

Encapsulation of fibrinogen and thrombin with calcium carbonate for hemorrhage control

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Abstract: Hemorrhage remains the major cause of death in combat and civilian trauma, although significant advances in hemostatic agents and blood products have enhanced damage control resuscitation and reduced mortality. Currently, few hemostats are available for cessation of non-compressible torso hemorrhage, e.g., intra-abdominal hemorrhage. To address this hard-to-solve problem, self-propelling hemostatic particles composed of coagulation factors e.g., fibrinogen, thrombin, CaCO_3 and protonated tranexamic acid (TXA^+) that can move against blood flow and promote clot formation have been developed. This paper describes the preparation and characterization of CaCO_3 -encapsulated fibrinogen/thrombin particles. The particles were prepared by interfacial reaction method using water-oil-water emulsion under different conditions that varied in the concentrations of the coagulation factor and ammonium carbonate solutions, amounts of surfactants, mixing speed, volume ratio between water and oil phases. The resulting CaCO_3 encapsulated fibrinogen/thrombin particles were characterized via light microscopy for morphology, gel electrophoresis for presence of fibrinogen and thrombin, rotational thromboelastometry for hemostatic effects and reaction with TXA^+ for self-propulsion test. It was found that CaCO_3 particles had spherical structure with less than 10 μm in diameter, could encapsulate fibrinogen and thrombin, enhance blood coagulation, and generate bubbles for propulsion by reacting with TXA^+ . Moreover, when particles were combined with TXA^+ , a synergistic hemostatic effect was obtained. These hemostatic and self-propelling properties could be optimized via changes to preparation method and composition. Further studies in animal bleeding models are warranted.

Keywords: calcium carbonate; hemorrhage control; hemostatic particle; fibrinogen; self-propulsion; thrombin; tranexamic acid; trauma

1. Introduction

Despite significant advances in hemorrhage control with local hemostatic agents and hemostatic resuscitation with blood products over the last twenty years, uncontrolled bleeding with accompanying



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trauma-induced coagulopathy (TIC) remains the leading cause of preventable death in combat trauma [1,2] and secondary cause of death in civilian trauma [3]. It is also the leading cause of preventable death for 30%-40% of six million trauma victims annually, one-half of whom die in the pre-hospital setting [4,5]. In addition, 87% of military personnel died of their injuries before reaching a definitive medical treatment facility in Afghanistan [6].

Hemostatic dressings and techniques have been made for prehospital control of life-threatening hemorrhage [7-9]. Current hemostatic dressings such as Combat Gauze (Z-Medica), which contains kaolin, do not deliver pharmacologically active hemostatic agents. Combat Gauze is the first-line hemostatic dressing recommended by Tactical Combat Casualty Care (TCCC), and is carried in the packs of US Army soldiers [10]. It is effective for severe bleeding when applied with 3 minutes of manual compression [11].

A major limitation of existing hemostatic agents is that they are lacking adequate clotting capacity to stop the acute hemorrhage within seconds even under conditions where blood clotting mechanisms are impaired, and are largely based on gauze delivery platforms applied to bleeding sites with manual pressure. Without proper compression, high blood flow flushes topical hemostats out of wounds and decreases the efficacy of many hemostatic dressings [12]. These dressings are not ideal for either under-fire combat settings where capacity to tightly pack and manually compress wounds is compromised, especially for lesser trained medics, or in instances of buddy care or self-care [13] or internal, non-compressible hemorrhage due to anatomical location and inaccessibility (e.g., torso) [14]. In addition, they are not rapidly biodegradable nor bioabsorbable, and thus must be removed after the emergency use, which may cause re-bleeding.

Because of their nano/micro sizes, hemostatic agents in a powder form are not limited by the size, shape, or location of a wound, making them suitable for irregular or deep wounds [15]. Large surface area to volume ratio and porous structure can lead to strong fluid absorption ability and water swelling, extensively contact the hemorrhagic sites, and reinforce the contact system activation and blood cells adhesion, biodegradability and safety in medical uses [16,17]. However, one limitation of the topical hemostatic powders is that they are rapidly pushed away by pressurized blood flow, preventing their delivery and delaying initiation of clotting at injured vessels, especially when bleeding originates from severe injuries, damaged vessels cannot be located, or when wounds cannot be compressed [12].

To address the aforementioned issues, self-propelling particles that can move against blood flow, delivering coagulation factors directly into damaged blood vessels, have been developed [18,19]. The particles are composed of fibrinogen- and/or thrombin-bound calcium carbonate (CaCO_3) and protonated tranexamic acid (TXA^+). On contact with blood, self-propulsion occurs as CaCO_3 and TXA^+ are combined in a neutralization reaction to generate propulsive CO_2 gas. The gas would push outwards and hold procoagulants fibrinogen and thrombin in place against the outflow of blood with enough force to allow them to react and create the fibrin clot necessary to seal the wounds of a trauma patient. TXA prevents the resultant clot from fibrinolysis and thus make it more effective at stopping hemorrhage. By-products of this reaction should all be bio-friendly and easily absorbed by the body without any potential harm: in fact, the calcium ion may further promote coagulation.

Calcium carbonate is a versatile inorganic mineral that is abundant in biological and geological systems [20], and can be synthesized and functionalized by a variety of methods [21]. Nano- and micro- CaCO_3 particles are biocompatible and ideal for biomedical applications [22], such as bubble-generating

drug carriers for localized controlled release [23] and various advanced CaCO_3 -based delivery systems [24]. Despite of these properties, the use of CaCO_3 particles alone will not stop bleeding quickly [25], and thus has been used with other hemostatic materials, such as chitosan [26].

