

Inhibition of Self-Assembling Peptide Fibrils Formation Using Thioflavin T as a Photosensitizer

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Abstract. Misfolded proteins produce fibrillar aggregates, which contain β -sheet higher order structures. The oligomers, protofibrils, and fibrils generated during protein aggregation process are cytotoxic and can cause various neurodegenerative diseases. Recently the photo-active materials, the photosensitizers, have attracted increased attention in the study and treatment of amyloid-related diseases. Here, we studied the photodynamic effect of the amyloid-specific fluorescence dye Thioflavin T on the formation of self-assembled peptide hydrogel. It was demonstrated that the gelation process under irradiation inhibits significantly, at that the structural and mechanical properties of mature fibrils change notably suggesting that ThT could be regarded as a theranostic probe. The developed peptide model allows for quantification of the photodynamic agent's efficiency in preventing aggregation, thus paving the way for a high-throughput test system for screening of light-responsive theranostic agents. © 2023 Journal of Biomedical Photonics & Engineering.

Keywords: hydrogel; peptide self-assembly; photosensitizer; fibrillation; scanning ion conductance microscopy; Thioflavin T; Fmoc-FF.

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1 Introduction

Protein conformation disorders accompany disruptions in nervous system functioning and are involved in etiology of neurodegenerative diseases [1, 2]. Misfolding and abnormal aggregation of β -amyloid, tau, α -synuclein, and polyglutamine containing proteins may result in the plaques deposition in brain, and cytotoxicity of protein aggregates, both fibrils and oligomers on the fibrillation pathway, is considered to be among the factors initiating

Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis [3–6].

Development of amyloid formation inhibitors has been the subject of numerous investigations, where both “chemical” and “physical” mechanisms of action were considered. While chemical approaches employ the use of specific biomolecules as amyloid inhibitors [7], physical strategies imply application of high hydrostatic pressure, low temperature, and irradiation with light [8]. In the latter case irradiation is supposed to induce

generation of reactive oxygen species (ROS, e.g., H_2O_2 , O_2^- , $\text{OH}^\cdot$, $^1\text{O}_2$) by photosensitizers such as light-responsive nanomaterials (fullerene, polyoxometalate, $\text{g-C}_3\text{N}_4$, etc.), organic dyes (riboflavin, rose bengal, porphyrins, etc.), metal oxide photocatalysts (Ag-TiO_2 , BiVO_4 , etc.) [9–12]. Among different photosensitizers, the methylene blue (MB) has been extensively used for photodynamic treatment of cancer cells due to its high solubility in aqueous media, low toxicity, ability to cross the blood-brain barrier, and high quantum yield of singlet molecular oxygen generation [13]. Lee et al. performed *in vivo* experiments where MB was supplied with food into the *Drosophila* organisms with model Alzheimer's disease, which was further illuminated by red LEDs. The high effectiveness of photodynamic treatment with MB as a sensitizer in suppressing synaptic toxicity and enhancing locomotion was demonstrated [14].

Another study demonstrated that the fluorescent dye Thioflavin T (ThT), which exhibits high affinity to amyloid fibrils [15, 16], could be used as a photosensitizer to destroy amyloid fibrils formed of the keratoepithelin peptide [17]. In the experiment, ThT molecules were incorporated into the fibrils and excited with pulsed 442 nm laser light, that led to the generation of ROS, inhibition of growth and destruction of fibrils.

ThT acts as a molecular rotor upon excitation, i.e., photon absorption results in the mutual rotation of two ThT fragments – benzothiazole ring and benzene ring, see Fig. 1 (benzothiazole ring is marked with blue color, benzene ring with yellow color) [18, 19]. Calculations

demonstrate that in the ground state the angle between these two fragments, Φ , is 37° . Upon light absorption, ThT is excited to a local excited state (LE) with the same geometry, from where intramolecular ultrafast relaxation process takes place: the angle Φ increases from 37° to 90° , resulting in the formation of twisted internal charge-transfer state (TICT), which is responsible for the ultrafast (~ 1 ps) relaxation of excitation. Hence, triplet yield and, consequently, singlet oxygen generation rate are negligible for free ThT in aqueous solution. On the contrary, upon binding to amyloid fibrils, a significant reduction of ThT flexibility occurs, thereby preventing intramolecular rotation and TICT state formation. Hence, ThT binding to fibrils leads to a 1000-fold increase of fluorescence lifetime (up to a few ns) and makes the population of the triplet state more efficient [20, 21]. As the result, only bound ThT molecules are efficient as photosensitizers. This fact suggests that ThT could be used as a theranostic agent: it stains fibrils selectively and potentially allows for their partial destruction via photodynamic action (Fig. 1). The aim of the paper was to verify this concept based on a robust and minimalistic model system.

For this purpose, we used the fibrillar hydrogel formed as the result of N-fluorenylmethoxycarbonyl diphenylalanine peptide (Fmoc-FF) self-assembly (Fig. 1A) [22–24]. Fmoc-FF is one of the most studied peptides for hydrogel preparation that alone or in combination with other substances can be used as a 3D scaffold for cartilage and bone tissue engineering [25].

