

Controlling Supramolecular Fiber Formation of Nucleopeptide by Guanosine Triphosphate

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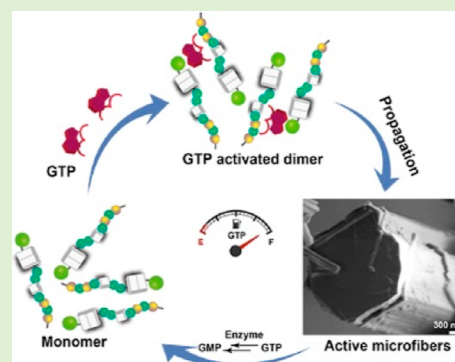


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Supporting Information

ABSTRACT: Cells use dynamic self-assembly to construct functional structures for maintaining cellular homeostasis. However, using a natural biological small molecule to mimic this phenomenon remains challenging. This work reports the dynamic microfiber formation of nucleopeptide driven by guanosine triphosphate, the small molecule that controls microtubule polymerization in living cells. Deactivation of GTP by enzyme dissociates the fibers, which could be reactivated by adding GTP. Molecular dynamic simulation unveils the mystery of microfiber formation of GBM-1 and GTP. Moreover, the microfiber formation can also be controlled by diffusion-driven GTP gradients across a semipermeable membrane in bulk conditions and the microfluidic method in the defined droplets. This study provides a new platform to construct dynamic self-assembly materials of molecular building blocks driven by GTP.



INTRODUCTION

Dynamic self-assembly is a ubiquitous phenomenon in living organisms, which requires multiple factors (e.g., the reversible transformation or continuous supply of building blocks) to maintain the dynamics of the higher-order assembled state.¹ The classic example is the formation of active self-assembled structures of the cytoskeleton (e.g., actin filaments and microtubule) driven by the ATP and GTP to perform sophisticated functions.^{2–4} For example, GTP activates tubulin dimer formation and coassembles to form the microtubule.⁵ The hydrolysis of GTP deactivates tubulin dimers to disassemble the microtubule. Such dynamic processes control the polymerization of the microtubule, resulting in diverse functions in living cells.³ These reversible and fascinating self-assembly properties in living organisms have inspired the development of dynamic materials with controllable functions.^{6–23}

To achieve dynamic control of the self-assembly of small molecules, suitable triggers with the ability to switch the formation of assemblies play essential roles. For example, phosphatase and kinase have been applied to control the reversible peptide self-assembly in one component.^{10,24,25} Light is another trigger to control the reversible self-assembly of small molecules.^{26,27} Very recently, van Esch and co-workers reported that alkylating agent activates the building block by methylation to form a nanofibrous network, and the hydrolysis of the methylated building block disassembles the fibers.²⁸ The transformation could cycle again by adding an alkylating agent. Xu group used two enzymes to control the peptide assembly and disassembly processes for targeting down regulation in

cancer cells.²⁹ By intelligent design, the group of Ulijn has developed a biocatalytic dynamic self-assembled system that uses chemical energy stored in a methyl ester precursor.¹⁸ Inspired by the pioneering work, we hypothesized that small molecules could be developed by rational design to form adaptive assemblies that respond to bimolecular molecules (e.g., GTP) reversibly controlled in situ.

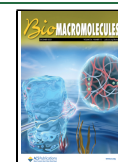
In this work, we reported a dynamic self-assembly system that could be controlled by the addition of GTP and enzymes. As shown in Figure 1, GTP activates nucleopeptide GBM-1 to self-assemble into microfibers with microscale size. Hydrolysis of the GTP by the enzyme deactivates GBM-1 and dissociates the microfibers, which can be activated again by adding fresh GTP. The results indicate that GTP could coassemble with GBM-1 to form a dimer, which could elongate to form microfibers. MD simulations and amino acid mutation experiments suggest that the interactions between cytosine from GBM-1 and guanine from GTP and the electrostatic interactions between lysine residues and phosphate groups stabilize the resulting microfibers. The fiber formation can also be controlled by diffusion-driven GTP gradients across a semipermeable membrane in bulk conditions and the microfluidic method in the defined droplets.

