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Using F_0F_1-ATPase motors as micro-mixers accelerates thrombolysis

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ABSTRACT

We have developed a novel micro-mixer using a biological molecular ATP motor. The micro-mixer was constructed from arrays of chromatophore-embedded δ-free F_0F_1-ATPases, where the δ-free F_1 part acted as a rotator to mix solutions, and the F_0 part was driven by light. Confocal microscope studies indicated that the micro-mixer did not touch directly on the fibrin labeled with FITC. The nanomechanical force generated by the motor induced drug movement in the solution and accelerated the fibrinolysis process. All results strongly suggest that the micro-mixers generated a nanomechanical force which accelerated the fibrinolysis process in the presence of lower concentrations of lumbrokinase.

F_0F_1-ATPase is a key enzyme in the biological world and one of the most ubiquitous proteins in nature [1–5]. ATP synthase is the universal enzyme that synthesizes ATP, the universal fuel that powers most cellular processes. The enzyme consists of two parts; the F_1 motor generates a mechanical torque using the hydrolysis energy of ATP and the F_0 motor generates a rotary torque in the opposite direction using a transmembrane proton motive force. These two motors are connected by flexible coupling, and each motor can be reversed: the F_0 motor can drive the F_1 motor to synthesize ATP, and the F_1 motor can drive the F_0 motor to pump protons.

In *Escherichia coli,* the F_1 and F_0 motors are composed of $\alpha_3\beta_3\gamma\delta\epsilon$ and ab_2c_n *subunits,* respectively [6]. These two parts of the enzyme are structurally linked by two stalks, a central stalk of the c and ϵ subunits that links to the c subunit ring, and an outer stalk of the δ b_2 subunits linking the $\alpha_3\beta_3$ subunits to the a subunit. If the δ subunit is removed only one central 'stalk' (ϵ, γ) remains in the F_0F_1-ATPase and the 'rotator' and 'stator' will be reorganized (see supporting information). In this δ-free F_0F_1-ATPase motor, the stator will consist of the b and a subunits, and the rotator will consist of the c ring, and the γ, ϵ and $\alpha_3\beta_3$ subunits. When this δ-free F_0F_1-ATPase is embedded within the chromatophore it is a fascinating structure that allows light energy to be converted into proton motive force and stored to drive the ATPase motor [7]. This enzyme is a good model for construction of second generation light-driven molecular motors [8,9], as we have demonstrated in previous publications [10–12]. However, the force provided by a single motor is too small to carry out certain tasks. Integration of many biomolecular motors into an artificial device would enable exploration of new applications for nanotechnology.

At the present time, cardiovascular disease is the number one cause of death around the world [13]. In traditional thrombolytic therapy, thrombolysis is slow as the thrombolytic process depends entirely on the action of drugs which can only be administered at relative low concentrations because of the increased risk of bleeding associated with them [14]. A mechanically assisted therapy has been reported [15], but difficulties with the mechanical device have prevented its use for medical applications.

Here we describe a straightforward approach: a micro-mixer of ordered chromatophore-embedded δ-free F_0F_1-ATPase was constructed on the surface of glass. The chromatophore-embedded δ-free F_0F_1-ATPase micro-mixer arrays generated a cooperative effect with low dose lumbrokinase [16] and accelerated the fibrinolysis process, as observed by fluorescence microscopy.

Materials and methods

Materials. FITC was purchased from Molecular Probes (Eugene, Oregon, USA). Ni-NTA magnetic beads were purchased from Micromod Partikeltechnologie GmbH (Germany). ATP and streptavidin were purchased from Sigma. Biotin-$(AC5)_2$Sulfo-OSu was purchased from Dojindo (Japan). Lipid-biotin was purchased from Avanti. All other reagents were of analytical grade and were purchased locally.

Labeling of fibrinogen with FITC and fibrin formation. Fibrinogen (1 ml, 0.03 mM) was dissolved in PBS buffered saline containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 8.5.