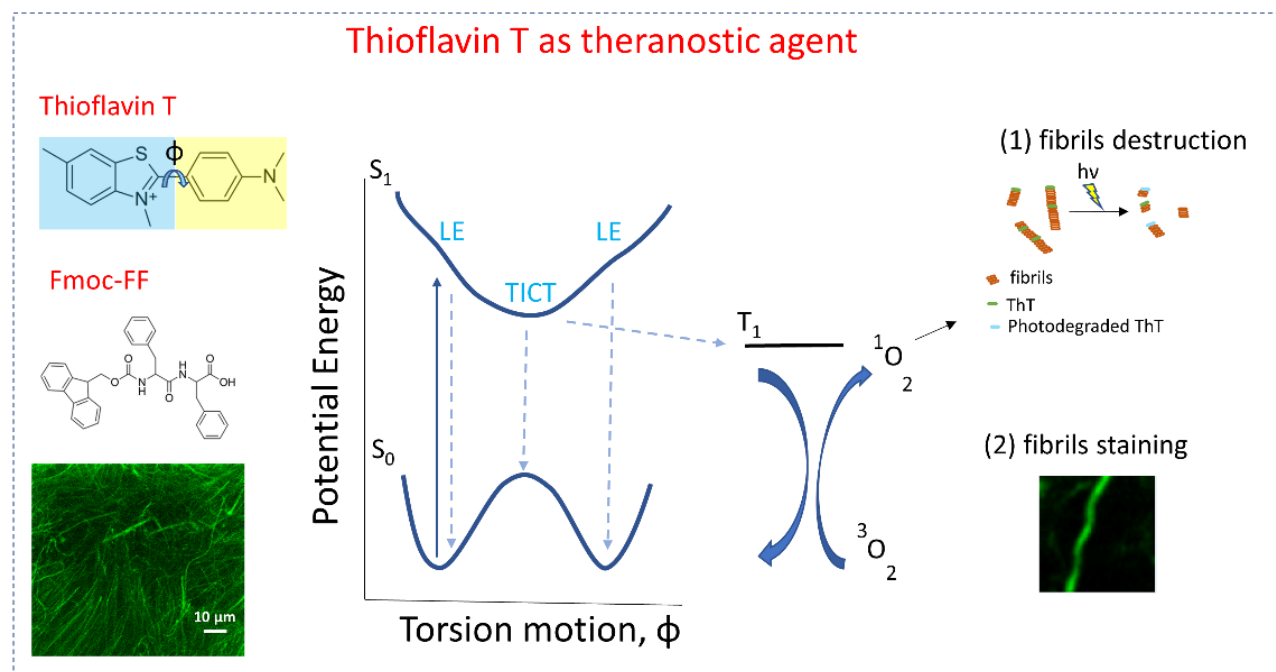


Fig. 1 The structure of ThT and Fmoc-FF (left) and schematic representation of the photophysical processes in ThT according to Ref. [18]: upon excitation into the local excited (LE) state, relative rotation of two fragments in a free ThT molecule (marked with blue and yellow rectangles in the ThT structure) occurs, leading to the emergence of the twisted internal charge transfer (TICT) state. The TICT state exhibits ultrafast relaxation to the ground state on a ~ 1 ps scale. Upon ThT binding to fibrils, its internal rotation is restricted, thus preventing the formation of the TICT state, leading to prolonged excited state relaxation (~ 1 ns) and increasing the efficiency of singlet-triplet conversion and singlet oxygen generation. Hence, ThT potentially allows for both staining and destruction of fibrils via the photosensibilisation effect.

It was shown that ThT not only could stain mature Fmoc-FF fibrils, but also could bind to the Fmoc-FF aggregates formed on the initial stages of hydrogelation [26]. The use of the peptide hydrogels as a model system for photodynamic ThT assay is attractive due to reproducibility of the kinetics of gel formation and certain similarity of interaction mechanisms upon peptide self-assembly and protein fibrils formation (π - π stacking, hydrophobic interactions). Moreover, the duration of fibrillation in the case of Fmoc-FF can be precisely tuned by external conditions and be as short as a few minutes, while for protein fibrils the aggregation process lasts from a few hours to a few weeks [27, 28]. Hence, peptide hydrogels could be a prospective system for testing physical approaches to fibrils disruption, including the photodynamic treatment.

In this paper we investigated the influence of photosensitization effect on the fibrillar structures formation by self-assembling Fmoc-FF peptide by a set of methods including fluorescence spectroscopy, fluorescence lifetime imaging microscopy (FLIM), and scanning ion-conductance microscopy (SICM). The kinetics of self-assembly and related morphological alterations were studied upon irradiation of the system in the presence of ThT. The developed model allows for quantification of the photodynamic agent's efficiency in preventing aggregation, thus paving the way for a high-throughput test system for screening of light-responsive theranostic agent.

2 Materials and Methods

2.1 Sample Preparation

The N-fluorenylmethoxycarbonyl diphenylalanine peptide (Fmoc-FF) was purchased from GL Biochem (Shanghai, China). Thioflavin T (ThT) was obtained from Sigma-Aldrich (Steinheim, Germany). The Fmoc-FF hydrogel was prepared using the solvent-switch method: peptide stock solution in dimethyl sulfoxide, $C_{\text{Fmoc-FF}}(\text{stock}) = 100 \text{ mg/mL}$, was dissolved with double distilled water to a final concentration of $C_{\text{Fmoc-FF}} = 1 \text{ mg/mL}$ [29, 30]. The final pH value of the samples was set to 5.

To calculate the ThT concentration, the molar extinction coefficient of $31600 \text{ l}/(\text{M}\cdot\text{cm})$ at 410 nm was used [31]. The temperature in the experiments was controlled and set to 25°C . ThT concentration was $C_{\text{ThT}} = 70 \text{ }\mu\text{M}$ in all the experiments. So, the ratio of peptide to dye concentration in all the experiment was constant and equal to $C_{\text{Fmoc-FF}}/C_{\text{ThT}} = 26$.

Photosensitization was induced by irradiating the samples at 460 nm with a LED ($80 \text{ mW}/\text{cm}^2$).

The sample preparation for each method was as follows:

1) Turbidity measurements. The ratio of peptide to dye concentration was constant, $C_{\text{Fmoc-FF}}/C_{\text{ThT}} = 26$ ($C_{\text{Fmoc-FF}} = 1865 \text{ }\mu\text{M}$, $C_{\text{ThT}} = 70 \text{ }\mu\text{M}$). The irradiation time was varied (0, 5, 10, 40 min), corresponding to irradiation doses of 0, 24, 48, and $192 \text{ J}/\text{cm}^2$.

2) FLIM measurements. The ratio of peptide to dye concentration was constant, $C_{\text{Fmoc-FF}}/C_{\text{ThT}} = 26$ ($C_{\text{Fmoc-FF}} = 1865 \text{ }\mu\text{M}$, $C_{\text{ThT}} = 70 \text{ }\mu\text{M}$). Excitation was performed in the two-photon excitation regime, the excitation wavelength was set to 900 nm . The protocol was as follows: first, a ROI of $10\times 10 \text{ }\mu\text{m}$ was irradiated for a certain time, and then a scan of a larger field, including the irradiated ROI, was performed – hence, both irradiated and non-irradiated areas were assessed.