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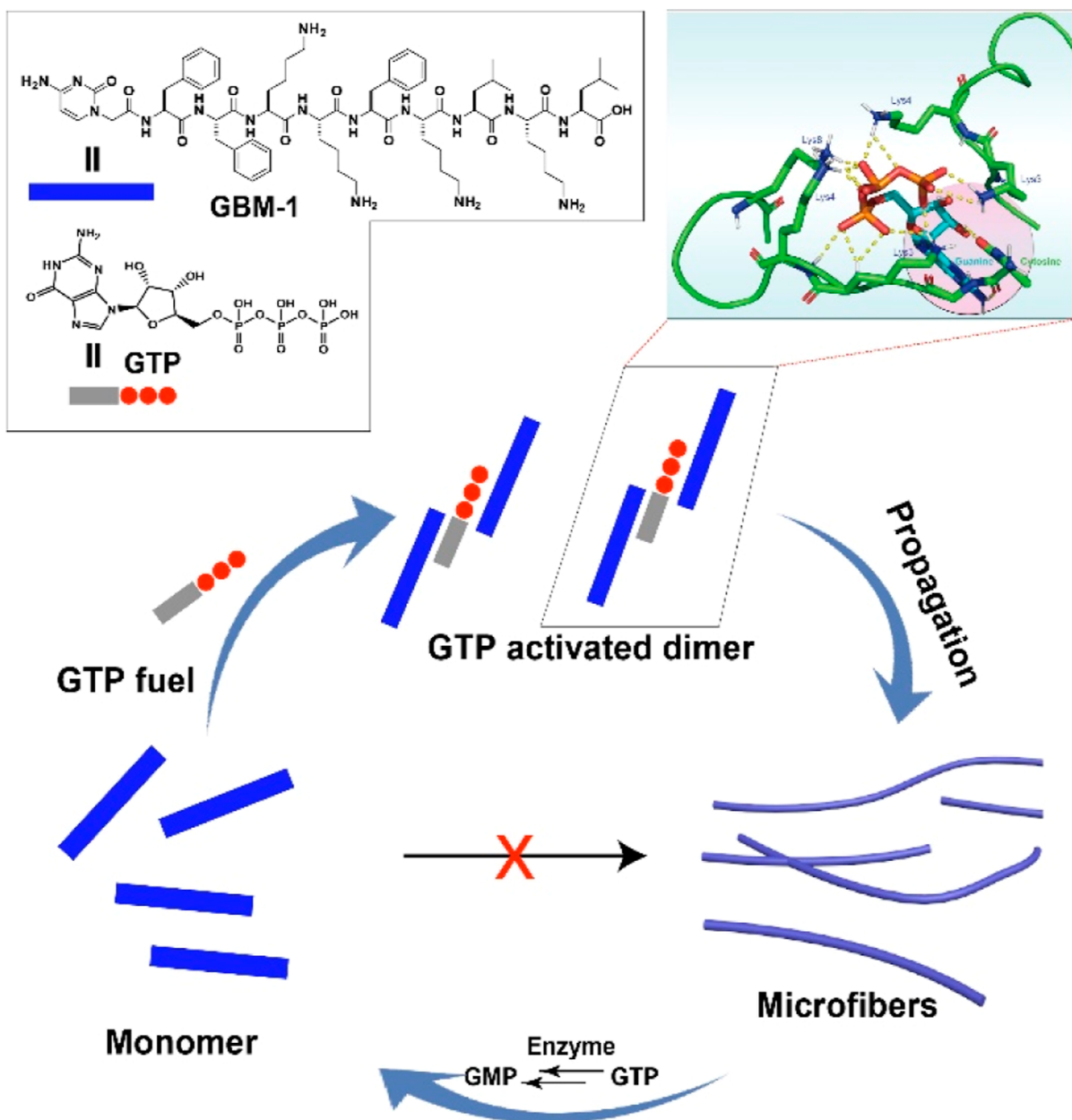


Figure 1. Molecular structures and the illustration of the processes of fiber formation.

METHODS

Formation and Optical Images of Microfibers. We put 200 μL of GBM-1 (1 mM in stock solution) into a test tube and treated with GTP (1 mM in stock solution) in HEPES solution (pH 7.4). The mixture of the solution was put at room temperature for 24 h. The final concentrations of GBM-1 and GTP are 0.5 mM, respectively.

Turbidity Assay. The microfibers were prepared as described in a 96-well plate. The UV-spectra of GBM-1 and GTP displayed that both of them showed no absorption peak at 405 nm (as shown in Figure S55). Then, the absorbance at 405 nm was measured every 30 s for 8 h by a multimode microplate reader (Varioskan LUX, Thermo Scientific, US).

Light Microscopy Images of Microfibers. After preparing the microfibers, we took 100 μL of solution into a 96-well plate and observed it under microscopy under bright field via EVOS FL Auto 2 (Thermo Fisher, US).

Determination of the Binding Stoichiometry between GBM-1 and GTP. The binding stoichiometry between GBM-1 and GTP was measured by Job's method and molar ratio method.³⁰

Briefly, the total concentration of GBM-1 and GTP was 2 mM and the concentration of GBM-1 increased while the concentration of GTP decreased (Job's method, Figure S56A). The concentration of GBM-1 was 1 mM and the concentration of GTP was gradually increased (molar ratio method, Figure S56B). The mixtures of these groups were coincubated for 24 h. The curve of intensity and $n(\text{GBM-1})/n(\text{GBM-1} + \text{GTP})$ was plotted and the cross point of the linear curves was the binding stoichiometry between GBM-1 and GTP.

Atomic Force Microscope. Twenty microliters of microfiber was dispersed on mica sheet, and the microfibers were dried using nitrogen purging. The morphology of the self-assemblies was recorded via an AFM (Cypher ES, US) with an AIST-NT Smart AFM system in tapping mode.

Polarized Light Microscopy Images of Microfibers. After the formation of the microfibers via the above-mentioned method, we put the microfibers on the objective stage and detected them by Oosight microscopy (Hamilton Thorne, US).

