Catalytic Microgelators for Decoupled Control of Gelation Rate and Rigidity of the Biological Gels

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A R T I C L E   I N F O

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A B S T R A C T

Fibrin gels have been extensively used for three-dimensional cell culture, bleeding control, and molecular and cell therapies because the fibrous networks facilitate biomolecular and cell transport. However, a small window for gelation makes it difficult to handle the gels for desired preparation and transport. Several methods developed to control gelation rates often alter the microstructure, thereby affecting the mechanical response. We hypothesized that a particle designed to discharge thrombin cargos in response to an external stimulus, such as H2O2, would provide control of the gelation rate over a broad range while strengthening the gel. We examined this hypothesis by assembling poly (lactic-co-glycolic acid) (PLGA) particles loaded with thrombin and MnO2 nanosheets that decompose H2O2 to O2 gas. The resulting particles named as catalytic microgelator were mixed with fibrinogen solution or blood containing 0.2 mM H2O2. Due to the increased internal pressure, these particles released a 3-fold larger mass of thrombin than PLGA particles loaded only with thrombin. As a consequence, catalytic microgelators increased the gelation time by one order of magnitude and the elastic modulus by a factor of two compared with the fibrin gel formed by directly mixing fibrinogen and thrombin in solution. These catalytic microgelators also served to clot blood, unlike PLGA particles loaded with thrombin. The resulting blood clot was also more rigid than the blood clot formed by thrombin solution. The results of this study would serve as a new paradigm in controlling gelation kinetics of pre-gel solution and mechanical properties of the post-gel matrix.

1. Introduction

Fibrinogen in the blood forms a fibrin gel that can control bleeding and further healing of wounds. In general, platelets activated in injuries initiate a coagulation cascade that leads to thrombin formation. Thrombin then converts the water-soluble fibrinogen to the insoluble fibrin by cleaving the fibrinopeptide at the center domain. The resulting active sites react with each other to form a three-dimensional fibrin fiber network. The gel is then further stabilized through chemical crosslinking reaction by factor XIII. [1,2,3,4,5] Fibrin associated with platelets forms blood clots in wounded tissue and acts as a provisional matrix that supports cellular activities essential for wound healing and tissue regeneration [6,7]. Fibrous networks of fibrin gels are advantageous to transporting biomolecules while sequestering cells. Therefore, fibrin gels prepared using isolated or recombinant fibrinogen have also been used for three-dimensional cell cultures, tissue glues, and molecular/cell therapies [8,9,10,11,12].

It is common to assemble fibrin gels by mixing the fibrinogen solution with thrombin molecules in a powder or solution form. [13,14] The quick gelation process leads to difficulties in handling the gel for...
cell encapsulation and implantation [15]. To resolve this challenge, several approaches have been exploited to tune the gelation rate. These approaches include alteration of the concentrations of fibrinogen, thrombin, calcium chloride, and Factor XIII [16,17,18,19]. For instance, decreasing the concentration of thrombin slows the gelation, thus providing users with an increased time window for delivery of the gel. However, the resulting gel presents a significant change in microstructure, where the fiber diameter increases and the branching points decrease. In addition, decreasing thrombin concentration increases the elastic modulus of the fibrin gel. However, a further decrease of the thrombin concentration below 0.25 NIHU/ml softens the gel [2,3].

Overall, it would be desirable to have a tool that provides a broader window for control of gelation while minimally altering the microstructure and mechanical properties of the fibrin gel. We hypothesized that particles discharging thrombin cargos in response to an external stimulus would allow us to decouple the dependency between the gelation time and the mechanics of the fibrin gel. This microparticle, referred to as a catalytic microgelator, would also allow us to avoid altering the concentrations of compounds used for the fibrin gel assembly that affects the microstructure and properties of the fibrin gel.

We examined this hypothesis by using poly(lactic-co-glycolic acid) (PLGA) particles loaded with thrombin and MnO₂ nanosheets, denoted as PLGA/MnO₂/thrombin particles. Manganese oxide nanosheets catalyze the decomposition of H₂O₂ to H₂O and O₂ gas. [20,21] Therefore, PLGA/MnO₂/thrombin particles suspended in a media containing H₂O₂ would increase the internal pressure by generating O₂ gas within their interiors. The increased pressure act as an outward force to release thrombin cargos. As a consequence, the increase in thrombin concentration around the particle surface would increase the number of fibrin fibers that sprout from the particle surface (Scheme 1).

In this study, we assessed the extents to which the catalytic microgelator modulates gelation time, microstructure, and mechanical properties of the fibrin gel and blood clot. Catalytic microgelators were mixed with 0.2 mM H₂O₂ solution to trigger the thrombin release before mixing them with fibrinogen solution [22,23]. First, we examined the H₂O₂ decomposition rate, internal pressure change, and thrombin release rate of catalytic microgelators. In parallel, we monitored the fibrin gel formation by confocal microscopy and small amplitude oscillatory shear (SAOS) measurements. We further characterized the resulting gel by imaging the fibrous structure and measuring the molecular diffusivity and the elastic modulus. To extend our study, we examine the extent to which the catalytic microgelators activate the porcine blood clotting response via SAOS and scanning electron microscopy (SEM). Finally, we employed a rabbit hemorrhage liver model to examine the hemostatic performance of the catalytic microgelators for potential biomedical applications. The results of this study improve the quality of fibrin-based gels used for both fundamental and applied bioscience studies.

2. Result and Discussion

2.1. Preparation and characterization of PLGA/MnO₂ particles encapsulating thrombin (PLGA/MnO₂/thrombin particles)

PLGA/MnO₂ particles that release thrombin in response to H₂O₂ were assembled by encapsulating MnO₂ nanosheets and thrombin molecules through the double emulsification. [24,25] Mixing KMnO₄ with alginate dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer in 1:1 ratio resulted in the MnO₂ nanosheets dispersed in water via reduction of KMnO₄ and simultaneous complexation with alginate (Figure S1). [20] The resulting spherical PLGA/MnO₂ particles possess a mean diameter of 1.6 ± 0.6 μm, as confirmed with optical microscope images (Fig. 1B and 1C). The incorporation of MnO₂ nanosheets in the PLGA particles did not result in a significant change in the diameter of particles and the molecular interaction [26,27] between the particles (Figure S2, S3). The presence of MnO₂ nanosheets in the PLGA particle was confirmed by complexing the nanosheets with the rhodamine-conjugated alginate (Fig. 1D). The spherical morphology of the PLGA particle was also confirmed by SEM (Fig. 1E).