3) Scanning ion-conductance microscopy (SICM). By this technique the structures of mature hydrogels were examined in the solution – the hydrogel that was formed without irradiation ($C_{\text{Fmoc-FF}} = 1865 \text{ }\mu\text{M}$) and the hydrogel that was formed after the sample was prepared and irradiated for 1 h with a 460 nm LED ($80 \text{ mW}/\text{cm}^2$, the irradiation dose $288 \text{ J}/\text{cm}^2$). The samples were prepared in a Petri dish and filled with acetate buffer, pH 5.

4) Atomic force microscopy (AFM) measurements. The method of sample preparation was the same as in the SICM measurements except for the moment that the samples were dried for 2 h after the hydrogels (with or without irradiation) were formed.

5) Measurement of the rheological properties of the samples. The mechanical properties of hydrogels were examined during its formation without irradiation ($C_{\text{Fmoc-FF}} = 1865 \text{ }\mu\text{M}$, $C_{\text{ThT}} = 70 \text{ }\mu\text{M}$) and when the hydrogel was irradiated for 40 min with a LED (460 nm , $80 \text{ mW}/\text{cm}^2$, the irradiation dose $192 \text{ J}/\text{cm}^2$).

So, turbidity, FLIM and rheological measurements were carried out during the process of hydrogel formation, whereas the microscopy studies – AFM and SICM, were carried out when the hydrogel had been already formed to analyze the final structure of investigated system.

2.2 Turbidity Measurements

Turbidity measurements were performed by measuring the transmission of a 633 nm laser through the cuvette (with a 2 mm optical path) with a spectrometer Maya 2000 PRO (Ocean Optics, USA) in a 180° geometry. The exposure time was set to 100 ms for each measurement point.

2.3 Fluorescence Lifetime Imaging

Microscopy (FLIM) Measurements

For FLIM measurements, a custom setup equipped with a Simple Tau 152 TCSPC FLIM module (Becker & Hickl GmbH, Berlin, Germany) was used. Fluorescence was excited by radiation from a TOPOL parametric femtosecond generator (Avesta, Moscow, Russia). Two-photon excitation was carried out at 900 nm with a pulse repetition rate of 80 MHz . The duration of one pulse was 100 fs . A lens with a $60\times$ magnification $\text{NA} = 1.4$ (CFI Plan Apochromat Lambda $60\times$, Nikon, Japan) was used.

2.4 Measurement of the Rheological Properties of the Samples

The Physica MCR 302 rheometer (Anton Paar GmbH, Graz, Austria) was used to study the mechanical properties of hydrogel with a parallel plate geometry (25 mm in diameter). Time sweep oscillatory measurements were carried out immediately after preparation of samples. The measurements were carried out at 25 °C.

2.5 Scanning Ion-Conductance Microscopy

The SICM (ICAPPIC Limited, United Kingdom) was used to investigate the samples topography. In the experiments the inverted optical microscope Eclipse Ti-2 (Nikon, Japan) was used. The feedback control and piezo positioning was controlled by the ICAPPIC Universal Controller and Piezo Control System (ICAPPIC Limited, United Kingdom). A P-2000 laser puller (Sutter Instruments, USA) was used to fabricate the pipettes with inner radius 40–50 nm. The approach rate was 120 $\mu\text{m}\cdot\text{s}^{-1}$. The set point was 0.5% for topography studies.

In SICM, the scanning probe is a glass pipette that is filled with buffer with a radius on the order of several tens of nanometers. The main Ag/AgCl electrode is

placed inside the pipette whereas the reference electrode is in the Petri dish where the sample is situated. The ion current begins to flow through the pipette tip when the potential bias is applied between Ag/AgCl electrodes. Away from the sample surface the ion current has a constant value at that it starts to decrease when the tip-sample surface distance is smaller than the probe tip radius. To obtain the topography of the sample, a feedback system is used based on the registration of the ion current flowing through the pipette and the coordinates of the piezo actuators [32].

2.6 Atomic Force Microscopy Measurements

Atomic force microscopy (AFM) photos of the dried hydrogels were obtained using a Bruker Bioscope Resolve AFM (Bruker, USA). The Peak Force Tapping mode was used to minimize the tip tapping force. We used the ScanAsyst Air cantilevers (Bruker AFM Probes, Camarillo, CA, USA) with a nominal spring constant of 0.4 N/m and a tip radius of 2 nm. $10 \times 10 \mu\text{m}$ and $3 \times 3 \mu\text{m}$ images were obtained at a maximal force $< 2 \text{ nN}$, at a resolution of 256×256 or 512×512 pixels. The measurements were carried out at 25 °C for dried samples.