Laser Scanning Confocal Microscopy. The microfiber formation of 0.5 mM GBM-1/GBM-NBD (GBM-1/GBM-NBD =

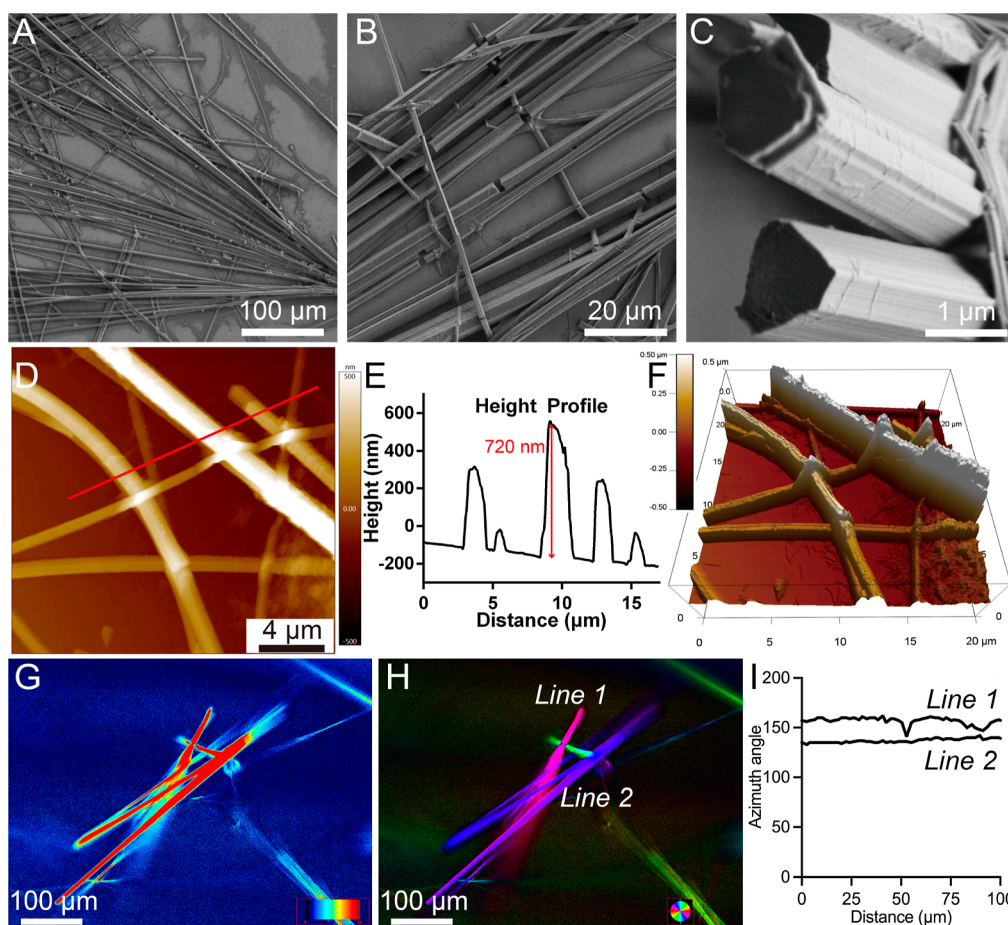


Figure 2. SEM images (A–C), AFM (D) images, height distribution (E), 3D images (F), and POM images (G,H) of fibers formed by GBM-1 (0.5 mM) and GTP (0.5 mM) for 24 h. The intensity of birefringence along the microfibers was shown as a colored rectangle in (G) and the azimuth of slow axis orientation was shown as a colored circle in (H). (I) Analysis of the azimuth angle of fibers using lines (100 μm) in (H).

19:1) and GTP/Mant-GTP (GTP: Mant-GTP = 29:1) for 24 h was imaged by LSM800 CLSM (Zeiss, Germany) using 488 nm (GBM-NBD) and 405 nm (Mant-GTP) wavelength channels.

Dynamic Formation of Microfibers. GBM-1 (0.5 mM) and GTP (0.5 mM) were put into a 96-well plate and detected by light microscopy (EVOS FL Auto 2, Thermo Fisher, US) every 5 min. After 24 h incubation, we added CIAP (0.5 U/mL) to above solution and detected for another 12 h. After that, we added the CIAP inhibitors (100 μM NaF and 100 μM Na_3VO_4) and another 0.5 mM GTP to the above solution and detected them by microscopy for another 24 h.

MD Simulations. All-atom MD simulations: the peptides and GTP were built by using visual MD (VMD). AA-MD simulations were carried out using the GROMACS 2019.6 software.³¹ The 11 peptides and 6 GTP were randomly placed in a 7.8 nm $\times$ 8.7 nm $\times$ 7.4 nm box and filled with standard simple point charge water with periodic boundary conditions. In order to ensure the accuracy of simulation, the gaff force field (GTP) and amber force field (peptide) were used for simulation. The simulation was run for 500 ns with an output frequency of 20 ps. The hydrogen bonding interactions were constrained by LINCS with an integration step of 2 fs. Electrostatic interactions were treated with Particle-mesh Ewald (PME) method.³² Nonbonded pairs were updated every 10 integration steps, with a cutoff of 10 Å. The temperature and pressure were constant using the Nose–Hoover method^{33,34} and Parrinello–Rahman method,^{35,36} respectively. Analysis was performed in the final 100 ns.