The mass of MnO₂ nanosheets loaded in 1 mg of particles could be controlled from 0.0625 to 6.25 μg by altering the initial concentration of MnO₂ nanosheets added to the aqueous media during the double emulsification. The loading mass of thrombin was minimally affected by the loading mass MnO₂ nanosheets in the particles. According to the thrombin ELISA kit, 1 mg of PLGA and PLGA/MnO₂ particles carried 72 ± 1.6 ng of thrombin. The small loading error range influenced the drug release profile insignificantly. We also evaluated the potential toxicity of the PLGA and PLGA/MnO₂ particles by measuring the

Scheme 1. Schematic illustration of the process to form fibrin networks in which fibrin fibers are interconnected with PLGA/MnO₂/thrombin particles (i.e., PLGA particles loaded with catalytic MnO₂ nanosheets and thrombin). H₂O₂ triggers continuous release of thrombin cargos from PLGA/MnO₂/thrombin particles and, in turn, increases the internal pressure of particles. The released thrombin initiates polymerization of fibrinogen on the PLGA particle surface and promotes the sprouting of fibrin fibers.
metabolic activity of endothelial cells incubated with these particles for 24 hrs. Up to the particle concentration of 10 mg/mL, more than 70% of cells remained metabolically active (Fig. 1F).

2.2. Kinetic study of H₂O₂ decomposition by PLGA/MnO₂ particles

We examined the extent to which PLGA/MnO₂ particles take up and decompose H₂O₂ by measuring peroxide-induced oxidation of ferrous to ferric ions. Both fresh and lyophilized PLGA/MnO₂ particles decreased H₂O₂ concentration from 0.2 to 0.15 mM continuously over 7 hours (Fig. 2A). In contrast, the direct addition of MnO₂ nanosheets to the H₂O₂ solution resulted in rapid decomposition, as reported before. [20,28,29] The H₂O₂ concentration decreased from 0.2 mM to almost 0 mM within an hour. In addition, blank PLGA microparticles did not decompose H₂O₂.

We calculated the amount of O₂ generated within the particle by using the stoichiometric relationship between decomposed H₂O₂ and generated O₂ (Figure S4). According to the calculation, both fresh and lyophilized PLGA/MnO₂ particles produced O₂ gas continuously up to 7 hours. The lyophilized PLGA/MnO₂ particles generated O₂ gas more actively than fresh ones, likely due to the more rapid diffusion of H₂O₂ into the particle.

We further examined the kinetics of H₂O₂ decomposition by monitoring visible absorbance at 454 nm, after titration with a Cu⁻² and neocuproine (Fig. 2B) [30]. The initial H₂O₂ decomposition rate was quantified with a slope of the linear portion of each curve shown in Fig. 2B. The H₂O₂ decomposition rate by lyophilized PLGA/MnO₂ particles was twice as high as that observed by fresh PLGA/MnO₂ particles (Fig. 2C). This result also suggests that H₂O₂ diffuses into lyophilized PLGA/MnO₂ particles more rapidly than fresh particles due to pore formation in the particle shell after lyophilization. [31,32] The decomposition rate by blank PLGA particles was zero.

In addition, we evaluated the degree that H₂O₂ decomposition rate depends on the H₂O₂ concentration during reactions on MnO₂ nanosheets (Fig. 2D and Figure S5). In this study, we compared the decomposition activity of lyophilized PLGA/MnO₂ particles with that of free MnO₂ nanosheets. The decomposition rate exhibited a nearly linear dependence on H₂O₂ concentration for both free MnO₂ nanosheets (rate \( \sim [H_2O_2]^{0.9 \pm 0.04} \)) and lyophilized PLGA/MnO₂ particles (rate \( \sim [H_2O_2]^{0.9 \pm 0.05} \)).

The activation energy for H₂O₂ decomposition (\( E_a \)) was calculated by fitting the curve of the decomposition rate constant (k) calculated by assuming a pseudo-first-order rate expression on H₂O₂ concentration versus inverse temperature to the Arrhenius equation.

\[
k = v e^{-E_a/RT}
\]

Where v is the pre-exponential factor, R is the ideal gas constant, and T is the absolute temperature (Fig. 2E and Figure S6). The activation energy with free MnO₂ nanosheets (44 ± 4 kJ/mol) is comparable to \( E_a \) attained with lyophilized PLGA/MnO₂ particles (49 ± 5 kJ/mol). The similarity of the H₂O₂ decomposition rates, power-law dependencies, and activation energies suggest that the integration of MnO₂ nanosheets into PLGA particles does not significantly affect the rate or the mechanism for H₂O₂ decomposition on these materials.

The lyophilized PLGA/MnO₂ particles were able to protect cells from H₂O₂ (Fig. 2F). We exposed endothelial cells to the H₂O₂ or the mixture of H₂O₂ and PLGA/MnO₂ particles for 24 hours and examined the metabolic activity of cells. 90% of cells lost metabolic activity in the media containing 0.3 and 0.6 mM H₂O₂. In contrast, approximately 90% of cells incubated with PLGA/MnO₂ particles remained metabolically active up to 0.6 mM H₂O₂ solutions. This protective effect is due to the uptake and decomposition of H₂O₂ by the MnO₂ within the PLGA/MnO₂ particles.

2.3. Analysis of thrombin release from particles

The ability of thrombin-encapsulating PLGA and PLGA/MnO₂ particles to release thrombin cargos was evaluated by measuring the mass of thrombin discharged from the particles (Fig. 3A). The particles were incubated in neutral PBS with 0.2 mM H₂O₂. The 0.2 mM concentration
was chosen because it is sufficient to trigger the active release of thrombin without damaging particles, according to our previous study. [20] The H$_2$O$_2$ level also falls in the range of the physiological concentration of H$_2$O$_2$ [22,23,33]. Thrombin molecules burst out within the first hour. Thrombin molecules partially embedded in the surface layer of the particles contribute mainly to this initial burst [34,35]. During 7 hours, fresh PLGA/MnO$_2$ particles, in which 6.25 μg of MnO$_2$ nanosheets were loaded into 1 mg of particles, released three times more thrombin than PLGA particles. Lyophilized PLGA/MnO$_2$ particles released thrombin faster than fresh particles. In contrast, PLGA/MnO$_2$ particles, in which 62 ng of MnO$_2$ nanosheets were loaded in 1 mg of particles, released thrombin cargos with a similar profile to PLGA particles. Thus, PLGA/MnO$_2$ particles (1 mg) encapsulated with 6.25 μg MnO$_2$ nanosheets were used in the rest of the study.

The H$_2$O$_2$-responsive release of thrombin is attributed to the generation of oxygen which increases the internal pressure of particles. [20] The total flux of thrombin across PLGA particles ($J_t$) after the initial burst (1-20 hour) was correlated to the pressure difference between inside and outside particles ($\Delta P$). The $\Delta P$ value of particle was calculated for each group using the ideal gas law for O$_2$ gas. The mass of O$_2$ shown in Figure S2 was used in the calculation. (Calculation of $\Delta P$ is detailed in the Supporting Information). The calculated $\Delta P$ values for lyophilized and fresh PLGA/MnO$_2$ were 10.3 mmHg and 9.8 mmHg, respectively. In contrast, PLGA particles free of MnO$_2$ only exhibited a minimal pressure difference of 2.1 mmHg (Table S1). As shown in Fig. 3B, the release rate ($J_t$) of thrombin molecules was linearly related to the calculated $\Delta P$.