Studies have demonstrated that the dressings loaded with the self-propelled thrombin- CaCO_3 particles and TXA^+ reduced blood loss and mortality more effectively than Combat Gauze in severe bleeding swine models when applied without manual pressure [27]. The gauze loaded with CaCO_3 and TXA^+ was more effective at inhibiting fibrinolysis than that loaded with CaCO_3 and non-protonated TXA [28]. Recently, a self-propelling thrombin-containing CaCO_3 and TXA^+ powder that could be delivered percutaneously into the closed abdomen using a spray system increased survival in a swine model of non-compressible intra-abdominal hemorrhage [29,30].

Furthermore, given the importance of fibrinogen in traumatic hemorrhage as the first clotting factor to reach critically low levels and association of low fibrinogen levels with increased bleeding, coagulopathy and poor clinical outcomes [31], we have incorporated fibrinogen into the CaCO_3 particle and shown the particle's hemostatic efficacy, without compromising self-propelling property when mixed with TXA^+ [19].

On the other hand, in all current formulation of fibrinogen- and thrombin- CaCO_3 particles, the coagulation factor was likely not carried completely by CaCO_3 to terminal bleeding sites due to loose interaction between them as these particles were prepared by simple mixing CaCO_3 particle with the coagulation factor.

This report describes preparation and characterization of a new type of fibrinogen- and thrombin- CaCO_3 particles, i.e., CaCO_3 -encapsulated fibrinogen and thrombin particles. Encapsulation of the coagulation factors inside CaCO_3 particles would provide better self-propulsion and delivery into deep bleeding sites, activating blood coagulation at underlying bleeding sites to achieve hemostasis of the entire wound. As a first step, we studied the feasibility of producing CaCO_3 -encapsulated fibrinogen and thrombin particles, respectively with hemostatic and self-propelling properties considering their differences in molecular sizes and therapeutic doses. Specifically, we prepared the particles using various methods and characterized the properties of resultant particles to identify possible parameter values to optimize the formulation. We also demonstrate a synergistic effect when the CaCO_3 -encapsulated thrombin particle and TXA^+ were combined, and discuss future work.

2. Methods

2.1. Preparation of self-propelling particles

Both CaCO_3 -encapsulated fibrinogen and thrombin particles were obtained. Two fibrinogen (9 and 15 mg/mL) and three thrombin concentrations (4.5, 8.9 and 17.8 mg/mL) were selected for further investigation of other variables. In addition to the encapsulation method, precipitation and absorption methods as previously reported [18] were examined for comparison.

The physical properties (e.g., morphology, particle size), hemostatic effects and self-propelling behavior of the particles could be altered by variations in the preparation, such as solution concentration, amount of surfactants, mixing speed and oil-to-water volume ratio. Various excipients (trehalose, polyvinylpyrrolidone and tannic acid) were also incorporated to increase fibrinogen stability [32] and thrombin loading [33] given their unique biomedical properties including hemostatic functions.

2.1.1 CaCO₃-encapsulated fibrinogen particle

The particle was prepared by a similar interfacial reaction method using water-oil-water (W/O/W) emulsion as reported [34] with minor changes. Typically, in one beaker, 3 M of ammonium carbonate ((NH₄)₂CO₃) (ACS grade, Fisher Scientific, Ottawa, ON, Canada) solution was prepared in 4 mL Milli-Q water. In another beaker, 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (99%, Fisher Scientific, Ottawa, ON, Canada) solution was made in 3 mL Milli-Q water, after which 63 mg of fibrinogen concentrate was added for reconstitution and spun for 5 min; the solution was subsequently added to the carbonate solution. Additionally, 6 mL hexane, 83.7 mg Tween 80, 41.3 mg Span 80 (Fisher Scientific, Ottawa, ON, Canada) was homogenized in a glass vial by vortex for 1 min. A water/oil mixture was obtained by adding the fibrinogen/carbonate solution to the hexane emulsion. The mixture was then stirred using a magnetic stirring bar at 200/400/990 rotations per minute (RPM) depending on the condition prescribed in Table 1 for 5 min. In addition, trehalose dihydrate (Fisher BioReagents, Fisher Scientific, Ottawa, ON, Canada) and polyvinylpyrrolidone with a molecular weight of 8000 (VWR International, Mississauga, ON, Canada) were added to the fibrinogen/carbonate solution, respectively in some conditions. Finally, this mixture was added to 80 mL 0.3 M CaCl₂ aqueous solution and spun at 200 /400 RPM for 10 min, leading to the formation of CaCO₃-encapsulated fibrinogen particles. The precipitated particles were collected by centrifugation at 3000 RPM for 10 min and washed three times with water. The particle and supernatant in the aqueous phase were lyophilized under vacuum, respectively. Control particles were prepared accordingly in the absence of fibrinogen.

Using a different carbonate, specifically Na₂CO₃ was attempted, due to its previous success in providing self-propelling particles through a precipitation process [19]. However, when fibrinogen was added to 3 M Na₂CO₃, the solution instantly gelled and clumped. This was most likely due to the high molar concentration of carbonate being used compared to the precipitation method (3 vs. 0.33 M). This highly salty and basic solution (pH > 13) likely resulted in denaturing and precipitation of fibrinogen. As such, the experiment condition was discarded, and the particles were solely made using the (NH₄)₂CO₃ solution (pH ~9.5).

For comparison, the fibrinogen-CaCO₃ particle was also prepared by the precipitation method as previously reported [19]. Briefly, fibrinogen was reconstituted in Milli-Q water at 20 mg/mL followed by dissolution of 10 mM of HEPES and 0.33 M Na₂CO₃, after which it was poured into equal volume of 0.33 M of CaCl₂ aqueous solution and stirred at 200 RPM for 2 h, leading to the precipitation of fibrinogen-CaCO₃ particles. The precipitated particles were collected by centrifugation and lyophilized under vacuum.

