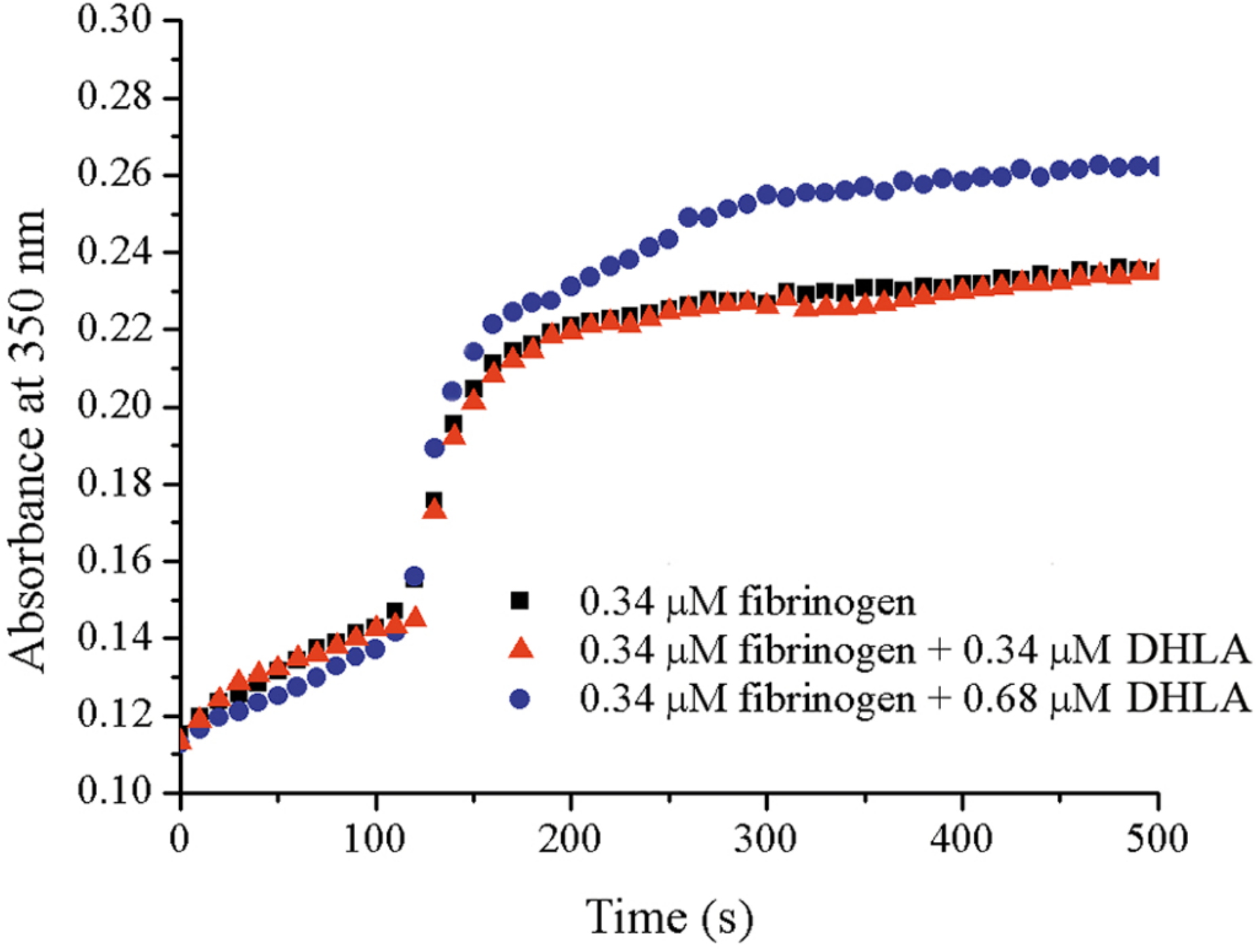
Absorbance at 350 nm
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0
100
200
300
400
500
Time (s)
0.34 μM fibrinogen
0.34 μM fibrinogen + 0.34 μM DHLA
0.34 μM fibrinogen + 0.68 μM DHLA