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FITC was added to the fibrinogen solution under intensive stirring to a final concentration of 50 mg/ml [17]. The fibrin fiber obtained from fibrinogen (10 μl) was polymerized with thrombin (0.5 U/μl) and fixed on the glass surface. After incubation at 37 °C for 30 min, a fibrin network with an approximate length of 200 μm was formed.

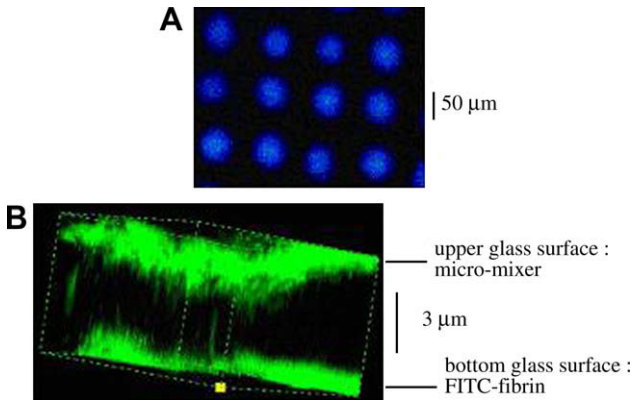

Fig. 1. Micro-mixer construction. (A) Chromatophore-embedded δ-free F_0F_1-ATPase micro-arrays observed by fluorescence microscopy. (B) Fluorescence image of patterned chromatophores and FITC-fibrin. F-DHPE-micro-mixer pattern was fixed on the upper glass surface and FITC-fibrin was fixed on the lower glass surface. The distance between glass surfaces was about 3–5 μm.

Construction of the micro-mixer. The chromatophore-embedded δ-free F_0F_1-ATPase was constructed according to a previously published method [11]. The micro-mixer was constructed in ordered arrays of chromatophore-embedded δ-free F_0F_1-ATPase using polydimethylsiloxane (PDMS) as described previously [18].

Observation of the fibrinolysis process by fluorescence microscopy. Lumbrokinase (thrombolytic drug) at a concentration of 30 U/μl was dissolved in high buffer capacity PBS (137 mM NaCl, 3 mM KCl, 80 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.4). This buffer system was used to keep pH constant throughout the whole reaction process.

After the micro-mixer was fixed on the surface of glass, the glass was placed in buffer (10 mM Tricine–NaOH, pH 6.5, 2 mM $MgCl_2$, 50 mM KCl, 5 mM NaN_3, and 2 mM ATP) and then illuminated with light through a 570 nm filter for 30 min at 4 °C, initiating proton transfer from the outside of chromatophores to the inside. Once proton transfer was initiated, the micro-mixer was sealed in an incubator at 37 °C. Thirty microliters of lumbrokinase (30 U/μl) was then added to dissolve the fibrin. The fibrinolysis process was observed with an Olympus IX71 fluorescence microscope, and recorded with a digital CCD camera (Princeton Scientific).

Ultra-weak luminescence analysis. The system used here was the same as that reported previously [19].