Coarse-Grained Simulations. To obtain large-sized aggregates and better understand the formation of microfibers, we also used Martini coarse-grained (CG) field^{37,38} to conduct large-scale MDs simulation of aggregates formed by 55 of GBM-1 and 30 of GTP

molecules and 28,414 CG water molecules for 1.8 μs . In order to ensure the accuracy of the CG field, we divided the CG particles of GTP molecules according to the classification of particle types that followed the work by Furuta and co-workers.³⁹ The temperature of simulation is 320 K, coupled by the V-Rescale method, and the pressure was 1 atm, maintained by the Parrinello–Rahman method.^{35,36} The simulated step size is 0.03 ps, and the data are saved every 10,000 steps. The settings of other parameters are set according to the MDP file on the official Web site without any modification.

RESULTS AND DISCUSSION

Molecular Design. We started to screen a short library of peptides with the N-terminal modification of cytosine (Tables S1 and S2) by native eye and light microscopy methods (Figures S51 and S52). We chose cytosine as the N-capping group because the cytosine interacts with the guanine group of GTP through strong hydrogen bonds. To demonstrate the importance of cytosine, we also used the groups of acetyl (Ac), guanine, and thymine to replace cytosine at the N-terminus of the peptide. After synthesizing Bis-Boc-protected cytosine carboxylic acid via four steps (Scheme S1), we used standard solid-phase peptide synthesis to obtain the final molecules (Scheme S2) and purify them by HPLC. The identity of all the molecules was confirmed by NMR and MS (Figures S2–S48). Our results indicate that Cytosine-FFKKFKLKL (GBM-1, Figure 1) is the optimized molecule for interacting with GTP selectively in HEPES solution (pH = 7.4). A single component

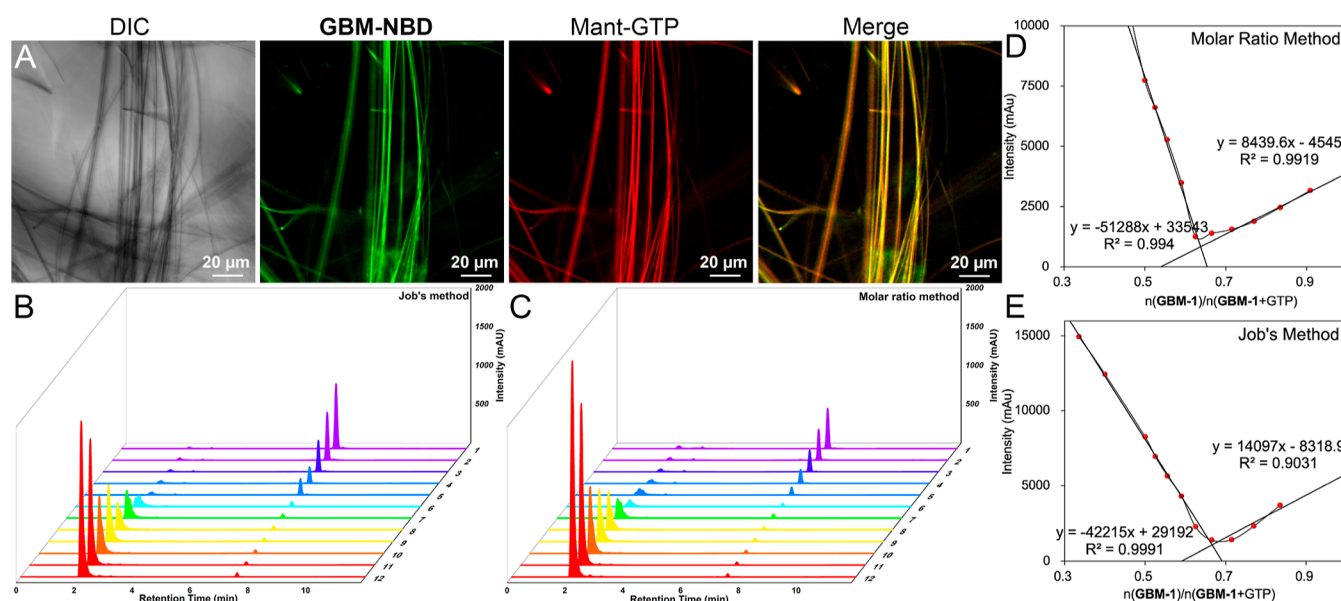


Figure 3. (A) CLSM images of fiber formation formed by 0.5 mM GBM-1/GBM-NBD (GBM-1/GBM-NBD = 19:1) and GTP/Mant-GTP (GTP: Mant-GTP = 29:1) for 24 h. Scale bar: 20 μm. The UV spectra of the mixtures of GBM-1 and GTP in Job's method (B) and the molar ratio method (C) after 24 h via analytical HPLC at the wavelength of 254 nm. Molar ratio (D) and Job's method (E) of continuous variation for determination of molar ratio of GBM-1 and GTP.

of GBM-1 or GTP could not form any observable structures under the detection of light microscopy (Figure S49). The addition of ATP to the solution of GBM-1 resulted in a clear solution, indicating the selectively induced self-assembly of our system toward GTP (Figure S50) and replacing dipeptide FF with GG or removing FF from GBM-1 results in the formation of a solution by adding GTP, which suggests the critical role of the self-assembly motif for the formation of microfibers. Using a different number of glycine (1 to 3) as a linker to conjugate cytosine and FF could not form fibers regardless of the presence of GTP. Specifically, putting one glycine between cytosine and FF results in aggregates. Two or three glycine as a linker could only form a solution without any detectable morphologies, indicating that the spacer between cytosine and FF significantly influences the formation of fibers. Our results also indicated that mutation of all the lysine to arginine results in cytosine-FFRRFRLRL, which could only form aggregates in the presence of GTP. Moreover, we also found that modifying lysine with the acetyl group of GBM-1 at different positions could form aggregate instead of fibers, suggesting the importance of electrostatic interactions between GBM-1 and GTP for the stability of fibers.