The linear dependence of $J_t$ on $\Delta P$ confirms that enhanced release of thrombin cargos from PLGA/MnO$_2$/thrombin particles is primarily due to the $\Delta P$, which is well-described by the Kedem-Katchalsky equation. [36,37] The equation relates $J_t$ to the flux driven by the hydraulic pressure difference and that induced by the diffusion as shown in Eq. (2).

$$J_t = a\Delta c + b\Delta P$$

Where $a = RT/\omega \sigma (1 - \sigma)\bar{C}_{L_p}$ and $b = (1 - \sigma)\bar{C}_{L_p}$ are constant values, $\Delta c$ is the concentration difference of thrombin between particle interior and exterior, and $\Delta P$ is the hydraulic pressure difference between inside and outside of the particle. $R$ represents the gas constant, $T$ the temperature, and $\sigma$ is the average concentration of thrombin across the particle. $L_p$, $\lambda$, and $\bar{C}$ are transport coefficients of filtration, solute permeability, and reflection coefficients of PLGA particle, respectively. In Eq. (2), $\Delta c$ was approximated as a constant, equal to the concentration of thrombin inside the particle because the concentration of thrombin within the particle was several orders of magnitude higher than that outside the particle. Thus, $J_t$ becomes primarily dependent on $\Delta P$ [20].

To confirm the O$_2$-induced release of particles, we examined the spatial distribution of fluorescently-labeled MnO$_2$ nanosheets in the PLGA/MnO$_2$ particles upon exposure to H$_2$O$_2$ using confocal microscopy (Fig. 3C). MnO$_2$ nanosheets were distributed uniformly within the particle suspended in PBS. In contrast, the particles incubated in the PBS supplemented with 0.2 mM H$_2$O$_2$ displayed the localization of
MnO₂ nanosheets to the particle wall. This result indicates that PLGA/MnO₂ particles increase the internal pressure due to the oxygen generation. Most of the MnO₂ nanosheets remained in the particles during H₂O₂-triggered thrombin release, as confirmed by the lack of change in the red fluorescence over time (Figure S7).

2.4. Microstructural Analysis of Fibrin Gel Formation

We examined the extent to which the H₂O₂-mediated thrombin release rate modulates the fibrin gel formation by visualizing the fibrous network formation process (Figure 4A and B). We encapsulated 72 ng of thrombin into 1 mg of PLGA or PLGA/MnO₂ particles. The loading amount of MnO₂ nanosheets was kept constant at 6.25 µg per mg of particles. The particles were mixed with an aqueous mixture of fluorescein-labeled fibrinogen and H₂O₂. The concentration of H₂O₂ in the fibrinogen solution was kept constant at 0.2 mM.

According to the real-time confocal images, thrombin-encapsulating PLGA/MnO₂ particles (PLGA/MnO₂/thrombin particles) served to form fibrin networks more rapidly than thrombin-encapsulating PLGA particles (PLGA/thrombin particles) (Figure 4B). The quantitative image analysis conducted by counting the fibrin fibers disclosed that PLGA/MnO₂/thrombin particles increased the total number of fibrin fibers by a factor of two (Figure 4C). PLGA/MnO₂/thrombin particles associated with the fibrin network, while PLGA/thrombin particles clustered together (Figure 4D-1 and D-2). The aggregation of PLGA/thrombin particles in the fibrin gel likely results from the hydrophobic interaction between the particles that do not associate with fibrin fibers. [38] According to the SEM images of the gel, PLGA/MnO₂/thrombin particles resulted in the fibrin gel with similar microstructure to the gel made using the thrombin solution, as evaluated with the fiber diameter and the number and density of branching points (Figure 5). In addition, PLGA/MnO₂/thrombin particles were connected with multiple fibrin fibers (Figure 5E). In contrast, the fibrin gel formed by PLGA/thrombin particles (Figure 5C and 5D) showed a thicker fibrin fiber and a smaller number of branching points than two other conditions (Figure 5D).

We propose that PLGA/MnO₂/thrombin particles release thrombin rapidly to increase the fibrinogen cleavage rate, compared with PLGA/thrombin particles. The resulting fibrinogen monomers serve to increase the number of branching points of fibrin networks instead of promoting longitudinal and lateral growth [3]. In addition, the anchorage of fibrin fibers to PLGA/MnO₂/thrombin particles suggests that thrombin released from the particles activate the polymerization of fibrinogen on the particle surface and promote the sprouting of fibrin fibers (Figure 5E). In contrast, PLGA/thrombin particles cleave fibrinopeptide at a lower rate than PLGA/MnO₂/thrombin particles due to the slow release of thrombin. As a consequence, fibrinogen monomers tend to polymerize into long fibers and then associate laterally to form thick fibers, as shown in Figure 5F.

Permeability of the gel was examined by measuring a diffusion coefficient of dextran within the gel (Figures 6A and 6B) [39, 40]. Dextran probes labeled with fluorescein were loaded into the fibrin gel for the FRAP assay. The diffusion coefficient of dextran was calculated from the fluorescence recovery rate in the photobleached spot (details are in the experimental section). The fibrin gel prepared with PLGA/MnO₂/
thrombin particles decreased the diffusion coefficient of the dextran by a factor of two compared with PLGA/thrombin particles (Fig. 6C).

We further evaluated the bio-adhesion of the fibrin gel to tissue by filling the wound-mimicking hole introduced in a porcine skin explant with the gel (Figure S8). Then, we observed infiltration of fibrin networks to the skin tissue using SEM. The gels prepared using the PLGA/MnO$_2$/thrombin particle and PLGA/thrombin particles anchored to the skin explant similar to the gel prepared with a mixture of fibrinogen and thrombin. We also characterized the surface hydrophilicity of the gels by measuring the water contact angle [41,42]. The thrombin released from PLGA particles did not alter the water contact angle on the fibrin gel surface significantly compared with the thrombin solution mixed with fibrinogen solution (Figure S9).

2.5. Rheological Characterization of Fibrin Gel Formation

One major advantage of using PLGA/MnO$_2$/thrombin particle-based gelator in the preparation of fibrin gels is to broaden the time window for gel preparation without reducing gel rigidity. This process is different from the conventional preparation method of the fibrin gel in which fibrinogen solution is mixed with the thrombin solution or powders. Branching points of the fibrin gel are responsible for the elastic response of the gel [2]. We, therefore, monitored the gelation process by measuring the storage modulus of the gel over time (Fig. 7A). Notably, mixing fibrinogen with the thrombin solution increased the storage modulus to 180 Pa within 5 minutes, with no significant increase after 5 minutes (Figure 7B-1). Mixing fibrinogen, thrombin, and blank PLGA particles exhibited a limited increase in the storage modulus to 3 Pa. This result implies that blank PLGA particles that are not releasing thrombin molecules do not participate in forming the gel network. Instead, blank PLGA particles act as a physical barrier of the fibrin gel formation.