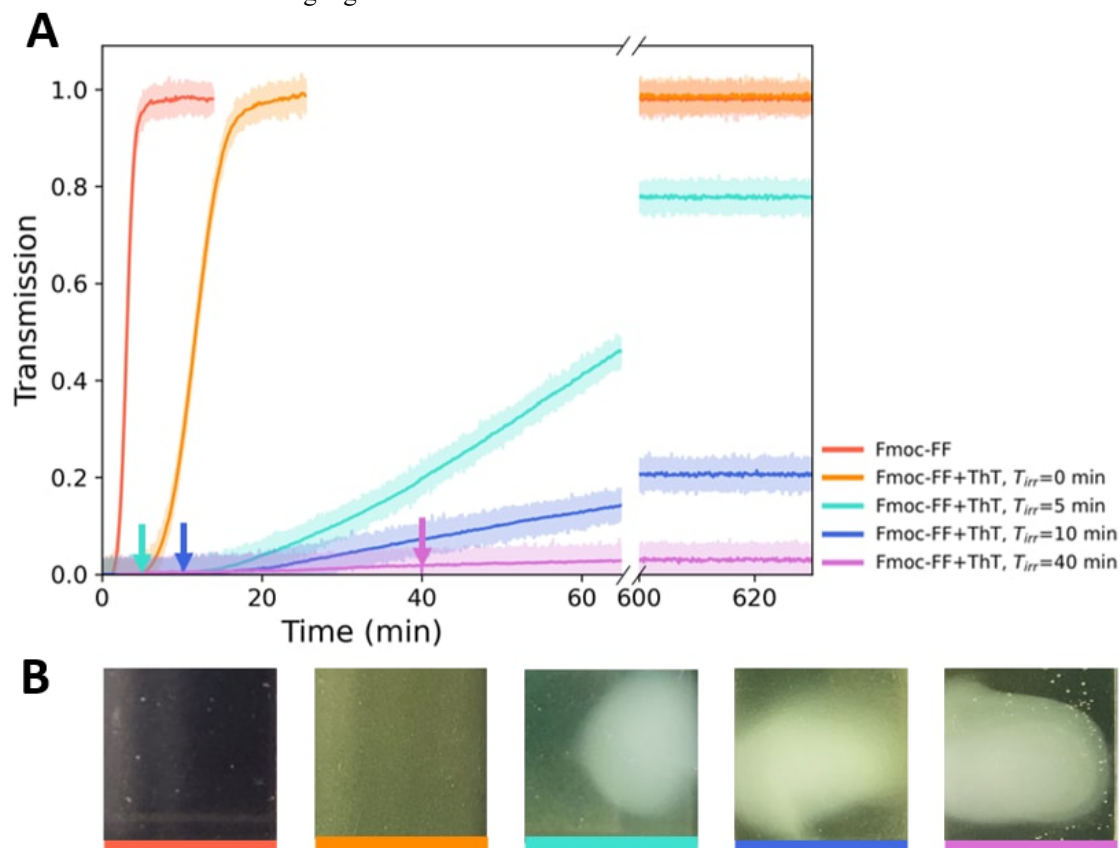


Fig. 2 (A) Irradiation time dependence of the Fmoc-FF+ThT gelation kinetics as monitored by light transmission. $C_{\text{Fmoc-FF}} = 1865 \mu\text{M}$, $C_{\text{ThT}} = 70 \mu\text{M}$ ($C_{\text{Fmoc-FF}}/C_{\text{ThT}} = 26$). The irradiation power was constant, $I = 80 \text{ mW}/\text{cm}^2$. The irradiation wavelength was $\lambda = 460 \text{ nm}$. (B) The images of the Fmoc-FF systems after their transmittance curves reached a plateau and the hydrogel was formed. Red, orange, greenish-blue, blue, and violet lines correspond to Fmoc-FF in the absence of ThT and Fmoc-FF in the presence of ThT upon irradiation during 0, 2.5, 5, 10, and 40 min. The arrows indicate where irradiation was switched off for each curve.

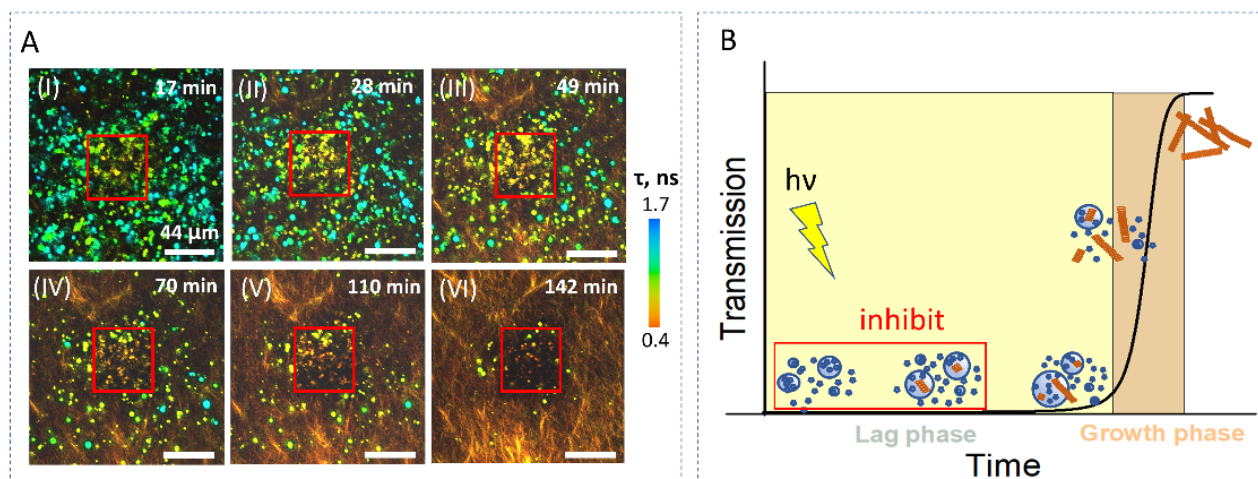


Fig. 3 (A) FLIM images of the Fmoc-FF hydrogelation process measured at different time. The red square depicts the area of irradiation. In the right upper corner of the images, the time from the beginning of the gelation can be seen. $C_{\text{Fmoc-FF}} = 1865 \mu\text{M}$, $C_{\text{ThT}} = 70 \mu\text{M}$ (Scale bar 44 μm). (B) Schematic of the Fmoc-FF peptide hydrogelation process tracked via transmission.

3 Results and Discussion

3.1 The Influence of Irradiation on Hydrogel Formation in the Presence of ThT

Turbidity measurements allow to observe the process of Fmoc-FF solution transition from the opaque into the optically transparent system during the hydrogel formation [27]. Hence, as the first step, the effect of the time irradiation on the Fmoc-FF self-assembly rate was assessed in the presence of ThT. Measuring the transmittance of the Fmoc-FF system under the irradiation revealed a gradual elongation of the hydrogelation kinetics, see Fig. 2A. The experiments performed at a fixed concentration of the peptide ($C_{\text{Fmoc-FF}} = 1865 \mu\text{M}$) and ThT ($C_{\text{ThT}} = 70 \mu\text{M}$) while varying irradiation time (0, 5, 10, and 40 min). Importantly, no effect of irradiation on the Fmoc-FF hydrogel formation without ThT was observed, see Fig. S1.

As it can be seen from Fig. 2B, the irradiated area doesn't become transparent, moreover, the gelation kinetics slows down strongly under irradiation (see Fig. 2A). At the same time in the unirradiated area the transparency is observed. So, the irradiation of peptide in the presence of ThT leads to the inhibition of self-assembly. The image of the Fmoc-FF system at the beginning of hydrogel formation can be seen in Fig. S2.