Figure 5

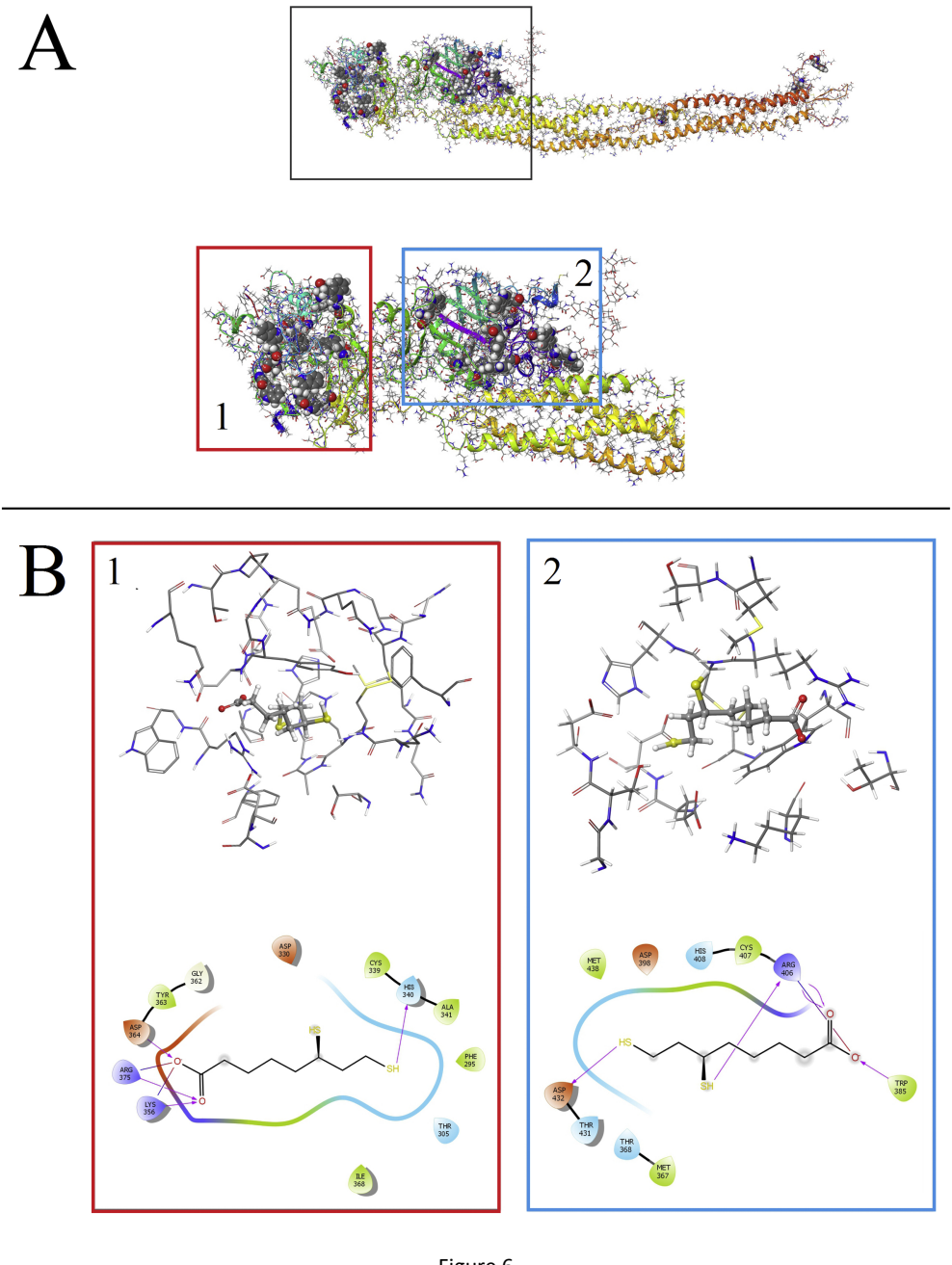
A
1
2
B
1
2
ASP 330
CYS 339
GLY 362
TYR 363
HIS 340
ALA 341
ASP 364
HS
SH
PHE 295
ARG 375
LYS 356
THR 305
ILE 368
MET 438
ASP 398
HIS 408
CYS 407
ARG 406
HS
SH
ASP 432
THR 431
THR 368
MET 367
TRP 385

Figure 6

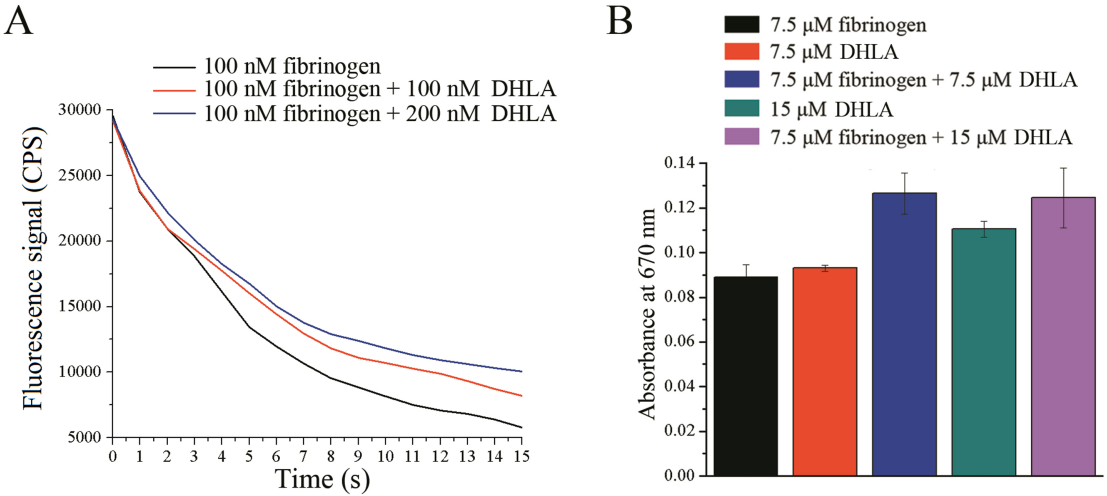
A
100 nM fibrinogen
100 nM fibrinogen + 100 nM DHLA
100 nM fibrinogen + 200 nM DHLA
Fluorescence signal (CPS)
30000
25000
20000
15000
10000
5000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (s)
B
7.5 μM fibrinogen
7.5 μM DHLA
7.5 μM fibrinogen + 7.5 μM DHLA
15 μM DHLA
7.5 μM fibrinogen + 15 μM DHLA
Absorbance at 670 nm
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

Figure 7