2.1.2 CaCO₃-encapsulated thrombin particle

The particle was prepared using the same method as that for the CaCO₃-encapsulated fibrinogen particle under different conditions as detailed in Table 2. Typically, in one beaker, 3 M of (NH₄)₂CO₃ solution was prepared in 4 mL Milli-Q water. In another beaker, 10 mM of HEPES was made in 3 mL Milli-Q water, after which 62.5 mg of bovine thrombin (99.9 U/mg, VWR International, Mississauga, ON, Canada) was added for reconstitution and spun for 5 min; the solution was subsequently added to the carbonate solution. Additionally, 6 mL hexane, 83.7 mg Tween 80, 41.3 mg Span 80 (Fisher Scientific, Ottawa, ON, Canada) was homogenized in a glass vial by vortex for 1 min. A water/oil mixture was

obtained by adding the hexane emulsion to the thrombin/carbonate solution. The mixture was then stirred at 990 RPM for 5 min. Finally, this mixture was added to 0.3 M of CaCl_2 in 80 mL Milli-Q water and spun at 400/990 RPM for 10 min, leading to the formation of CaCO_3 -encapsulated thrombin particles. In addition, tannic acid (TA) (VWR International, Mississauga, ON, Canada), was added to the CaCl_2 solution at 0.1 mg/mL in an attempt to increase thrombin loading as reported [33]. TA has also been used as an effective hemostatic material due to its interaction with plasma proteins as well as a biocompatible cross-linker to stabilize microparticles [35]. The precipitated particles were collected by centrifugation at 3000 RPM for 10 min and washed three times with water. The particle and supernatant in the aqueous phase were lyophilized under vacuum, respectively. Control particles were prepared accordingly in the absence of thrombin.

The absorption method was also used to prepare thrombin- CaCO_3 particle with or without TA as reported [33] for comparison. First, CaCO_3 was synthesized by combining 0.33 M of CaCl_2 aqueous solution with equal volume of 0.33 M Na_2CO_3 in Milli-Q water. The solution was stirred at 450 RPM for 40 sec at room temperature and the resultant particles were then left undisturbed for 15 min. These particles were then centrifuged at 800 RPM for 5 min and washed with water three times. TA was added to the CaCl_2 solution to obtain TA-containing CaCO_3 particle. These particles were either freeze-dried under vacuum or used as is, to prepare the thrombin-absorbed CaCO_3 particle as follows: 100 mg of the CaCO_3 particle was dispersed in 5 mL of thrombin-HEPES solution (60 mg of thrombin, and 10 mM HEPES). The sample was shaken at 4°C for 2 h, and these particles were then centrifuged, washed with water and freeze-dried under vacuum.

2.1.3 Protonation of TXA

Protonated TXA was prepared by first dissolving TXA at its unprotonated form (98+%, ACROS Organics™, Fisher Scientific, Ottawa, ON, Canada) into Milli-Q water at 10% weight to volume ratio. To ensure TXA dissolved completely, the mixture was stirred with a stir bar at a constant speed of 200 RPM until no more crystals could be observed. This process typically took approximately 5 min to complete. One mL of stock hydrochloric acid (HCl) was added into the solution to reach a pH of around 4.5, then any necessary fine tuning of pH was performed by adding 100 μL of HCl at a time to further decrease pH or sufficient water to increase pH. The target acidity of the solution was pH 4.3 as described elsewhere [18]. This solution was lyophilized under vacuum to collect solid TXA in a protonated form, TXA^+ .

2.2. Characterization of self-propelling particles

The morphologies of CaCO_3 -encapsulated fibrinogen and thrombin particles were characterized by light microscopy. As essential requirements for their use in non-compressible hemorrhage control, the hemostatic and self-propelling properties of the particles were measured by a number of methods as described below. The presence and hemostatic effects of fibrinogen and thrombin were quantified by gel electrophoresis and rotational thromboelastometry (ROTEM), respectively. Both methods have been used to analyze fibrinogen [36]. Self-propelling ability of the particle when mixed with TXA^+ in water was measured following the method as previously reported [19]. Furthermore, self-propulsion phenomena of the particles were video recorded in real time and quantitatively analyzed for their response time and moving speed.

2.2.1 Light microscopy

Microscopy images were acquired by ZEISS LSM 800 – Airyscan monitored by ZEN BLUE (Carl Zeiss Canada Ltd., North York, ON, Canada) for all the particles synthesized. A small sample of particle was spread onto a glass slide, and then shaken to remove excess particle. It was then placed onto the microscope stage, and the analysis was performed via a computer (ZEISS LSM 800, Carl Zeiss Canada Ltd.).