3.2 Hydrogel Structural Phase Transition and Its Dependence on the Irradiation Time as Revealed by FLIM

To study how irradiation affects the structure of Fmoc-FF aggregates in the presence of ThT, the FLIM measurements were carried out. We note that FLIM allows both for imaging of aggregates in the system as

well as for mapping the parameters of ThT microenvironment (mainly, viscosity) by mapping the ThT fluorescence lifetime [33, 34]. Fig. 3A demonstrates representative FLIM images obtained during the Fmoc-FF hydrogelation, where the red square depicts the irradiated area.

A number of stages could be observed under the Fmoc-FF hydrogel formation. When a dilution of the Fmoc-FF stock solution in water occurs, spherical aggregates can be seen (Fig. 3A(I–II)), that evolve into larger spheres after a while. Then, the transition from spheres to fibers was observed (Fig. 3A(III–IV)), at that the mature fibrillar gel was the terminal stage of the self-assembly process (Fig. 3A(V–VI)). The FLIM image for the final hydrogel with the irradiation of the central part, like Fig. 3A(VI) with smaller scale can be seen in Fig. S3, where the significant difference between irradiated and non-irradiated part of the hydrogel can be seen.

Fig. 3A(I) demonstrated that in 17 min the hydrogel had not been formed in the presence of ThT, see the non-irradiated area (out of red square). This points on the fact that ThT has a strong effect on the gelation process. This data is in accordance with work where it was revealed that ThT has a direct effect on the structural properties of the Fmoc-FF hydrogel. The kinetics of gelation changed significantly in the presence of dye: the growth rate decreases and increase of the lag phase duration were observed.

FLIM demonstrated that irradiation of the Fmoc-FF system in the presence of ThT effects the lag phase of hydrogel formation. Fig. 3A(VI) shows that while the Fmoc-FF formed fibrillar structures outside the area of irradiation, the irradiated area (red square) still contains only spherical aggregates that corresponds to the lag-phase of the Fmoc-FF peptide hydrogelation process (schematically shown in Fig. 3B).

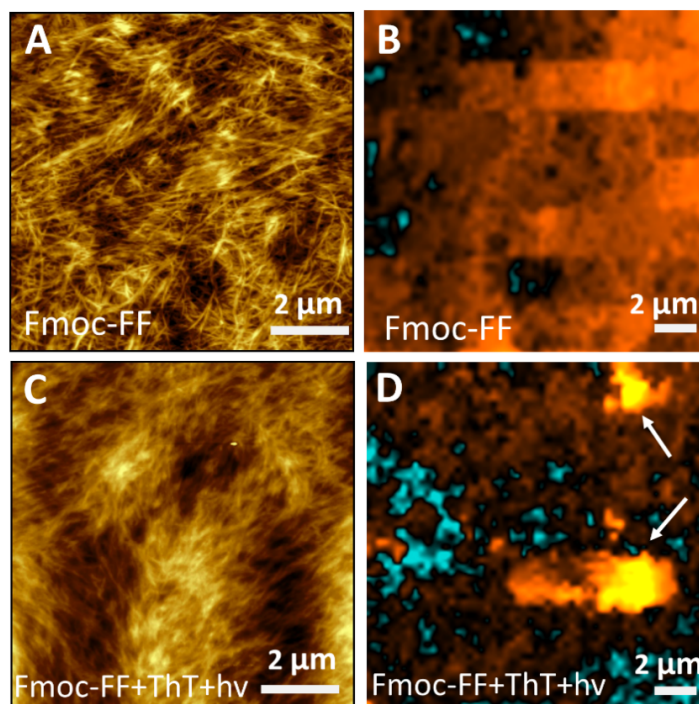


Fig. 4 Structural analysis of Fmoc-FF aggregates in the presence of ThT formed during self-assembly in the absence and presence of irradiation. AFM images for (A) Fmoc-FF hydrogel (C) Fmoc-FF aggregates formed in the presence of ThT under irradiation. SICM images for (B) Fmoc-FF hydrogel (D) Fmoc-FF hydrogel formed in the presence of ThT under the irradiation. By arrows the peptide aggregates are shown in the formed hydrogel. $C_{\text{Fmoc-FF}} = 1865 \mu\text{M}$, $C_{\text{ThT}} = 70 \mu\text{M}$. The samples were irradiated for 1 h with a diode, $\lambda_{\text{ex}} = 460 \text{ nm}$, $I = 80 \text{ mW/cm}^2$.

3.3 The Effect of Photosensitizer on the Morphology and Structure of Fibers in the Fmoc-FF Hydrogel

We also assessed the morphology of the formed Fmoc-FF aggregates using two techniques – AFM and SICM. AFM measurements were performed on dried samples (Fig. 4A, C), while the SICM method allowed to obtain the topography of the surface of the sample under the native conditions (in aqueous solution) in a non-contact mode (Fig. 4B, D) [35–37].

Both methods revealed topography with high resolution – the fibrils were characterized by several hundred nanometers thickness and several micrometers length. In the case of SICM technique the mature fibrils are thicker as they “swelled” in aqueous solution in comparison with dried AFM samples, see Fig. 4A, B, correspondingly. The average thickness of fibrils that were dried overnight and obtained by AFM technique was $d = 0.33 \mu\text{m}$, while for fibrils that were studied in aqueous solution by SICM method this value was $d = 0.43 \mu\text{m}$.

According to the obtained data it can be stated that the fibers formed in the presence of photosensitizer (ThT) under the irradiation are thicker and shorter (see Fig. 4C, D) compared to the fibers formed at the absence of ThT and irradiation (see Fig. 4A, B). Also, the irradiated Fmoc-FF+ThT system demonstrated the presence of large spherical aggregated, that are marked

with arrows ($\sim 2\text{--}3 \mu\text{m}$, Fig. 3D), which are responsible for the turbidity of the irradiated area (Fig. 2A).