Characterization of Microfibers. Scanning electron microscopy (SEM) indicated that the microfibers formed by GBM-1 and GTP are several hundred micrometers long and about 1–2 μm in width, and the microfibers are asymmetric quadrilateral in cross sections with a uniform diameter (Figure 2A–C). The cross-sectional height profiles and 3D images from the AFM experiment further confirmed the dominance of large-sized microfibers with different heights (Figure 2D–F). The wide and flat plateau in the height profile (Figure 2E) is consistent with their morphologies in SEM images. Polarized-light microscopy (POM) results showed that microfibers formed by GBM-1 and GTP have a strong intensity of birefringence, indicating the anisotropy of the microfibers (Figure 2G–I).⁴⁰ The pseudoorientation images display the degree of polarization (Figure 2G) and the polarization

azimuth angle (Figure 2H) in microfibers. The fiber growth direction is perpendicular to the azimuth direction, suggesting that the molecular arrangement in the fibers is perpendicular to the growth direction. We used lines along fibers to measure the azimuth angle of fibers. The azimuth angle distribution in the birefringent regions is significantly uniform, indicating that the fibers present a highly linear arrangement (Figure 2I). Furthermore, we monitored the dynamic changes of absorbance at a wavelength of 405 nm by a multimode microplate reader since the fibers have detectable absorbance at this wavelength. The single component molecule has no obvious absorbance at this wavelength (Figure S54), which could not affect the results of the turbidity experiment. The turbidity experiment reveals the dynamic processes of fiber growth (Figure S55). The turbidity curve reached a plateau about 6 h after coincubating GBM-1 with GTP, suggesting that the GTP-induced fiber growth is moderate. Moreover, light microscopy results also showed that the microfibers did not change with a change in GTP concentration (Figure S60) at a fixed concentration of GBM-1 (0.5 mM).

Interactions between GBM-1 and GTP. To directly visualize GTP-induced fiber growth and the interactions between GBM-1 and GTP, we synthesized nitrobenzoxadiazole (NBD, an environment-sensitive fluorophore^{41,42})-labeled GBM-1 at its N-terminus to result in GBM-NBD (Figures S47 and S48). Since the fluorescent molecules (GBM-NBD and Mant-GTP) were involved in the coassembly, the introduction of fluorescent molecules should not disrupt the formation of the assembly. Thus, we chose the optimized ratio of compounds because such a ratio could not destroy the assembly structure. Confocal laser scanning microscopy (CLSM) indicated that introducing NBD in the molecule does not influence the GTP-induced fiber growth of GBM-NBD (Figure S57 and Video S1). CLSM results (Figure 3A) also showed that adding a commercially available fluorophore-labeled GTP (Mant-GTP, Figure S1) could form fluorescent fibers with cytosine-labeled peptide, evidenced by the

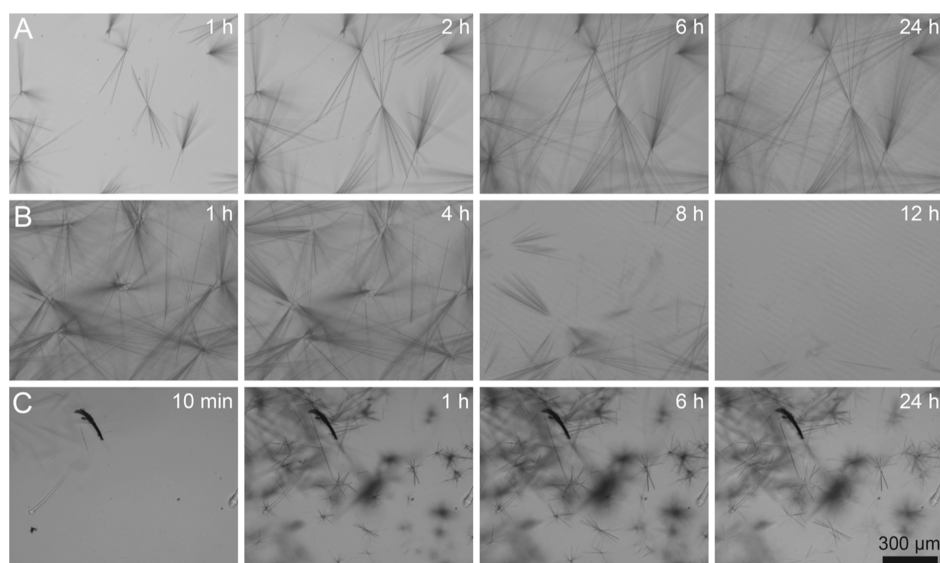


Figure 4. (A) The light microscopy images of fibers formed by **GBM-1** (0.5 mM) and GTP (0.5 mM) for 24 h. (B) The light microscopy images of fibers degraded by adding CIAP (0.5 U/mL) for another 12 h. (C) The light microscopy images of fibers were reformed by ALP inhibitors (100 μ M NaF and 100 μ M Na_3VO_4) and another 0.5 mM GTP for 24 h. Scale bar: 300 μ m.

observation of colocalization of green fluorescence from **GBM-NBD** with red fluorescence from Mant-GTP. These results suggested that GTP induced fiber formation by coassembling with **GBM-1** noncovalently.