2.2.2 Gel electrophoresis

Twenty mg particle samples were rocked in 1 mL pH 4.3 TXA⁺ for 2 h to dissolve the calcium carbonate and free any fibrinogen or thrombin. The samples were centrifuged and the obtained supernatants (10–20 µL) was mixed with 30 µL of sodium dodecyl sulphate (SDS) buffer (Fisher Scientific, Ottawa, ON, Canada), 40 µL of Milli-Q water, and in case of fibrinogen, 8–10 mg of Dithiothreitol as a sulfide bond reducer (Fisher Scientific, Ottawa, ON, Canada). Moreover, the fibrinogen and thrombin samples were prepared at 10 mg/mL in TXA⁺ solution as a positive control, respectively. Albumin from human serum (Sigma-Aldrich, Inc., Saint Louis, MO, USA) was prepared at 10 mg/mL in TXA⁺ solution as a negative control. The mixture was heated at 96°C for 5 min and analyzed by continuous SDS-polyacrylamide gel (Novex™ WedgeWell™ Tris-Glycine mini gels) electrophoresis on an Invitrogen™ Mini Gel Tank (Fisher Scientific, Ottawa, ON, Canada). The gel was run at 125 V and 50 mA for 100 min, and stained with coomassie blue (Fisher Scientific, Ottawa, ON, Canada). Prestained protein ladder composed of a mixture of 12 proteins ranging from 10 to 205 kDa (10, 16, 26, 30, 33, 42, 52, 70, 80, 86, 121 and 205 kDa) (Fisher Scientific, Ottawa, ON, Canada) was run as molecular weight standards (Mw STD).

The aforementioned preparation and characterization methods demonstrate the feasibility of encapsulating fibrinogen and thrombin inside CaCO₃ particles with hemostatic and self-propelling properties tested below.

2.2.3 Rotational Thromboelastometry (ROTEM)

ROTEM has been widely used to measure hemostatic effects of materials in various forms [37]. ROTEM was performed in the presence of the particles to measure the effect on hemostasis, with both plasma with a low concentration of fibrinogen and normal human plasma, at 37°C with a ROTEM Delta machine (Instrumentation Laboratory, Bedford, MA, USA). Typically, 6 mg of fibrinogen or thrombin-containing CaCO₃ or blank CaCO₃ particles were added to a ROTEM cup, followed by addition of 20 µL star-tem (0.2 M CaCl₂) and 300 µL citrated plasma. ROTEM was also performed in the absence of any CaCO₃ particles according to manufacturer's standard procedure and designated as NATEM. All tests were run for more than 60 min. The ROTEM variables assessed included coagulation time (CT) and maximum clot firmness (MCF).

2.2.4 Self-propulsion test

The fibrinogen/thrombin loaded or unloaded CaCO₃ particles and TXA⁺ were well mixed in a predetermined ratio, typically 12:10 mass ratio (12 mg CaCO₃ particles and 10 mg TXA⁺). The mixture was placed on an aluminum weigh boat and aliquoted into 10 approximately equal piles.

One-mL pipette was filled with a predetermined volume of water (0.6 mL), then the top was sealed with Parafilm and the open end was applied to the sample. At this point the CaCO_3 and TXA^+ mixture would dissolve and react, generating bubbles. Particle agglomerations of varying sizes could typically be observed self-propelling up the length of the pipette. Using Tracker, a program designed for motion tracking (downloadable from <https://physlets.org/tracker/>), the movement of these particle agglomerations was quantified over time. Although technically speaking the tracked masses were composed of many individual “particles” insofar as particles are individual CaCO_3 microspheres or TXA^+ fragments, they were in of themselves called “particles” for convenience.

Two primary metrics were derived from the self-propulsion tracking. The first, known as “lag time,” is measured in seconds (sec) and denotes the amount of time between the pipette tip encountering the sample and the movement of a given tracked particle. The second, particle speed, is measured in cm/sec and denotes the speed the particle moves over the first 20 data points measured from its initial separation from the weigh boat.

2.2.5 Estimation of encapsulation efficiency (EE) and loading capacity (LC)

Ten mg particle samples were rocked in 0.5 mL pH 4.3 TXA^+ to dissolve the calcium carbonate and free any fibrinogen or thrombin. The samples were centrifuged and the obtained supernatants (20 μL) was added to plasma (380 μL). The mixture was analyzed by ROTEM to quantify the amount of fibrinogen and thrombin, respectively and calculate LC for each preparation using the following equations derived from a linear relationship between plasma fibrinogen or thrombin concentration and ROTEM MCF or CT, respectively:

$$\frac{\text{mg fibrinogen}}{\text{mg CaCO}_3} = \left(\frac{\text{MCF} - 0.2911}{7.7299} - 0.81 \right) * 10/10$$

$$\frac{\text{mg thrombin}}{\text{mg CaCO}_3} = \left(\frac{\text{CT} - 944.88}{-339829} \right) * 10/10$$

The EE was estimated based on the LC and amounts of CaCO_3 particles produced and fibrinogen or thrombin used in each condition, assuming complete reaction between $(\text{NH}_4)_2\text{CO}_3$ and CaCl_2 .

2.2.6 Release study of CaCO_3 -encapsulated thrombin particles

Ten mg particle samples were rocked in 0.5 mL pH 4.3 TXA^+ and phosphate buffer saline (PBS) solution in an Eppendorf tube on a shaker, respectively. An aliquot of 10 μL sample was taken over time and analyzed by ROTEM performed with each sample-spiked plasma. The percentage change in CT relative to corresponding control was calculated to assess the thrombin release profile.

2.2.7 Statistical analysis

Data points were expressed as mean $\pm$ standard deviation (SD, $n=3$) unless specified. Intergroup analysis was performed using independent t test. All statistical analyses were conducted using SPSS Statistics 28 (IBM Corporation, Armonk, NY, USA). A p value of less than 0.05 was considered significant.