In the presence of H$_2$O$_2$, catalytic PLGA/MnO$_2$/thrombin particles served to increase the storage modulus of the gel continuously over 100 minutes. The storage modulus eventually reached approximately 250 Pa after 2.5 hours. In contrast, non-catalytic PLGA/thrombin particles led to a softer network than PLGA/MnO$_2$ particles, as indicated by the lower storage modulus throughout the measurements (Figure 7B-2). The storage modulus at 3.75 hours after initiation of gelation was quantified as the equilibrium storage modulus, denoted as $G'_e$, because no significant increase occurred beyond this time. The equilibrium storage modulus of the gel formed by mixing thrombin solution with fibrinogen was defined as the storage modulus at 25 minutes after initiation of gelation. $G'_e$ of the gel prepared by the PLGA/MnO$_2$/thrombin particles was 33% higher than the fibrin gel prepared by mixing fibrinogen and thrombin solution, 71% higher than that of the fibrin gel prepared with the PLGA/thrombin particles, and 350% higher than that of the fibrin gel prepared by mixing fibrinogen, thrombin, and
blank PLGA (Figure 7C). We propose that the difference between these conditions is attributed to the short half-life of thrombin. In particular, the PLGA/MnO₂/thrombin particles serve to release biologically active thrombin and create reactive sites of fibrinogen to polymerize continuously, while the thrombin dissolved in solution loses bioactivity quickly [43]. Furthermore, due to the connection of particles with interconnected fibrin fibers, the equilibrium storage module of the gel formed by thrombin releasing particles (e.g., PLGA/MnO₂/thrombin particles and PLGA/thrombin particles) are significantly higher than the storage modulus of the fibrin gel made with a mixture of fibrinogen, thrombin and blank PLGA particles.

In parallel, the time required to reach a stiffness equal to one half of Gₚ, denoted t₁/₂, was quantified to assess the time window required for gel fabrication (Fig. 7D). While the t₁/₂ of PLGA/MnO₂/thrombin particles was five times larger than the mixture of fibrinogen and thrombin, t₁/₂ was significantly smaller than that attained with the PLGA/thrombin particles. The difference in t₁/₂ between PLGA/MnO₂/thrombin and PLGA/thrombin particles is in accordance with the thrombin release and kinetic study, where the faster release of thrombin leads to smaller t₁/₂. t₁/₂ for the mixture of fibrinogen, thrombin, and PLGA particles was comparable to that with the PLGA/thrombin particles. This result indicates that the fibrin gelation process can be modulated by tuning the thrombin release profile from the particles. The accelerated release of thrombin from the PLGA/MnO₂/thrombin particles led to a decrease in the gelation time [3]. We propose that a further increase in particle concentration will facilitate the gelation process due to the increase in total mass of thrombin in the pre-gelled fibrinogen solution. However, excess particles can act as physical barriers for the growth of fibrin fibers, which is implicated in Fig. 7C. More in-depth relationship between particle concentration and gel stiffness will be of interest for future study.

We further fitted the storage modulus-time curves in Fig. 7B to a simple model proposed for a blood coagulation process (Eq. (3)) [44],

\[ G' (t) = G'_v \exp (-\beta / t) \]  

in which \( G'_v \) represents the equilibrium storage modulus, \( \beta \) is the characteristic time, and \( t \) indicates the time scale of gelation (Figure S10). This single exponential model gave a good fitting quality (R² = 0.994) for the gelation process (Table 1). PLGA/MnO₂/thrombin particles resulted in the five-fold increase in \( \beta \) compared with the mixture of fibrinogen and thrombin solutions where the smaller \( \beta \) represents faster gelation. The PLGA/MnO₂/thrombin particles led to the smaller \( \beta \) than PLGA/thrombin particles. However, the relatively low R² value with PLGA/MnO₂/thrombin particles (i.e., 0.93) indicates that the single exponential model may not sufficiently describe the gelation caused by the microparticles.

Therefore, the evolution of the storage modulus was decomposed into two reaction phases, as proposed by Kaibara. [45] The first phase corresponds to the elastic response caused mainly by the polymerization and branching of fibrin fibers, while the other represents the elastic response associated with the lateral association of fibrin polymers. As shown in Fig. 7, the storage modulus increases more steeply during the first phase. To separate the distinct contributions, we exploited the double exponential model [45] (Eq. (4)),

\[ G(t) = G'_v + G_1 (1 - \exp (-\frac{1}{\tau_1})) + G_2 (1 - \exp (-\frac{1}{\tau_2})) \]  

in which \( G'_v \) is the storage modulus of the pre-gelled solutions, \( G_1 \) is the storage modulus contributed by the first branching and crosslinking reaction phase, \( G_2 \) is the storage modulus caused by the second lateral growth of fibrin polymers, and \( 1/\tau_1 \) and \( 1/\tau_2 \) are the characteristic rates for branching and lateral growth, respectively (Fig. S11).

As shown in Table 2, PLGA/MnO₂/thrombin particles showed slower rates of branching (1/\( \tau_1 \)) and lateral growth (1/\( \tau_2 \)) than the mixture of fibrinogen and thrombin by one and two orders of magnitude, respectively. Combined with the higher \( G_1 \) and \( G_2 \) values, this result confirms the advantages of using PLGA/MnO₂/thrombin particles to form a stiffer network while expanding the time window for gelation. In addition, PLGA/MnO₂/thrombin particles led to a faster branching rate (1/\( \tau_2 \)) than PLGA/thrombin particles due to the faster release of
thrombin molecules. The rate of lateral growth (1/z) had no significant difference. This result suggests that thrombin released from PLGA/MnO$_2$ particles contributed to increasing the number of branching points of fibrin networks.

We further examined whether the fibrin gel formed using the PLGA/MnO$_2$/thrombin particles modulate activities of endothelial cells to form blood vessel-like lumens similar to the gel formed using the thrombin solution [46]. In this study, we loaded human umbilical vein endothelial cells (HUVECs) within fibrin gels of interests installed in the center channel of a microfluidic chip assembled for neovascularization study (Figure S12). As the vascular endothelial growth factors (VEGF) diffuse from the outer channels and to the HUVEC-laden fibrin gel, cells assembled to form interconnected endothelial lumens. Likely due to the similar fibrous structure between two fibrin gel systems, the length and the number of resulting endothelial lumens were similar to each other.