To investigate the binding stoichiometry between **GBM-1** and GTP, we used an Agilent 1260 Infinity II LC System with reverse-phased (RP) C18 column to determine the retention time and UV absorption peak areas of **GBM-1** and GTP in the mixtures (Figure 3B,C). The y -axis indicates the UV absorbance of the supernatant of the mixtures after the binding of **GBM-1** and GTP. The results of molar ratio and Job's method suggested that the binding stoichiometry between **GBM-1** and GTP is about 2:1 (Figure 3D,E), indicating that GTP activates **GBM-1** to form a dimer and then grows to form the fibers.

Controlling of Dynamic Processes of Microfibers. To investigate whether the microfibers' growth could be controlled by hydrolysis of GTP, we chose the enzyme of calf intestinal alkaline phosphatase (CIAP)⁴³ to perform depolymerization studies because of its high activity to catalyze the hydrolysis of phosphate termini from GTP. Light microscopy results show that the fibers disassembled in the presence of CIAP (0.5 U/mL, Figure 4B). With the increase of enzyme concentration, the hydrolysis rate of GTP becomes faster, and thus, the fibers disappear much faster (Figure S58). After depolymerizing the fiber with CIAP, we readdded GTP and CIAP inhibitors to the above solution. The results indicated that the fiber gradually regrew over time (Figure 4C).

To track the entire cyclic processes of the active fiber formation over time, we performed time-dependent fiber growth using light microscopy. The results (Figure 4A and Video S2) showed that the fibers started to grow after adding GTP for a short time. Interestingly, the fiber growth started from one central point and extended to several directions centered on this point. The fibers became stable after the addition of GTP for 6 h. Microscopic level observation also indicated that the disassembly of fibers starts from the distal end to the central point (Figure 4B and Video S3). Moreover, the fibers regrew by reading GTP (Figure 4C and Video S4).

The dynamic formation of fibers induced by GTP is similar to the behavior of (de)polymerization of the microtubule, suggesting the feasibility of the strategy to construct active materials by GTP.

MD Simulations. We next performed MD simulations to gain more insights into GTP-induced microfiber formation.³¹ All-atom MD results reveal the detailed interactions between **GBM-1** and GTP. To facilitate the description of the interaction force type, we divided the structural formulas of the nucleopeptide and GTP into different parts (Figure 5A,B). The simulation is run for 500 ns in total (Video S6). To understand the folding state of **GBM-1** in the aggregate, the cluster analysis is conducted on the conformation of $2501 \times 11 = 27,511$ **GBM-1** in 2501 frames at the range of 400–500 ns. We obtained the 22,507 conformations of the large cluster and 5 small clusters, and the intermediate structure of the large cluster is shown in Figure 5C. Figure 5D showed that the hydrogen bonds between guanine and cytosine (a-i, ii) and electrostatic interactions between lysine from **GBM-1** and phosphate group from GTP (c-iii) are the major contributors to the formation of microfibers, agreeing with experimental results that are using acetyl lysine to replace K3, K4, K6, or K8 fail to form the microfibers (Figure S53 and Table S2). Figure 5E exhibits hydrogen bonds that are involved in the formation of fibers. **GBM-1** and GTP have different charges, which contribute to the formation of hydrogen bonds. Further analysis indicates that the balance of noncovalent interactions from the intermolecular interaction of **GBM-1**, van der Waals interactions, and coulomb energy together facilitate a more stable microfiber (Figure 5F).

To understand the self-assembly of the larger-sized aggregates, we also performed Martini CG field^{37,38} to conduct a large-scale MDs simulation of aggregates for 1.8 μ s (Figure 5J and Video S5). To ensure the accuracy of the CG field, we divided the CG particles of **GBM-1** and GTP molecules into different colors of beads, as shown in Figure 5G–I. The results showed that **GBM-1** and GTP were randomly dispersed in the box and rapidly aggregated into small clusters after the 15 ns simulation. The small clusters further interact with each other