Table 1. Preparation of CaCO₃-encapsulated fibrinogen particles under various conditions

Batch ^a	Ammonium carbonate ((NH ₄) ₂ CO ₃) concentration (M)	Fibrinogen concentration in (NH ₄) ₂ CO ₃ solution (mg/mL)	Hexane amount added (mL)	Mixing speed of W and O (RPM)	Mixing speed of W/O and W	Any additives (concentration in fibrinogen/(NH ₄) ₂ CO ₃ solution)	Encapsulation efficiency (%)	Loading capacity (mg fibrinogen/mg particle)
Pre	0.33 M Na ₂ CO ₃	20 in Na ₂ CO ₃ solution	NA	NA	200 ^b		NA	0.022
Enc-Initial	3	9	6	400	200		20.3	0.0107
Enc-Fib _{high}	3	15	6	990	400		7.4	0.004
Enc-AC _{low}	2	9	6	990	400		13.8	0.0108
Enc-Fib _{high} -AC _{low}	2	15	6	990	400		11	0.0144
Enc-No Fib-AC _{low}	2	0	6	990	400		NA	NA
Enc-Tre _{high}	3	9	6	990	400	Tre (200 mg/mL)	20.3	0.107
Enc-Tre _{low}	3	9	6	990	400	Tre (50 mg/mL)	9.6	0.0005
Enc-PVP	3	9	6	990	400	PVP (10mM)	20.8	0.0109

^a Each batch was coded as initial condition (Enc-Initial) and altered variables with changes relative to the initial condition; AC for ammonium carbonate, Fib for fibrinogen, Enc for encapsulation method, NA for not applicable, O for oil, Tre for trehalose dehydrate, Pre for precipitation method, PVP for polyvinylpyrrolidone with a molecular weight of 8000, W for water; ^b Mixed with equal volume of 0.33 M CaCl₂ aqueous solution.

Table 2. Preparation of CaCO₃-encapsulated thrombin particles under various conditions

Batch ^a	Ammonium carbonate ((NH ₄) ₂ CO ₃) concentration (M)	Thrombin concentration in (NH ₄) ₂ CO ₃ solution (mg/mL)	Surfactant amount added (Span 80/Tween 80) (mg)	Hexane amount added (mL)	Mixing speed of W and O (RPM)	Mixing speed of W/O and W (RPM)	Additives (concentration in CaCl ₂ solution)	Encapsulation efficiency (%)	Loading capacity (mg thrombin/mg particle)
Enc-Initial	3	8.9	41.3/83.7	6	990	400		43.9	0.0231
Enc-Thr _{low}	3	4.5	41.3/83.7	6	990	400		40	0.0105
Enc-Thr _{high}	3	17.8	41.3/83.7	6	990	400		24.5	0.0258
Enc-AC _{low}	2	8.9	41.3/83.7	6	990	400		33.1	0.0261
Enc-Surf _{high}	3	8.9	82.8/166.4	6	990	400		49.9	0.0262
Enc-Mix _{high}	3	8.9	41.3/83.7	6	990	990		49	0.0257
Enc-Hex2x	3	8.9	41.3/83.7	14	990	400		48.7	0.0256
Enc-Hex4x	3	8.9	41.3/83.7	28	990	400		49	0.0257
Enc-TA	3	8.9	41.3/83.7	6	990	400	TA (0.1 mg/mL)	47	0.0247
Enc-TA-No Thr	3	0	41.3/83.7	6	990	400	TA (0.1 mg/mL)	NA	NA
Abs-Initial	NA	6 b	NA	NA	NA	450 c		NA	0.0265
Abs-TA	NA	6 b	NA	NA	NA	450 c	TA (0.1 mg/mL)	NA	0.0264
Abs-TA _{FD}	NA	8.9 b	NA	NA	NA	450 c	TA (0.1 mg/mL)	NA	0.0262
Abs-TA-No Thr	NA	0	NA	NA	NA	450 c	TA (0.1 mg/mL)	NA	NA
Abs-TA _{Enc}	2	8.9 b	41.3/83.7	6	990	400	TA (0.1 mg/mL)	NA	0.0263

^a Each batch was coded as preparation method (Enc or Abs), initial condition and altered variables with changes relative to the initial condition; Abs for absorption method, AC for ammonium carbonate, Enc for encapsulation method, Hex for hexane, Mix for mixing speed of W/O with W, NA for not applicable, O for oil, Sur for surfactant, TA for tannic acid, TA_{Enc} for CaCO₃-TA particle prepared by the encapsulation method, TA_{FD} for freeze-dried CaCO₃-TA particle, Thr for thrombin, W for water; ^b In 10 mM HEPES solution mixed with 100 mg CaCO₃ prepared either by the encapsulation method or from equal mole of 0.33 M Na₂CO₃ and 0.33 M of CaCl₂ in the absence and presence of TA; ^c Mixing 0.33 M Na₂CO₃ with equal volume of 0.33 M CaCl₂

Table 3. Microscopy images of the CaCO₃-encapsulated fibrinogen particles at 40× magnification. Refer to Table 1 for sample details.