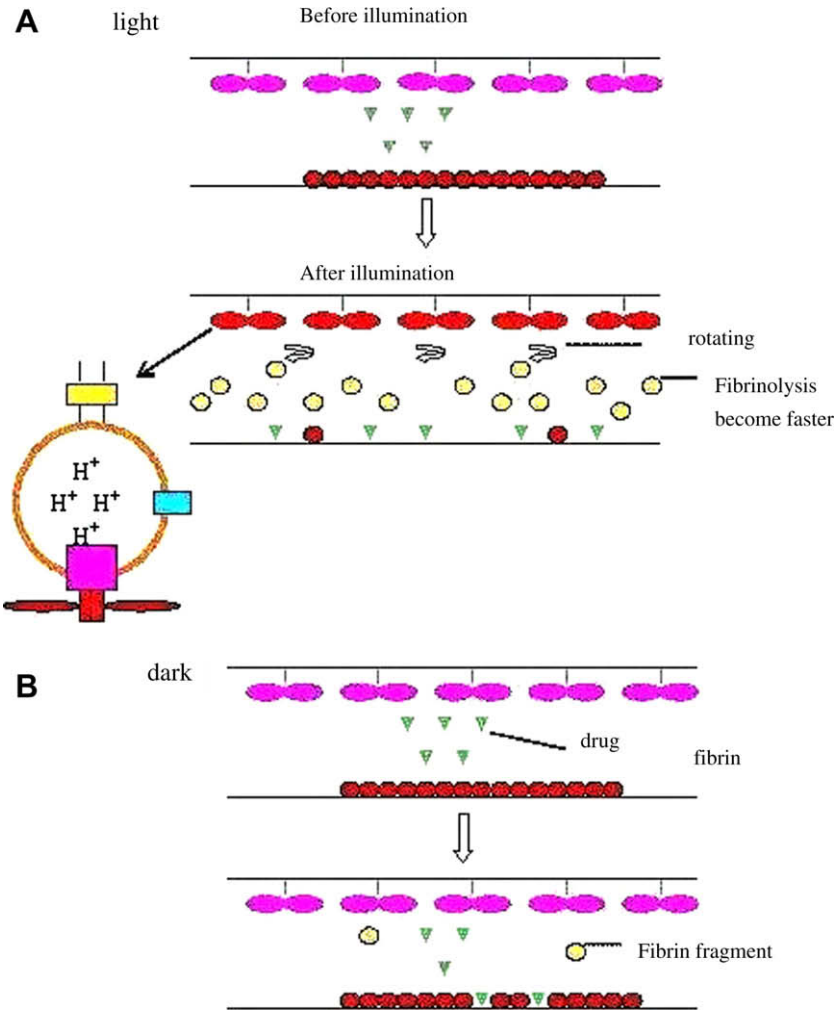

Fig. 2. Experimental scheme of the fibrinolysis process. (A) After illumination, the fibrinolysis process is driven by the micro-mixers. (B) Control (no illumination).

Statistical analysis. The Kruskal–Wallis test was used to analyse experimental results as the variance within groups was not equal.

Results and discussion

Chromatophore-embedded δ-free F_0F_1-ATPase arrays were attached to a glass surface through molecular recognition of biotin-streptavidin-biotin as described previously [18]. Chromatophores are vesicle made of lipid-protein complexes and have an average diameter of 70 ± 15 nm. The detailed structure of chromatophores was determined by Geyer and Helms [8]: each chromatophore contains one F_0F_1-ATPase and LH_1, LH_2, bc1 and RC complexes.

Micro-mixers observed under a fluorescence microscope are shown in Fig. 1A. The working principle of the micro-mixer is as follows: F_1, with two attached antibodies, acted as the rotor for mixing the solution, while F_0 acted as the force generator and was driven by the proton gradient. The chromatophore membrane acted as a reservoir to store the proton energy that was converted from light energy. When chromatophores were illuminated at low temperature, a proton gradient was generated. Addition of NaN_3/ATP not only inhibited the hydrolysis activity of the F_0F_1-ATPase, but also helped bind β_3 and γ tightly together [20]. This micro-mixer is a new model combining the use of chromatophore-embedded δ-free F_0F_1-ATPase, and the principle of light energy transfer into mechanical energy.

In our experimental system, the chromatophore-embedded δ-free F_0F_1-ATPase micro-mixer labeled with F-DHPE was attached to the upper glass surface, while fiber-FITC was attached to the lower glass surface. The distance between the glass surfaces was about 3–5 μm and was filled with lumbrokinase solution (Fig. 1B). The micro-mixer worked by molecular motor rotation. Although it did not touch the fiber-FITC, it generated a nanomechanical force that kept the lumbrokinase solution moving, thus accelerating the fibrinolysis process.

When chromatophore-embedded δ-free F_0F_1-ATPases are arranged in ordered arrays, they work in a cooperative manner. Fig. 2 explains how the rotation of the micro-mixers was in a cooperative manner in our experimental system. In order to observe the effect of the rotation of the micro-mixers we used a low dose of drug (5%). On illumination of the micro-mixers (Fig. 2A), the rotation of the molecular motors was driven by the PMF. During the fibrinolysis process, the cooperative effect of the rotation of the motors and the lumbrokinase resulted in accelerated fibrin dissolution relative to the control (without illumination) (Fig. 2B). The rate of the fibrinolysis process depended not only on the effect of the drug (lumbrokinase), but also on the effect of rotation driven by the PMF.