2.6. Rheological Characterization of Blood Clotting Promoted by PLGA/MnO$_2$/thrombin Particles

We further evaluated the efficacy of PLGA/MnO$_2$/thrombin microparticles to activate the blood clotting in response to H$_2$O$_2$. In this study, 3.2% buffered sodium citrate was added to porcine blood to prevent instantaneous clotting after blood collection. The PLGA/MnO$_2$/thrombin particles were mixed with H$_2$O$_2$ solution (0.2 mM) to activate the release of thrombin cargos. Then, the particles were added to the blood. The blood mixed with the PLGA/MnO$_2$/thrombin particles formed a clot within 10 minutes. Therefore, the mixture stayed at the top of the tube after flipping. In contrast, the blood mixed with PLGA/thrombin particles failed to form a clot even after 30 minutes (Fig. 8A).

In parallel, we monitored the strengthened network of the blood mixture via SAOS rheology. The blood mixed with PLGA/MnO$_2$/thrombin particles displayed a continuous increase of the storage modulus up to 10 Pa through 70 minutes. (Fig. 8B). The storage modulus of the resulting blood clot was twice as large as that of the blood clot formed by thrombin solution (Fig. 8C and Figure S13). In contrast, the blood mixed with PLGA/thrombin particles showed almost no increase in the storage modulus. This result indicated that PLGA/MnO$_2$/thrombin particles were able to discharge promptly and polymerize fibrinogen in the blood even with the presence of sodium citrate.

We also observed the microstructure of the blood clot formed by PLGA/MnO$_2$/thrombin particles with SEM (Fig. 8D). The images show an interconnected and highly branched fibrous network, similar to the pure fibrin gel prepared with PLGA/MnO$_2$/thrombin particles. The
Table 1
Gelation curve fitting parameters obtained from the gelation curves in Figures 7B-1 and B-2 using Eq. (1). \( G_{\text{eq}} \) represents the final equilibrium storage modulus, and \( \beta \) is the characteristic time for gelation.

<table>
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<th>Parameters</th>
<th>( G_{\text{eq}} ) [Pa]</th>
<th>( \beta ) [min]</th>
<th>( R^2 )</th>
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<td>PLGA/thrombin particles</td>
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<td>Mixture of thrombin and blank PLGA particles</td>
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Table 2
Gelation curve fitting parameters obtained from the gelation curves in Figures 7B-1 and B-2 using Eq. (2). \( G_1 \) is the storage modulus contributed by the branching and crosslinking reaction phase, and \( G_2 \) represents the storage modulus result from the second lateral growth of fibrin fibers, and \( 1/\tau_1 \) and \( 1/\tau_2 \) are the characteristic rates for branching and lateral growth, respectively.

<table>
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<th>Parameters</th>
<th>( G_1 ) [Pa]</th>
<th>( 1/\tau_1 ) [1/min]</th>
<th>( G_2 ) [Pa]</th>
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<td>PLGA/thrombin particles</td>
<td>72.63</td>
<td>0.052</td>
<td>80.87</td>
<td>0.0089</td>
<td>0.9985</td>
</tr>
<tr>
<td>Mixture of thrombin and blank PLGA particles</td>
<td>4.34</td>
<td>0.052</td>
<td>3.642</td>
<td>0.005</td>
<td>0.9994</td>
</tr>
</tbody>
</table>

Magnified image shown in Figure 8D-2 confirms that PLGA/MnO\(_2\) particles are connected with fibrin fibers likely because the polymerization of fibrinogen occurred actively on the particle surface (yellow arrows in Fig. 8D-2).

Based on the results, we propose that our catalytic microgellers is a promising ingredient of the hemostatic agents by reproducing two major hemostasis processes: platelet-fiber association and thrombin-activated fibrin network formation [47]. Success in recapitulating the hemostasis process relies on the ability to expedite the discharge of thrombin cargos from microparticles. Otherwise, the microparticles fail to form the desired fibrin networks promptly, as demonstrated with PLGA particles loaded with thrombin only. In contrast, the catalytic microgellers exposed to H\(_2\)O\(_2\) discharge thrombin actively into the blood and, in turn, promote fibrin network formation. In addition, as exhibited with SEM images and the mechanical test, PLGA/MnO\(_2\)/thrombin particles associated with fibrin fibers serve to stiffen the blood
2.7. In Vivo Hemostatic Performance Analysis

Finally, we examined the extent to which PLGA/MnO$_2$/thrombin particles can act as a hemostatic agent by measuring time to hemostasis with a rabbit liver bleeding model (Fig. 9, details are described in the Experimental Section) [48]. Briefly, a 3 mm-diameter defect was created on the rabbit liver using a biopsy punch, and time to hemostasis was measured. The bleeding stopped after 225 seconds without any treatment. The repeated administration of PLGA/thrombin particles into the defect minimally influenced the bleeding (Fig. 9A, Video 2). After 90 seconds, the degree of bleeding was not significantly different from the untreated condition (Fig. 9A & Video 1). Following continued administration of PLGA/thrombin particles, the bleeding stopped after 187 seconds. During this period, 50 drops of particle suspension were administered to the bleeding site. The insignificant difference of the time to hemostasis between these two groups indicated that the slow release of thrombin from the PLGA/thrombin particle is not sufficient to facilitate hemostasis. In contrast, the PLGA/MnO$_2$/thrombin particles mixed with 0.2 mM H$_2$O$_2$ solution at a concentration of 40 mg/ml could stop bleeding after 90 seconds (Figure 9A & B, Video 3). During this period, ten drops of particle suspension were used to control bleeding. This accelerated hemostasis is attributed to the active release of thrombin triggered by H$_2$O$_2$.

In the past, a series of hemostatic particles were reported to treat a severe injury that is not amenable to hemostasis. These particles were designed to reproduce the biological function of natural platelets that adhere to the bleeding site and aggregate [47,49,50,51,52,53]. For instance, liposomes were decorated with Glycoprotein Ia-IIa that adhere to the Von Willebrand factor (vWF) or collagen to promote adhesion to a bleeding site [54]. To reproduce aggregation effect of the platelets, fibrinogen or the fibrinogen-binding peptide molecules have been incorporated into the surface of microparticles [50]. However, bleeding often washes out coagulation factors, including thrombin, thus limiting fibrin network formation. In this regard, the design principle of the catalytic microgelator would be broadly useful for improving the quality of current hemostatic particles by promoting particle-fiber connection and reinforcing blood clots.