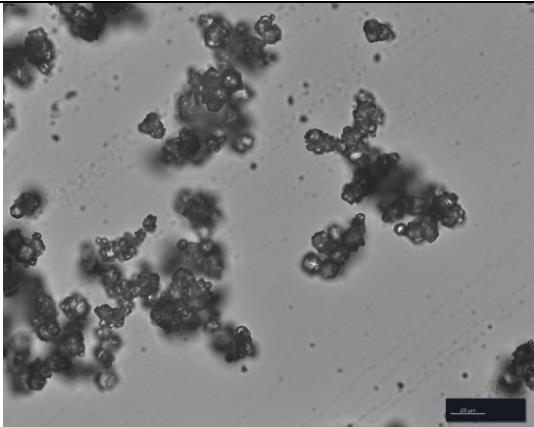
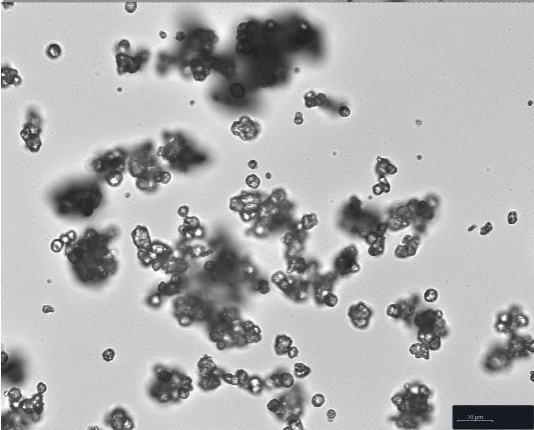
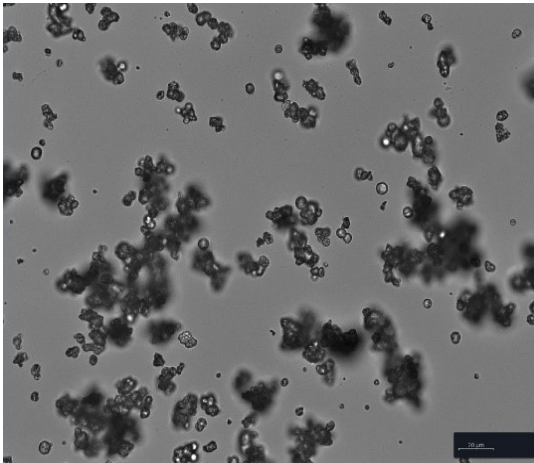
Sample	Image (each scale bar represents 20 μm)	Average particle size ± SD (μm) (n=5)
Enc-AC _{low} -No Fib		12.41±2.48
Enc-Fib _{high}		9.33 ±1.78
Enc-AC _{low}		6.67 ± 1.36

Table 3. Cont.

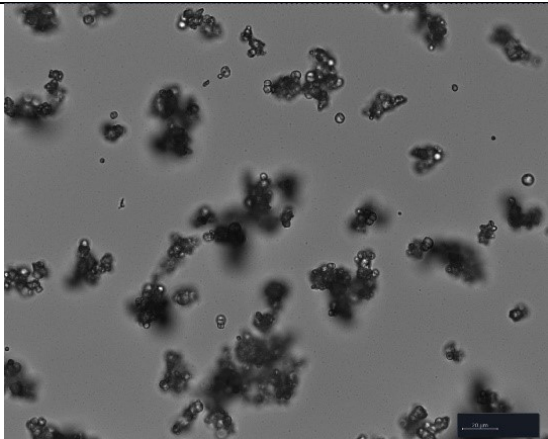
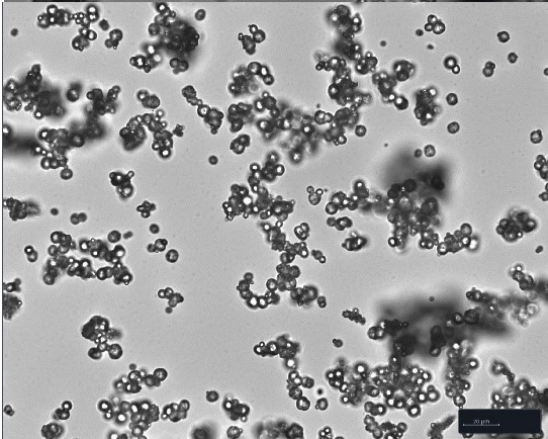
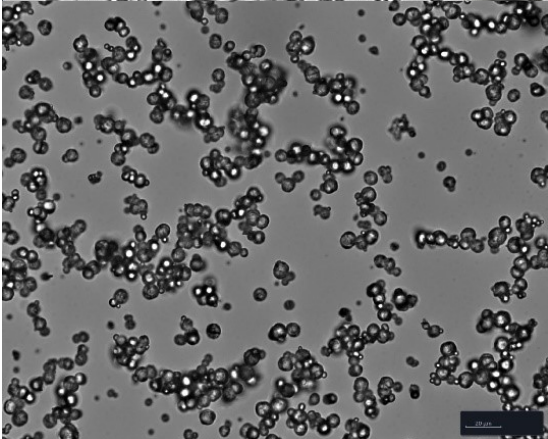
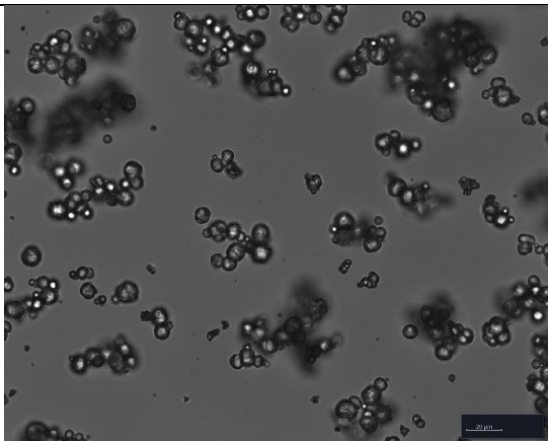
Sample	Image (each scale bar represents 20 μm)	Average particle size ± SD (μm) (n=5)
Enc-Fib _{high} -AC _{low}		6.27 ± 1.26
Enc-Tre _{high}		6.27 ± 1.07
Enc-Tre _{low}		6.933 ± 1.28

Table 3. Cont.

Sample	Image (each scale bar represents 20 μm)	Average particle size $\pm$ SD (μm) (n=5)
Enc-PVP		10.00 ± 1.20

3. Results

Self-propelling particles unloaded and loaded with fibrinogen and thrombin have been prepared with different encapsulation efficacies and loading capacities as summarized in Tables 1 and 2. These particles were then characterized using light microscopy, gel electrophoresis, ROTEM and self-propulsion test, respectively.