So, summarizing microscopy measurements it can be said that FLIM investigations demonstrated that the irradiation influences namely the lag phase during hydrogel formation: irradiation leads to the situation when a great amount of peptide aggregates stays in the form of spheres and do not transform into the protofibrils. SICM and AFM studies provided information that when the irradiation is stopped the hydrogel is formed though its structure differs from the structure of hydrogel that is formed without irradiation: the mature irradiated hydrogel consists of shorter fibrils, also in its structure big peptide aggregates are observed.

3.4 The Effect of Photosensitizer on the Mechanical Properties of the Fmoc-FF Hydrogel

Herein the rheological measurements were carried out in order to study the effect of photosensitizer on the mechanical characteristics of self-assembled hydrogel. The dependence of the storage modulus G' on the gelation time for Fmoc-FF+ThT hydrogel without irradiation (black line) and with 40 min of irradiation (red line) (the dose of irradiation was $d = 192 \text{ J/m}^2$) is shown in Fig. 5A. The dependence of the storage modulus on time for pure Fmoc-FF system without irradiation can be seen in Fig. S4.

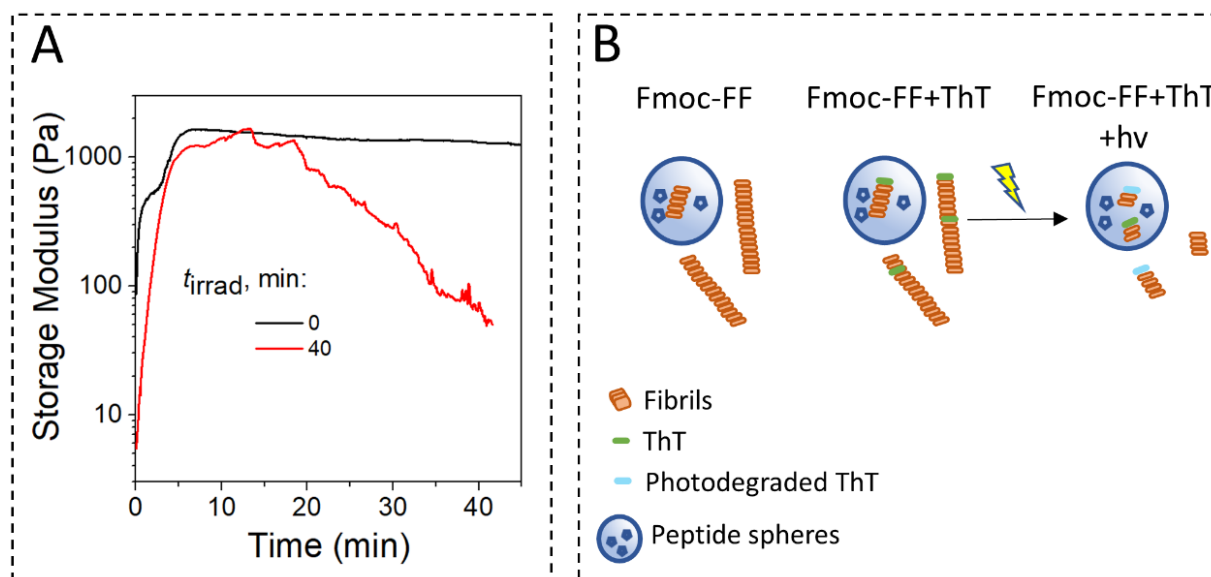


Fig. 5 (A) The dependence of storage modulus on time for Fmoc-FF + ThT hydrogel without irradiation (black line) and with 40 min of irradiation (red line), (B) An illustration of dissociation of hydrogel fibrils by photo-excited ThT.

The storage modulus G' for the investigated system reached a plateau of 1800 Pa after 5 min. The photo-excitation of photosensitizer leads to the situation when initially the storage modulus grows to the value 1800 Pa (like for Fmoc-FF+ThT system without irradiation) whereafter the softening of hydrogel structure under irradiation over time is observed (see green line in Fig. 5A). This process can be explained as follows: due to the photosensitization the oxidation of peptides occurs. This process can be a reason for the weakening of the interaction energy between peptides and further destruction of intermolecular peptides bonds that can lead to the decrease of systems' Young modulus, see Fig. 5B.

The destructive effect of ThT on different amyloid structures were demonstrated in literature. Ozawa et. al studied the irradiation of 442 nm laser with nm on β 2-microglobulin (β 2-m) in the presence of 10 μ M ThT and showed that irradiation inhibited fibrillation and led to partial destruction of preformed fibrils [38]. Also, the effect of laser beam on A β (1–40) fibrils in the presence of ThT was studied [39], where irradiation also led to partial destruction of fibrils. The effect of laser irradiation on keratoepithelin in the presence of Thioflavin T was demonstrated [17]. The generation of ROS from photoexcited fluorescence dye was a reason for fibril destruction. By Ahn et al. the chemical and mechanistic study of photodynamic inhibition of Alzheimer's amyloids in the presence of ThT was conducted [40].

The chemical modifications of main amino acid residues of due to photoexcited ThT were observed using blue LED light (450 nm). The quantitative chemical kinetics analysis suggested that the oxidized monomer species did not aggregate. The authors described the oxidation of aggregates by the generation of singlet oxygen by photoexcited ThT. These results are in

accordance with the data of the present work that shown that ThT can be regarded as a photosensitizer its presence has a direct effect on the structural and mechanical properties of the Fmoc-FF hydrogel.

4 Conclusions

The results of the present work show that ThT can be regarded as a photosensitizer its presence has an influence on Fmoc-FF hydrogel properties. The kinetics of hydrogel formation varied in the presence of photo-excited fluorescence dye: increase of the lag phase duration was observed. FLIM studies demonstrated that upon irradiation inhibition of hydrogel formation occurs due to the slow down of the metastable spheres transition to the protofibrils. Moreover, the irradiation of Fmoc-FF + ThT system leads to the shortening and softening of the final fibrillar structure of hydrogel suggesting that ThT can be regarded as a theranostic probe: (1) due to its presence in hydrogel the laser-induced destruction of fibrils was observed, (2) the ThT is a gold standard for amyloids staining.

Disclosures

Authors declare no competing interests.