3. Conclusion

The results of this study demonstrate that the catalytic microgelator made of PLGA particles, MnO$_2$ nanosheets, and thrombin cargos is advantageous to extending gelation time and increasing mechanical strength of fibrin gels and blood clots. PLGA/MnO$_2$/thrombin particles suspended in media or blood containing 0.2 mM H$_2$O$_2$ took up and decomposed H$_2$O$_2$ generated O$_2$ gas, increased internal pressure, and
finally ejected thrombin cargos much faster than PLGA/thrombin microparticles. The fibrinogen solution or blood mixed with PLGA/MnO₂/thrombin particles displayed the extended gelation time by one order of magnitude. Also, the mixture formed a more rigid and elastic fibrin network or blood clots than the gel formed by mixing thrombin and fibrinogen solution. These results are attributed to the PLGA/MnO₂/thrombin particles that serve to sustain the presence of thrombin molecules in the fibrinogen solution or blood. It is also likely that thrombin released from PLGA/MnO₂ particles promotes polymerization of fibrinogen on the particle surface and, in turn, increases the number of linkages between fibrin fibers and particles. As a consequence, PLGA/MnO₂ particles also act as reinforcing fillers. We propose that the results of this study provide a new paradigm in controlling gelation kinetics of pre-gel solution and mechanics of the post-fibrin gel in a sophisticated manner.

4. Experimental Section

4.1. Materials

Poly(lactic-co-glycolic acid) (PLGA) with an acid end group and molecular weights ranging from 6,000 to 10,000 g/mol (lactic acid: glycolic acid = 50:50) was purchased from LACTEL Absorbable Polymers, DURECT (U.S.A). Alginate (MW ≈ 250000 g mol⁻¹) was obtained from FMC Biopolymer. Cellulose filter was purchased from Millipore Inc. 2-(N-morpholino)ethanesulfonic acid (MES), potassium permanganate (KMnO₄), bovine serum albumin (BSA), poly(vinyl alcohol) (PVA, MW = 9000 – 10000), 1-hydroxybenzotriazole hydrate, fluorescein isothiocyanate-dextran (FITC-dextran), neocuproine (> 98%), thrombin from human plasma, fibrinogen from human plasma, and fluorescein isothiocyanate-dextran (FITC-dextran) were
4.2. Synthesis of Fluorescently Labeled Alginate and Water-Dispersible \( \text{MnO}_2 \) nanosheets

Alginate (0.5 mmol uronic acids) dissolved in MES buffer was labeled with lissamine rhodamine B ethylendiamine using aqueous carbodiimide chemistry. 1-Hydroxybenzotriazole hydrate (0.08 mmol), lissamine rhodamine B ethylendiamine (0.0008 mmol), and 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (0.16 mmol) were added sequentially to the alginate solution. The reaction mixture was stirred at room temperature and protected from light for 4 hours. Then, the fluorescently labeled alginate was purified by dialysis against DI water over 2 days and finally lyophilized. Filtered alginate was prepared by dissolving alginate in 0.2 M MES buffer (pH 6) at a concentration of 1.0% (w/v). Then, the alginate solution was filtered with 0.22 \( \mu \)m cellulose filter and freeze-dried. The water-dispersible \( \text{MnO}_2 \) nanosheets were prepared by mixing 2.0% (w/v) alginate solution prepared with 0.2 M MES buffer with 50 mM KMnO\(_4\) at 1:1 ratio. The mixture was sonicated for 30 min.

4.3. Assembly of PLGA Particles Loaded with Human Thrombin and MnO\(_2\) Nanosheets (PLGA/MnO\(_2\)/thrombin particles)

PLGA/MnO\(_2\)/thrombin particles were prepared via double emulsification (W\(_1\)/O/W\(_2\)). Water phase 1 (W\(_1\)) was prepared by adding 50 \( \mu \)L thrombin (100 NIHU/mL) to a 150 \( \mu \)L suspension of MnO\(_2\) nanosheets. The organic phase was prepared by dissolving 10 mg of PLGA in 0.75 mL of dichloromethane (DCM). Vortex mixing was conducted to form the W/O emulsion. This primary emulsion was then transferred to 6 ml of 1% (w/v) PVA solution (W\(_2\)). Then, the mixture was vortexed again to prepare the secondary emulsion (W\(_1\)/O/W\(_2\)). The mixture was continuously stirred for 4 hr to evaporate DCM. The resulting particles were washed three times and collected by centrifugation at 2,000 rpm for 10 min. In parallel, PLGA particles loading thrombin were assembled by following the same procedure in the absence of MnO\(_2\) nanosheets.

4.4. Characterization of PLGA/MnO\(_2\)/thrombin particles

The optical and confocal microscopic images of PLGA particles were obtained by the optical microscope (Leica DMIL) and confocal microscope (LSM 700, Zeiss), respectively. For scanning electron microscopy (SEM) imaging of particles, PLGA particle suspensions were frozen at -20 °C and then lyophilized. The SEM images of PLGA microparticles were obtained with the HITACHI S-4700 microscope operating at 5.0 kV. The elemental analysis of manganese within PLGA particles was conducted with the inductively coupled plasma atomic emission spectrometer (ICP/AES). The size distribution of particles was obtained by image analysis using the ImageJ software (NIH Image).

Molecular interactions between the PLGA, MnO\(_2\) nanosheets, and thrombin were investigated using vibrational spectroscopy. Samples (−3 mg) were pressed onto the diamond internal reflection element (IRE) of an attenuated total reflectance infrared spectrometer (ATR-IR; Bruker, Alpha). Spectra were recorded at ambient conditions (64 scans, 2 cm\(^{-1}\) resolution) with a clean diamond IRE as the background.

4.5. Characterization of Reactivity of PLGA/MnO\(_2\)/thrombin particles

Initial H\(_2\)O\(_2\) decomposition rates were measured using a batch reactor with a volume of 20 cm\(^3\). H\(_2\)O\(_2\) was added to PBS and heated to the desired temperature (300 - 320 K) while stirring the mixture at 800 rpm. H\(_2\)O\(_2\) decomposition was initiated by adding PLGA/MnO\(_2\) particles or MnO\(_2\) nanosheets. Small aliquots (−0.5 mL) were extracted through a 0.22 \( \mu \)m syringe filter as a function of time. These aliquots were immediately titrated with an aqueous solution of CuSO\(_4\) (8.3 mM), neocuprine (12 mM), and ethanol (25% v/v) [30]. The concentration of H\(_2\)O\(_2\) in each aliquot was quantified with the absorbance at 454 nm using a visible-light spectrophotometer (Spectronic, 20 Genesys). The absorbance was back-calculated to the concentration of H\(_2\)O\(_2\) using a standard curve made with solutions of known H\(_2\)O\(_2\) concentration.

The long-term H\(_2\)O\(_2\) decomposition over 7 hours was analyzed by adding 10 mg of PLGA particles into PBS with 0.2 mM H\(_2\)O\(_2\) and incubating them at 37 °C. The mixture was shaken at 100 rpm. At different time points (1, 3, 7, and 20 hours), the supernatant was collected by centrifuging PLGA particles. The H\(_2\)O\(_2\) concentration was quantified using the Pierce Quantitative Peroxide Assay Kit.