3.1. Light microscopy

Table 3 summarizes images of various batches of CaCO_3 -encapsulated fibrinogen particles prepared under different conditions, and the average particle sizes as measured in diameter. The CaCO_3 -encapsulated fibrinogen particles all showed a similar morphology of nearly spherical structures with diameters in a range of 6.27–10.00 μm , which is slightly smaller than the control CaCO_3 particle without fibrinogen (12.41 μm of Enc-AC_{low}-No Fib). Comparisons among the batches showed the effects of solution concentration and composition on particle morphology. The CaCO_3 -encapsulated fibrinogen particles prepared with low $(\text{NH}_4)_2\text{CO}_3$ concentration (Enc-AC_{low}) or trehalose (Enc-Tre_{high} and Enc-Tre_{low}) showed smaller, more uniform and spherical morphology, with a diameter of around 6 μm . The control CaCO_3 particle with no fibrinogen appears larger, more crystalline and irregular shape, coalesced into aggregations. The encapsulated fibrinogen particles showed bright centre regions surrounded by dark edges.

As shown in Table , the CaCO_3 -encapsulated thrombin particles showed a relatively complete spherical structure and the particle size was in the range of 4–10 μm , slightly smaller than that of the CaCO_3 -encapsulated fibrinogen particles. With an increase in the mixing speed of W/O emulsion with water, the particles showed a reduced size from 8.80 ± 1.03 μm to 5.28 ± 0.39 μm (Enc-Initial vs. Enc-Mix_{high}), while increasing amount of surfactants resulted in less reduction in the particle size (Enc-Initial vs. Enc-Sur_{high}) than expected given the significant role of surfactants in stabilizing and forming microparticles with different sizes *via* the W/O emulsion method [35]. The particles prepared with oil to

water volume ratio of 2 (Enc-Hex₂) showed the smallest size, more uniform and spherical morphology. In contrast, the control particles (Enc-Initial-No Thr) showed larger size and aggregation. Similar to the CaCO₃-encapsulated fibrinogen particles, the encapsulated thrombin particles showed bright centre regions surrounded by dark edges.

In comparison with the encapsulation method, the absorption method led to spherical and small thrombin-CaCO₃ particles with a diameter less than 4 µm, especially in the presence of TA (Enc-TA vs. Abs-TA and Abs-TA_{FD}). In the absorption method, TA may act as a crosslinker for CaCO₃ through interaction between TA hydroxyl groups and Ca²⁺ [38] and a linker to connect thrombin and CaCO₃ [33]. However, when the CaCO₃-TA particle was prepared by the encapsulation method (Abs-TA_{Enc}), a larger and less spherical thrombin-CaCO₃ particle with a diameter of 7.60 was observed.

Table 4. Microscopy images of the CaCO₃-encapsulated thrombin particles at 40× magnification. Refer to Table 2 for sample details.

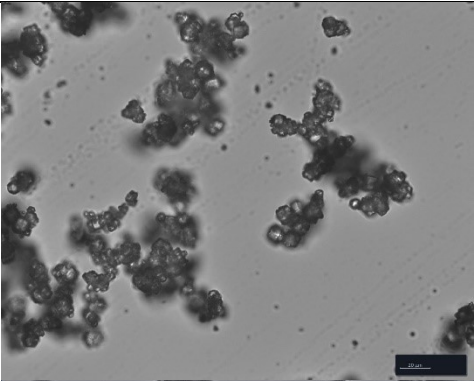
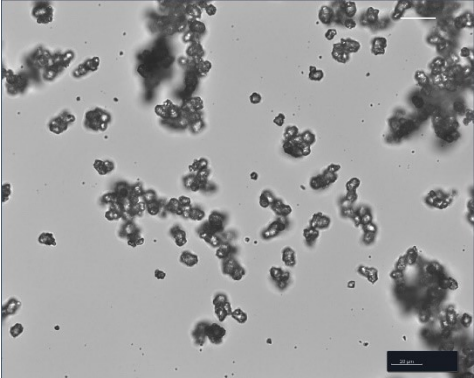
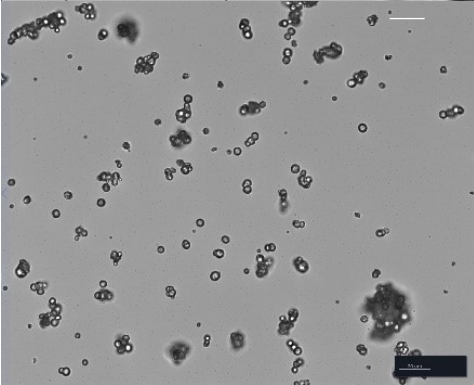
Sample	Image (each scale bar represents 20 µm)	Average particle size ± SD (µm) (n=5)
Enc-Initial-No Thr		—
Enc-Initial		8.80 ± 1.03
Enc-Thr _{high}		6.88 ± 0.64

Table 4. Cont.