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References

1. C. Soto, S. Pritzkow, "Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases," *Nature Neuroscience* 21(10), 1332–1340 (2018).
2. A. B. Diack, J. D. Alibhai, R. Barron, B. Bradford, P. Piccardo, and J. C. Manson, "Insights into mechanisms of chronic neurodegeneration," *International Journal of Molecular Sciences* 17(1), 82 (2016).
3. C. Peng, J. Q. Trojanowski, and V. M. Y. Lee, "Protein transmission in neurodegenerative disease," *Nature Reviews Neurology* 16(4), 199–212 (2020).
4. C. Di Scala, N. Yahi, S. Boutemour, A. Flores, L. Rodriguez, H. Chahinian, and J. Fantini, "Common molecular mechanism of amyloid pore formation by Alzheimer's β -amyloid peptide and α -synuclein," *Scientific Reports* 6(1), 28781 (2016).
5. C. A. Ross, M. A. Poirier, "What is the role of protein aggregation in neurodegeneration?" *Nature Reviews Molecular Cell Biology* 6(11), 891–898 (2005).
6. F. J. B uerlein, R. Fern andez-Busnadiego, and W. Baumeister, "Investigating the structure of neurotoxic protein aggregates inside cells," *Trends in Cell Biology* 30(12), 951–966 (2020).
7. P. Velander, L. Wu, F. Henderson, S. Zhang, D. R. Bevan, and B. Xu, "Natural product-based amyloid inhibitors," *Biochemical Pharmacology* 139, 40–55 (2017).
8. R. Liu, R. Su, M. Liang, R. Huang, M. Wang, W. Qi, and Z. He, "Physicochemical strategies for inhibition of amyloid fibril formation: an overview of recent advances," *Current Medicinal Chemistry* 19(24), 4157–4174 (2012).
9. B. I. Lee, Y. J. Chung, and C. B. Park, "Photosensitizing materials and platforms for light-triggered modulation of Alzheimer's β -amyloid self-assembly," *Biomaterials* 190, 121–132 (2019).
10. B. S. Dash, S. Das, and J. P. Chen, "Photosensitizer-functionalized nanocomposites for light-activated cancer theranostics," *International Journal of Molecular Sciences* 22(13), 6658 (2021).
11. K. Kannan, D. Radhika, K. K. Sadasivuni, K. R. Reddy, and A. V. Raghu, "Nanostructured metal oxides and its hybrids for photocatalytic and biomedical applications," *Advances in Colloid and Interface Science* 281, 102178 (2020).
12. J. S. Lee, B. I. Lee, and C. B. Park, "Photo-induced inhibition of Alzheimer's β -amyloid aggregation in vitro by rose bengal," *Biomaterials* 38, 43–49 (2015).
13. M. Oz, D. E. Lorke, M. Hasan, and G. A. Petroianu, "Cellular and molecular actions of Methylene Blue in the nervous system," *Medicinal Research Reviews* 31(1), 93–117 (2011).
14. B. I. Lee, Y. S. Suh, Y. J. Chung, K. Yu, and C. B. Park, "Shedding light on Alzheimer's β -amyloidosis: photosensitized methylene blue inhibits self-assembly of β -amyloid peptides and disintegrates their aggregates," *Scientific Reports* 7(1), 7523 (2017).
15. N. R. Rovnyagina, G. S. Budylin, Y. G. Vainer, T. N. Tikhonova, S. L. Vasin, A. A. Yakovlev, V. O. Kompanets, S. V. Chekalin, A. V. Priezzhev, and E. A. Shirshin, "Fluorescence lifetime and intensity of thioflavin T as reporters of different fibrillation stages: Insights obtained from fluorescence up-conversion and particle size distribution measurements," *International Journal of Molecular Sciences* 21(17), 6169 (2020).
16. B. Friege, L. Gremer, H. Heise, D. Willbold, and H. Gohlke, "Binding modes of thioflavin T and Congo red to the fibril structure of amyloid- β (1–42)," *Chemical Communications* 56(55), 7589–7592 (2020).
17. D. Ozawa, Y. Kaji, H. Yagi, K. Sakurai, T. Kawakami, H. Naiki, and Y. Goto, "Destruction of amyloid fibrils of keratoepithelin peptides by laser irradiation coupled with amyloid-specific thioflavin T," *Journal of Biological Chemistry* 286(12), 10856–10863 (2011).
18. V. I. Stsiapura, A. A. Maskevich, V. A. Kuzmitsky, V. N. Uversky, I. M. Kuznetsova, and K. K. Turoverov, "Thioflavin T as a molecular rotor: fluorescent properties of thioflavin T in solvents with different viscosity," *The Journal of Physical Chemistry B* 112(49), 15893–15902 (2008).
19. F. Y. Khusbu, X. Zhou, H. Chen, C. Ma, and K. Wang, "Thioflavin T as a fluorescence probe for biosensing applications," *Trends in Analytical Chemistry* 109, 1–18 (2018).
20. V. I. Stsiapura, A. A. Maskevich, S. A. Tikhomirov, and O. V. Baganov, "Charge transfer process determines ultrafast excited state deactivation of thioflavin T in low-viscosity solvents," *The Journal of Physical Chemistry A* 114(32), 8345–8350 (2010).
21. N. Amdursky, Y. Erez, and D. Huppert, "Molecular rotors: what lies behind the high sensitivity of the thioflavin-T fluorescent marker," *Accounts of Chemical Research* 45(9), 1548–1557 (2012).
22. A. M. Smith, R. J. Williams, C. Tang, P. Coppo, R. F. Collins, M. L. Turner, A. Saiani, and R. V. Ulijn, "Fmoc-diphenylalanine self assembles to a hydrogel via a novel architecture based on π - π interlocked β -sheets," *Advanced Materials* 20(1), 37–41 (2008).
23. C. Diaferia, M. Ghosh, T. Sibillano, E. Gallo, M. Stornaiuolo, C. Giannini, G. Morelli, L. Adler-Abramovich, and A. Accardo, "Fmoc-FF and hexapeptide-based multicomponent hydrogels as scaffold materials," *Soft Matter* 15(3), 487–496 (2019).