4.6. Characterization of Thrombin Release from Particles

The mass of thrombin released from PLGA/MnO\(_2\)/thrombin or PLGA/thrombin particles was measured by suspending 10 mg of particles in 1 mL of PBS mixed with 0.2 mM H\(_2\)O\(_2\) and incubating them at 37 °C. The mixture was shaken at 100 rpm. At different time points (1, 3, 7, and 20 hours), the supernatant was collected by centrifuging PLGA particles. The thrombin concentration was determined using a thrombin ELISA kit.

4.7. Microstructure and Bioadhesion Analysis of Fibrin Gel

The microstructure of the fibrin gel was analyzed by imaging the fibrinous structure using a scanning electron microscope (HITACHI, S-4700). Fibrin gels were prepared by mixing 500 \( \mu \)L fibrinogen (2.5 mg/ml)/H\(_2\)O\(_2\) (0.2 mM) solution with 5 mg PLGA/MnO\(_2\)/thrombin or PLGA/thrombin particles. Control fibrin gels were prepared by mixing thrombin and fibrinogen solutions. The thrombin concentration was adjusted to be same across conditions. After gelation overnight, the resulting fibrin gels were washed three times (5 min per wash) in DI water. Then the fibrin gels were dehydrated with an increasing percentage of ethanol. In particular, the gel samples were immersed in 30, 50, and 70% of ethanol sequentially for 1 hr. The fibrin gels were transferred to 100% ethanol and dehydrated overnight. After dehydration, the fibrin gels were dried with CO\(_2\) using a critical point dryer (Tousimis). SEM images of the gels coated with Pt were taken using the HITACHI S-4700 microscope operating at 5.0 kV.

The bioadhesion of the fibrin gel was analyzed by visualizing the gel-skin interface using a scanning electron microscope (FEI, Quanta FEG 450 ESEM). Fibrin gels were prepared by mixing fibrinogen with thrombin solution, PLGA/MnO\(_2\)/thrombin particles, or PLGA/thrombin particles using the concentration mentioned in the microstructure study. The gel was placed in the wound-mimicking hole punched on the porcine skin explant. After gelation for 1 hr, the resulting fibrin gel was dehydrated with an increasing percentage of ethanol. In particular, the gel samples were immersed in 30, 50, and 70% of ethanol sequentially for 1 hr. The fibrin gels were transferred to 100% ethanol and dehydrated overnight. After dehydration, the fibrin gels were dried with CO\(_2\) using a critical point dryer (Tousimis). SEM images of the gel-skin interface coated with Pt were taken using the FEI Quanta FEG 450 ESEM operating at 10.0 kV.

The water contact angle was measured using a contact angle goniometer (Rame-Hart). After leveling the instrument, 5 \( \mu \)L of deionized water droplet was placed on gel surface. The images of the droplets on the various surfaces were taken and the instrument software was used
to quantify the contact angle. Fresh samples were used for each replicate measurement.

4.8. In situ Confocal Imaging of Fibrin Gel Formation

The fibrous networks evolved during the gelation were imaged using a confocal microscope (LSM 700, Zeiss). The pre-gel solution consisting of fluorescently labeled fibrinogen (2.5 mg/mL) and H$_2$O$_2$ (0.2 mM) was mixed with rhodamine-labeled PLGA or PLGA/MnO$_2$ particles. In this study, fibrinogen was labeled with Alexa-488, while alginate coupled with MnO$_2$ nanosheets was labeled with rhodamine. PLGA particles free of MnO$_2$ nanosheets were labeled by loading rhodamine-labeled alginate via double-emulsification. The pre-gelled mixture was immediately transferred to a microscope slide with a 0.26 mm spacer and capped with a cover slide. The fibrin gel formation was monitored every 10 min for 1 hour by imaging green-colored fibrin fibers that developed over time.

4.9. Analysis of the Biomolecular Diffusion in the Fibrin Gel

The diffusion coefficient of dextran probes within the fibrin gel was measured using the FRAP assay. The pre-gelled mixture that contains fibrinogen (2.5 mg/mL), H$_2$O$_2$ (0.2 mM) and PLGA particles were mixed with 2 mg/mL fluorescein-conjugated dextran. Then, the solution was transferred to the microscope slide with a 0.26 mm spacer and capped with a cover slide. After gelation, the FRAP assay was performed using a multi-photon confocal microscope equipped with a FRAP module (LSM 710, Zeiss). A circle spot with a diameter of 0.2 mm was photobleached using a 488 nm argon laser. After photobleaching, the fluorescent image and intensity were recorded every 1 s for 150 sec. The fluorescence intensity ($F$) over time ($t$) was plotted and fitted using the Eq. (5)

$$F = A_1 + A_2 \exp\left(-\frac{2T}{t}\right)$$  \hspace{1cm} (5)

Where $A_1$ is the initial fluorescence intensity, $A_2$ is the modified Bessel function, and $T$ is the recovery time constant. The diffusion coefficient ($D$) was then calculated using the Eq. (6)

$$D = \frac{R^2}{4T}$$  \hspace{1cm} (6)

where $R$ is the radius of the photobleached spot.

4.10. Rheological Characterization of Gelation Kinetics and Mechanical Property of Fibrin Gel

The gelation process was monitored with small amplitude oscillatory shear rheology using an MCR702 rheometer (Anton Parr) with a parallel plate configuration (25 mm). Reaction mixtures comprised of fibrinogen (12 mg/mL) and H$_2$O$_2$ (0.2 mM) were mixed in a centrifuge tube with a total volume of 0.5 mL. After the addition of PLGA/MnO$_2$/thrombin particles (20 mg) or PLGA/thrombin particles (20 mg), the reaction components were thoroughly mixed with a pipette tip. Then, the mixture was quickly transferred from the tube to the rheometer bottom plate. Measurements of the storage and loss moduli were taken at 0.1 Hz under an imposed strain amplitude of 1%. Data points were recorded every 30 seconds, and the gelation process was monitored until the moduli reached a plateau. The temperature was maintained at 37°C using a Peltier heat exchanger. The control fibrin gel was prepared by mixing fibrinogen and thrombin solution. The thrombin concentration was kept the same as the amount of thrombin released from the PLGA/MnO$_2$/thrombin particles over 7 hours.