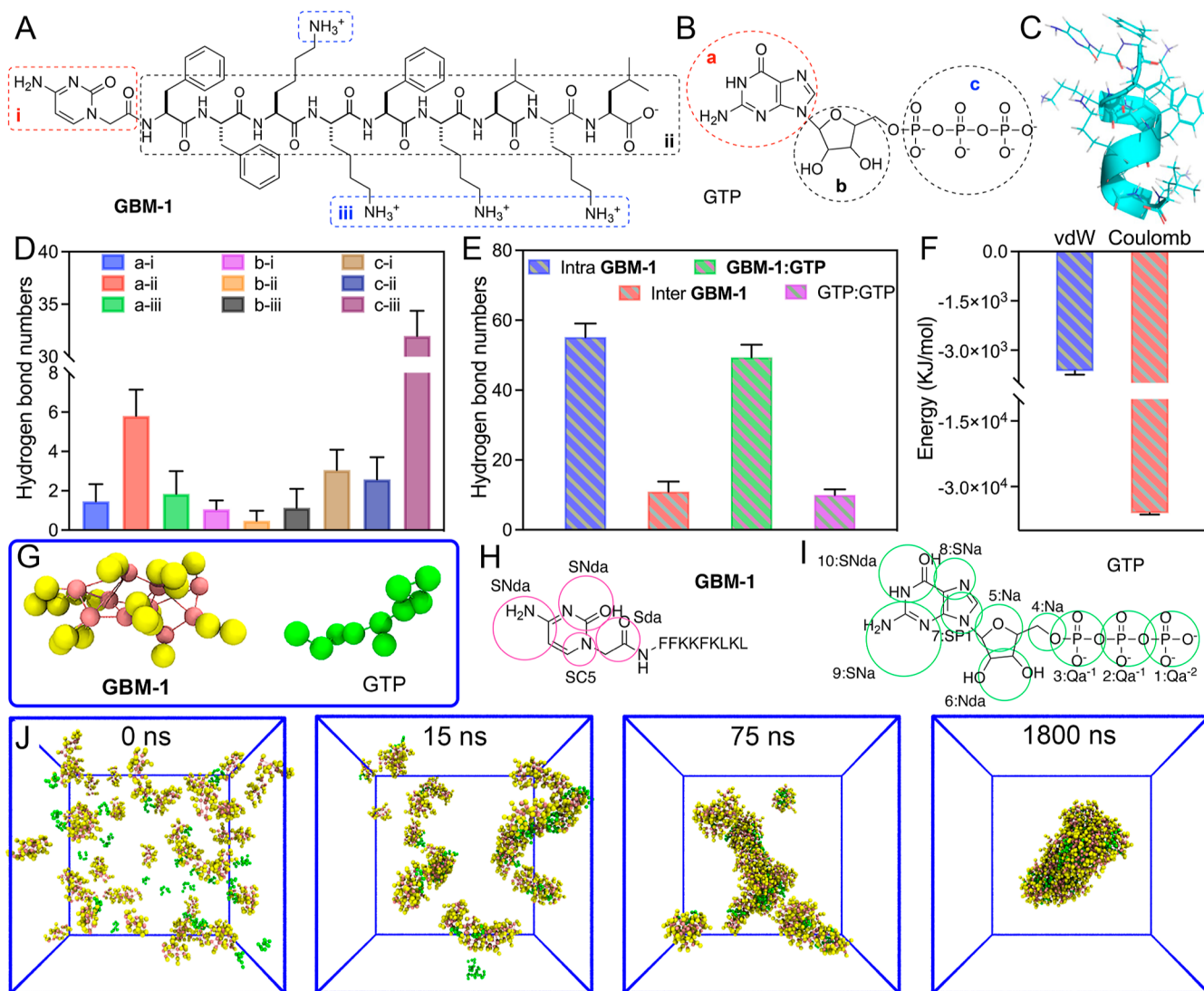


Figure 5. Analysis of all-atom (AA-MD) and CG-MD simulations of fiber formation by **GBM-1** and **GTP**. The protonated chemical structure of **GBM-1** (A) and **GTP** (B). (C) The intermediate structure of the cluster contains 22,507 **GBM-1** conformations. (D) Hydrogen bond types and distribution within the fibers. a, b, c and i, ii, and iii represented the divisions of **GTP** and **GBM-1**, respectively (as shown in A,B). (E) The numbers of hydrogen bonds of **GBM-1** and **GTP**. (F) van der Waals force and coulomb energy of clusters at 500 ns of the simulation. (G) Yellow-pink beads are **GBM-1** and green beads are **GTP**, respectively. The CG models of **GBM-1** (H) and **GTP** (I). (J) CG-MD simulation analysis of the growth of microfibers.

to form rodlike aggregates after 192 ns, agreeing with the experimental observations.

Microfiber Formation in Confined Conditions. Reaction–diffusion (RA) processes are an essential part of the living system, and compartmentation is the typical phenomenon in living cells.⁶ We hypothesized that microfiber growth could be controlled in a confined environment by mimicking membrane organelles (Figure 6A). We first put **GBM-1** (MW = 1350) in a semipermeable membrane with a molecular cutoff weight of 1000 Da, and **GTP** could diffuse easily through this membrane. The results (Figure 6B) showed that **GTP** diffused through the membrane and induced the microfiber growth of **GBM-1** only inside the membrane. The fibers could be easily collected for further applications. These results demonstrated that we could control the fiber formation in the confined microenvironment by reaction–diffusion processes.

Encouraged by these results, we further investigated the physicochemical consequences of **GTP**-induced polymeriza-

tion of **GBM-1** confined in aqueous microdroplets. In this setting, the microfluidic method was employed to establish the aqueous droplets of a water-in-oil (w/o) emulsion (Figures 6C and S59), one channel with **GBM-1**, and one channel with **GTP** in an aqueous solution to form a microdroplet through a mixing channel. The microfibers formed quickly inside the droplets. The results also show that proximal droplets tend to fuse to form stochastic coalescence through physical contact. These results demonstrate the feasibility of our system to construct active fibers in confined systems.⁴⁴

CONCLUSIONS

This work describes the **GTP**-induced dynamic self-assembly system for the construction of microfibers of the peptidic building block, which is achieved by rationally designed molecules that interact with **GTP** with balanced noncovalent interactions, as evidenced by experimental and computational results. Time-dependent experiments demonstrate that we