24. R. Orbach, I. Mironi-Harpaz, L. Adler-Abramovich, E. Mossou, E. P. Mitchell, V. T. Forsyth, E. Gazit, and D. Seliktar, "The rheological and structural properties of Fmoc-peptide-based hydrogels: the effect of aromatic molecular architecture on self-assembly and physical characteristics," *Langmuir* 28(4), 2015–2022 (2012).
25. E. Rosa, C. Diaferia, E. Gianolio, T. Sibillano, E. Gallo, G. Smaldone, M. Stornaiuolo, C. Giannini, G. Morelli, and A. Accardo, "Multicomponent Hydrogel Matrices of Fmoc-FF and Cationic Peptides for Application in Tissue Engineering," *Macromolecular Bioscience* 22(7), 2200128 (2022).
26. T. N. Tikhonova, N. R. Rovnyagina, Z. A. Arnon, B. P. Yakimov, Y. M. Efremov, D. Cohen-Gerassi, M. Halperin-Sternfeld, N. V. Kosheleva, V. P. Drachev, A. A. Svistunov, P. S. Timashev, L. Adler-Abramovich, and E. A. Shirshin, "Mechanical Enhancement and Kinetics Regulation of Fmoc-Diphenylalanine Hydrogels by Thioflavin T," *Angewandte Chemie International Edition* 60(48), 25339–25345 (2021).
27. K. Lundmark, G. T. Westermark, A. Olsén, and P. Westermark, "Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism," *Proceedings of the National Academy of Sciences* 102(17), 6098–6102 (2005).
28. C. Li, J. Adamcik, and R. Mezzenga, "Biodegradable nanocomposites of amyloid fibrils and graphene with shape-memory and enzyme-sensing properties," *Nature Nanotechnology* 7(7), 421–427 (2012).
29. V. Basavalingappa, T. Guterman, Y. Tang, S. Nir, J. Lei, P. Chakraborty, L. Schnaider, M. Reches, G. Wei, and E. Gazit, "Expanding the functional scope of the fmoc-diphenylalanine hydrogelator by introducing a rigidifying and chemically active urea backbone modification," *Advanced Science* 6(12), 1900218 (2019).
30. J. Raeburn, G. Pont, L. Chen, Y. Cesbron, R. Lévy, and D. J. Adams, "Fmoc-diphenylalanine hydrogels: understanding the variability in reported mechanical properties," *Soft Matter* 8(4), 1168–1174 (2012).
31. A. I. Sulatskaya, A. V. Lavysh, A. A. Maskevich, I. M. Kuznetsova, and K. K. Turoverov, "Thioflavin T fluoresces as excimer in highly concentrated aqueous solutions and as monomer being incorporated in amyloid fibrils," *Scientific Reports* 7(1), 2146 (2017).
32. A. Page, D. Perry, and P. R. Unwin, "Multifunctional scanning ion conductance microscopy," *Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences* 473(2200), 20160889 (2017).
33. B. Yakimov, A. Gayer, E. Maksimov, E. Mamonov, A. Maydykovsky, T. Murzina, V. Fadeev, and E. Shirshin, "Fluorescence saturation imaging microscopy: molecular fingerprinting in living cells using two-photon absorption cross section as a contrast mechanism," *Optics Letters* 47(17), 4455–4458 (2022).
34. S. Kundu, C. Banerjee, and N. Sarkar, "Inhibiting the fibrillation of serum albumin proteins in the presence of surface active ionic liquids (SAILs) at low pH: spectroscopic and microscopic study," *The Journal of Physical Chemistry B* 121(32), 7550–7560 (2017).
35. V. S. Kolmogorov, A. S. Erofeev, E. Woodcock, Y. M. Efremov, A. P. Iakovlev, N. A. Savin, A. V. Alova, S. V. Lavrushkina, I. I. Kireev, A. O. Prelovskaya, E. V. Sviderskaya, D. Scaini, N. L. Klyachko, P. S. Timashev, Y. Takahashi, S. V. Salikhov, Y. N. Parkhomenko, A. G. Majouga, C. R. W. Edwards, P. Novak, Y. E. Korchev, and P. V. Gorelkin, "Mapping mechanical properties of living cells at nanoscale using intrinsic nanopipette-sample force interactions," *Nanoscale* 13(13), 6558–6568 (2021).
36. Y. Zhang, Y. Takahashi, S. P. Hong, F. Liu, J. Bednarska, P. S. Goff, P. Novak, A. Shevchuk, S. Gopal, I. Barozzi, L. Magnani, H. Sakai, Y. Suguru, T. Fujii, A. Erofeev, P. Gorelkin, A. Majouga, D. J. Weiss, C. Edwards, A. P. Ivanov, D. Klennerman, E. V. Sviderskaya, J. B. Edel, and Y. Korchev, "High-resolution label-free 3D mapping of extracellular pH of single living cells," *Nature Communications* 10(1), 5610 (2019).
37. Y. Takahashi, A. I. Shevchuk, P. Novak, Y. Murakami, H. Shiku, Y. E. Korchev, and T. Matsue, "Simultaneous noncontact topography and electrochemical imaging by SECM/SICM featuring ion current feedback regulation," *Journal of the American Chemical Society* 132(29), 10118–10126 (2010).
38. D. Ozawa, H. Yagi, T. Ban, A. Kameda, T. Kawakami, H. Naiki, and Y. Goto, "Destruction of amyloid fibrils of a β 2-microglobulin fragment by laser beam irradiation," *Journal of Biological Chemistry* 284(2), 1009–1017 (2009).
39. H. Yagi, D. Ozawa, K. Sakurai, T. Kawakami, H. Kuyama, O. Nishimura, T. Shimanouchi, R. Kuboi, H. Naiki, and Y. Goto, "Laser-induced propagation and destruction of amyloid β fibrils," *Journal of Biological Chemistry* 285(25), 19660–19667 (2010).
40. M. Ahn, B. I. Lee, S. Chia, J. Habchi, J. R. Kumita, M. Vendruscolo, C. Dobson, and C. B. Park, "Chemical and mechanistic analysis of photodynamic inhibition of Alzheimer's β -amyloid aggregation," *Chemical Communications* 55(8), 1152–1155 (2019).