Fig. 3 shows the morphological features of the fibrinolysis process observed directly under a fluorescence microscope. At the outset fibrin fiber diameter, fiber length, fiber density, and branch point density were similar in the two samples (Fig. 3A and E). Acceleration of the fibrinolysis process by micro-mixers can be observed by comparing Fig. 3A–D. Fibrin diameter was clearly diminished, length shortened, and density decreased. After 30 min (Fig. 3D) the fibrinolysis process was almost complete, with little fibrin remaining (Fig. 3D). Meanwhile, in the control (Fig. 3E–H) it can be observed that during fibrin processing fiber density decreased to a lesser extent than with the micro-mixers. After 30 min (Fig. 3D and H), the fibrin fiber network was dissolved to a large degree with the micro-mixers to the extent that intact fibers were not observed. In the control (Fig. 3H), however, the outlines of the fibrin fibers can still be observed.

Fig. 4 shows that fibrin-FITC fluorescence intensity decreased with increasing illumination time during the time course 0, 10, 20, 30 min for the fibrinolysis process at 37 °C. Each group had

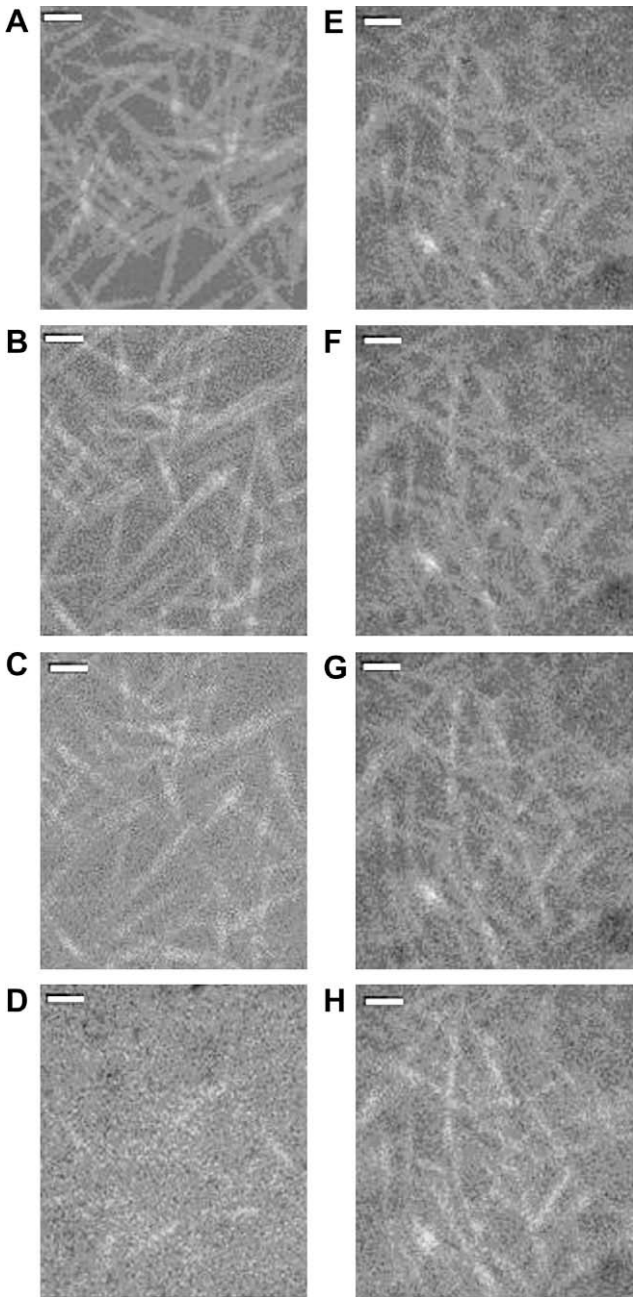

Fig. 3. The fibrinolysis process driven by micro-mixers is faster than that of drugs alone. Dynamic visualization of the fibrinolysis process by fluorescence microscopy.