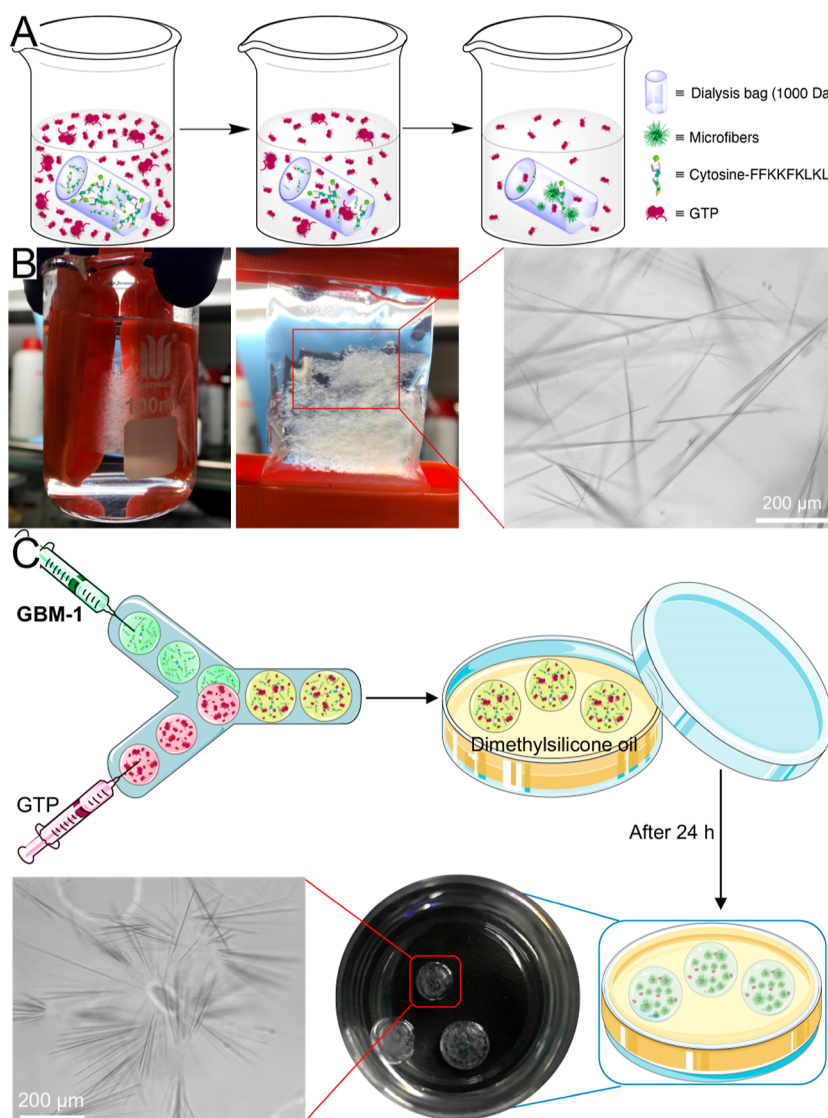


Figure 6. (A) The illustration of the microfiber formation in a semipermeable membrane. (B) The fibers can be observed in the inner membrane by native eye and light microscopy. The concentration of **GBM-1** and **GTP** is 0.5 mM. (C) The microfluidic method to construct the aqueous droplets of a water-in-oil (w/o) emulsion and the corresponding light microscopy results.

could kinetically track the whole cyclic processes of the active fiber formation by adding **GTP**. Moreover, the experiments in our work also showed that it is feasible to control the fiber formation in a confined environment, suggesting its potential applications in biomimetic systems.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biomac.3c00674>.

Details on experiment materials and instruments and synthesis of Bis-Boc protected cytosine carboxylic acid and **GBM-1**, and additional characterization including details of NMR spectra; optical and light microscopy images of peptides; UV–vis spectra; turbidity experiment; fluorescence images; microfluidic instruments, supporting tables (MS results and phenomenon of different peptides in the treatment of **GTP**) (PDF)

Real time visualization of **GTP** (0.5 mM) fueled dynamic formation of microfibers by **GBM-NBD** (0.5

mM) under fluorescence microscopy in HEPES buffer (pH 7.4) (MP4)

Real time visualization of **GTP** (0.5 mM) fueled dynamic assembly process of microfibers formed by the **GBM-1** (0.5 mM) under microscopy at in HEPES buffer (pH 7.4) (MP4)

Real time visualization of degradation of microfibers induced by consuming **GTP** (MP4)

Real time visualization of re-growth of microfiber by re-fuel **GTP** (MP4)

Coarse-grained (CG) simulations of the growth of microfibers in the Martini coarse-grained field by 55 of **GBM-1** and 30 of **GTP** molecules and 28414 coarse-grained water molecules (MP4)

All-atom (AA) simulations of the growth of microfibers in the cgenff force field by 11 of **GBM-1** and 6 of **GTP** molecules (MP4)

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Author Contributions

H.W. conceived this work. H.Z. performed the experiments and collected data. B.W. repeated some of microfibers formation experiments. X.Y. characterized some of compounds, such as SEM, CLSM, and so forth. T.X., J.L., J.W., and Z.Z. synthesized and characterized some of the compounds. H.Z. and H.W. analyzed the data and wrote the manuscript with the input from the other authors. All authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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