4.11. In vitro angiogenesis assay

HUVECs were suspended in the fibrinogen solution (2.5 mg/mL) containing H$_2$O$_2$ (0.2 mM) at a concentration of $4 \times 10^5$ cells/mL. PLGA/MnO$_2$/thrombin fibrin gel was prepared by mixing cell suspension with PLGA/MnO$_2$/thrombin particles. Then, the pre-gel solution was immediately introduced to the center HUVEC channel. The mass ratio between fibrinogen and particles was kept constant at 1:4. The control fibrin gel was prepared by mixing cell suspension with thrombin. The thrombin concentration was kept the same as the amount of thrombin released from the PLGA/MnO$_2$/thrombin particles over 7 hours. The cell suspension-gel constructs were allowed to gel for 5 min at room temperature. To fill the outer channel, the inlet reservoirs of the cell culture media channels were loaded with EGM-2 supplemented with 3 mg/mL of VEGF. The vacuum was then applied at the outlet reservoirs. Following the loading of all four reservoirs, the microfluidic platforms were incubated at 37°C and 5% CO$_2$. The cell culture medium was removed and refilled with fresh EGM-2 culture medium every 24 hr. After 3 days, cells were washed once with PBS and fixed in methanol-acetone solution (1:1 ratio) for 20 min at -20°C. After blocking with 3% BSA in PBS for 1 hr, samples were incubated overnight at 4°C with CD31 primary antibodies directly conjugated with a fluorescent marker (1:20). The cell nuclei were stained with DAPI (1:50) for 20 min at room temperature. The chips were washed three times and stored in PBS before imaging.

4.12. Rheological Characterization and Microstructure Analysis of Blood Clots

PLGA/MnO$_2$/thrombin particles (40 mg) or PLGA/thrombin particles (40 mg) were lyophilized and mixed with H$_2$O$_2$ (0.2 mM) before adding them into 1 mL of porcine blood. Blood was mixed with 3.2% citrate to prevent coagulation. The process of blood clotting was monitored by conducting SAOS rheology. After mixing of blood with particles of interest, 0.5 mL of the mixture was transferred to the bottom plate of the MCR 702 rheometer. For the control group, the pre-gel solution was prepared by mixing 1 mL of porcine blood with an equal amount of thrombin that was released by PLGA/MnO$_2$/thrombin particles over 7 hours.

To visualize the resulting blood clots, the sample mixtures mentioned above were pipetted into a centrifuge tube and flipped every 10 minutes. Scanning electron microscope (Hitachi, S-4800) was used to visualize the microstructure of the fully clotted blood clots formed by PLGA/MnO$_2$/thrombin particles. The sample was prepared in a cylindrical polydimethylsiloxane (PDMS) mold. After one hour, each sample was rinsed with PBS and fixed in the Karnovsky fixative overnight at 4°C. Then, the fibrin clots were rinsed with PBS and fixed with 1% osmium tetroxide for an hour. The clots were rinsed in distilled water and dehydrated by increasing concentration of ethanol gradually. The resulting clots were immersed in 30, 50, and 70% of ethanol sequentially for 1 hr per ethanol concentration. Then, the clot was transferred to 100% ethanol and dehydrated overnight. The dehydrated gel was dried with CO$_2$ using a critical point dryer (Tousimis). SEM images of the clots coated with Pt were taken with HITACHI S-4800 microscope operating at 2.0 kV.

4.13. In Vivo Hemostatic Performance Analysis

To examine the in vivo hemostatic ability of the PLGA/MnO$_2$/thrombin and PLGA/thrombin particles, a rabbit (healthy male New Zealand white rabbit, 3.2-3.5 kg) hemorrhaging liver model was used ($n = 3$ per group). Animal care and experimental procedures were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at Yonsei University Health System, Seoul, Korea (IACUC approval No. 2017-0351). Briefly, 5 mg/kg of xylazine and 15 mg/kg of Zoletil® was intramuscularly injected every 15 min as premedication. After intubation with a 3.0 or 3.5 mm endotracheal tube, 2.0% isoflurane was used to maintain inhalation anesthesia. All the animals received crystalloid solution (10 mL/kg/h) throughout the surgical procedure. After abdominal incision, the bleeding was induced using a biopsy punch.
(Integra MilTEX) to create a 3 mm (diameter) defect. As a negative control, time to hemostasis was measured without any treatment. PLGA/MnO₂/thrombin particles or PLGA/thrombin particles were lyophilized and mixed with H₂O₂ (0.2 mM) before applying on the bleeding site. 0.25 ml of particle solution at a concentration of 40 mg/ml were treated in each defect site. The time to hemostasis was measured with and without treatment of the particle solution.

4.14. Analysis of the Toxicity of Thrombin Carriers

Mouse endothelial cell line, C166 (ATCC CRL2581), was maintained in Dulbecco’s Modified Eagle Medium (DMEM) growth media supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin, and cultured at 37°C, under an atmosphere of 5% CO₂ and 95% humidified air. C166 cells were seeded onto 96-well plates at a density of 5000 cells per well one day before treatment. For a certain experiment, the cell culture media was mixed with H₂O₂ to make the H₂O₂ concentration being 0.2 mM. Different concentrations of PLGA or PLGA/MnO₂ particles were prepared by serial dilutions in the cell culture media. The cells were then incubated with particles. 0.1 ml of PLGA or PLGA/MnO₂ particle suspension was added to each well. After 24 hr, 0.1 ml of growth media and 0.05 ml of XTT cell proliferation assay kit working solution were then added to each well. The cells were incubated for 3 h at 37°C. The absorbance values of the solution at a wavelength of 490 and 630 nm were measured by using the microplate spectrophotometer (TECAN, Infinite 200 Pro). The relative metabolic activity of cells was quantified as [(A₄₉₀/A₆₃₀) of each group / (A₄₉₀/A₆₃₀) of control group] where A₄₉₀ and A₆₃₀ represent absorbance at 490 nm and 630 nm respectively. The control group represents the group in which only media was added to each well.

5. Statistical Analysis

Three samples were analyzed per condition, and the data were presented as mean ± standard deviation unless otherwise specified. To determine significance, comparisons between groups were performed by one-way ANOVA followed by Tukey’s post hoc analyses. Data were considered significant for p values less than 0.05.

6. Supporting Information

The Supporting Information is available free of charge. Water-dispersible MnO₂ nanosheets composed with alginate, SEM images of PLGA particles, FTIR spectra of PLGA particle, cumulative O₂ generated through the H₂O₂ decomposition, H₂O₂ decomposition curve under different conditions, theoretical calculation of the difference between internal pressure and external pressure of PLGA/MnO₂ particles, quantitative analysis of fluorescent-labeled MnO₂ intensity change over time, evaluation of the bioadhesion of fibrin gel, evaluation of surface water contact angle of fibrin gel, gelation curve fitting, in vitro angiogenesis assay, blood clot formation triggered by thrombin (PDF)

Author Contributions

Y.-T.H. and H.K. conceived the idea including designing the experiments. Y.-T.H., D.T.B., and D.W.F. performed the H2O2 decomposition experiment and analysis. Y.H., C.-W.L., and S.A.R. performed rheological measurements and analysis. D.-H.K., Y.-J.L., and H.-J.S. assisted with the in vivo bleeding control experiment. Y.-T.H. performed the angiogenesis experiment, and H.J assisted with the microfluidics design. L.G.S assisted with porcine blood acquisition. Y.-T.H. and Y.S developed the fabrication process. Y.-T.H. wrote the manuscript with input from all authors. The authors declare no competing financial interest.

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Appendix A. Supplementary data

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References