10 samples and data were analysed after normalization. In curve A, the fibrinolysis process was dependent on the effect of the drug alone (no illumination); the fibrin-fluorescence intensity ($\times 10^4$ U) decreased from 25.3, 24.6 ± 0.49; 24.1 ± 0.63 to 23.6 ± 0.69. In curve B, fibrin-FITC fluorescence decreased after the micro-mixer was illuminated for 15 min, driving rotation of the micro-mixers; the fibrin-fluorescence intensity decreased from 25.3, 23.7 ± 0.83; 22.3 ± 0.54 to 21.3 ± 0.74. In curve C, fibrin-FITC fluorescence decreased after the micro-mixer was illuminated for 30 min; the fibrin-fluorescence intensity decreased from 25.3, 21.9 ± 2.13, 20.5 ± 2.30 to 19.4 ± 2.36. In curve D, fibrin-FITC fluorescence decreased after the micro-mixer was illuminated for 45 min; the fibrin-fluorescence intensity decreased from 25.3, 19.4 ± 2.89, 16.4 ± 2.76 to 13.8 ± 2.42.

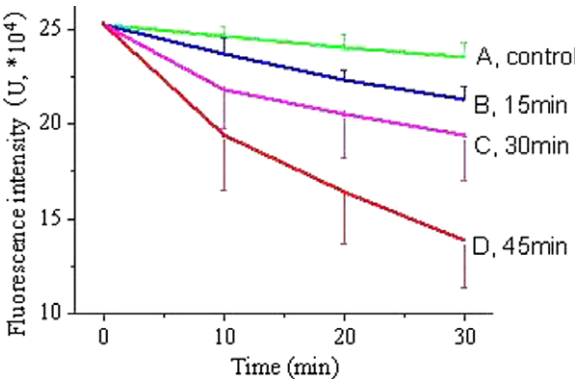

Fig. 4. Dose-effect of illumination on fibrinolysis rate.

In order to keep the fibrinolysis process dependent on only the rotary movement of δ-free F_0F_1-ATP, and to avoid fluorescent intensity decrease induced by pH changes in the solution, we used high pH buffer capacity PBS (80 mM), keeping the solution pH environment constant [21]. Therefore, the fluorescence intensity decrease observed represents a real dissolution of fibrin. Furthermore these results were confirmed by changes in fibrin shape and structure.

The chromatophore-embedded δ-free F_0F_1-ATPase rotary effect of these micro-mixers could make a greater contribution to the fibrinolysis process than that of drugs; in our experimental system the effect of micro-mixers and drugs together was five times higher than that of the drug alone.

Images were taken at 0 min (A and E), 10 min (B and F), 20 min (C and G) and 30 min (D and H). Panels A–D and E–H show the fibrinolysis process for micro-mixers and controls, respectively (scale bar: 50 μm). The concentration of lumbrokinase was 30 U/μl, 30 μl.

Dose effect of fibrin-FITC fluorescence intensity decreased with increasing illumination in the fibrinolysis time course 0, 10, 20, 30 min. Data were normalized before analysis. Lumbrokinase concentration was 30 U/μl in each group. Each data point is the average of 10 samples. Variances were not equal among groups (Levene's test was 9.368, $P = 0.001$), so the Kruskal–Wallis test was used. Differences among groups were significant ($\chi^2 = 33.649$, $P = 0.001$).

While the micro-mixer we have described has potential applications for thrombolytic processing *in vitro*, it has also attracted considerable attention for its potential applications for thrombolytic therapy *in vivo*. Biological molecular motors have a number of advantages, namely they are highly efficient, are potentially cheap to use, and are readily available in nature. No doubt, the decrease in drug side-effects and shorter therapy time required for thrombolytic therapy will be of great advantage in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.110.

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