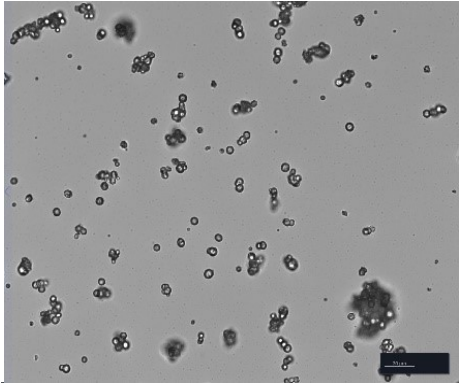
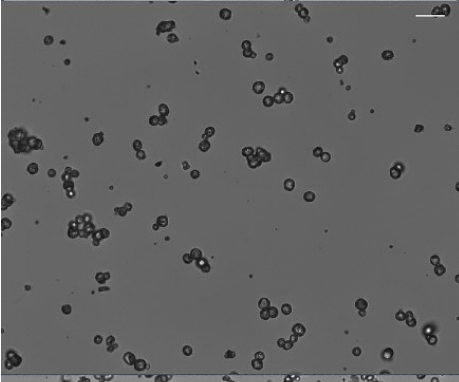
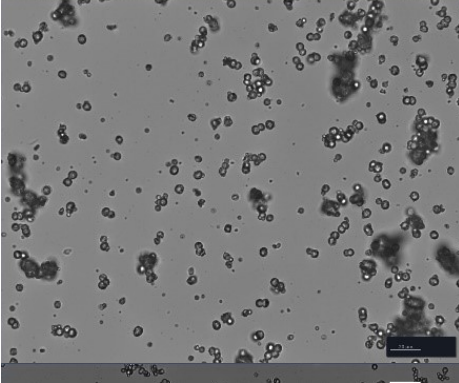
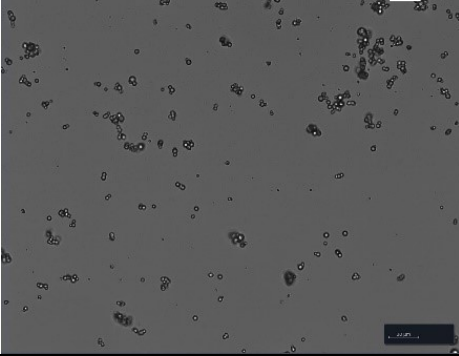
Sample	Image (each scale bar represents 20 μm)	Average particle size ± SD (μm) (n=5)
Enc-AC _{low}		7.04 ± 0.60
Enc-Sur _{high}		7.84 ± 0.32
Enc-Mix _{high}		5.28 ± 0.39
Enc-Hex _{2x}		4.00 ± 0.88

Table 4. Cont.

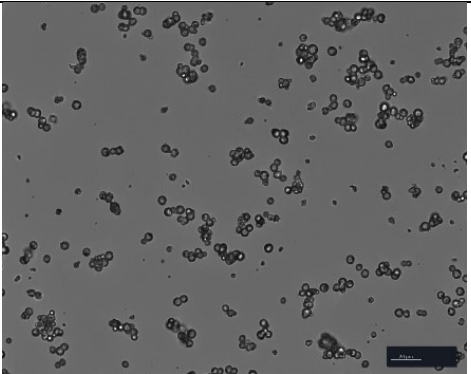
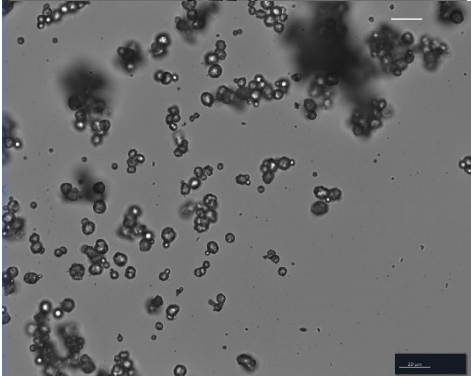
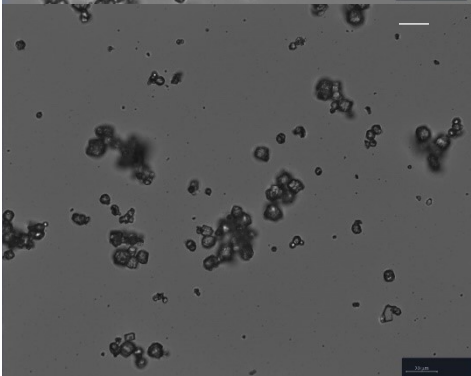
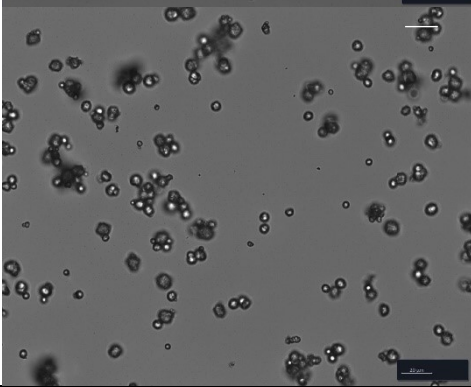
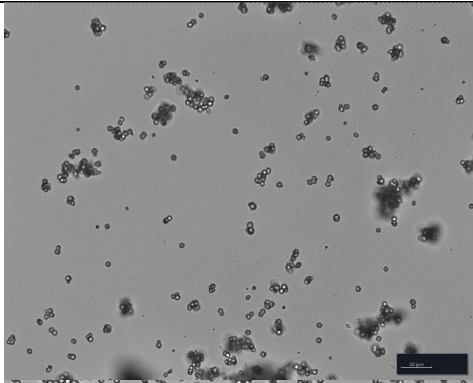
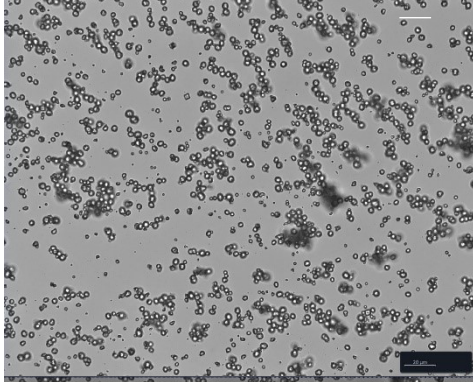
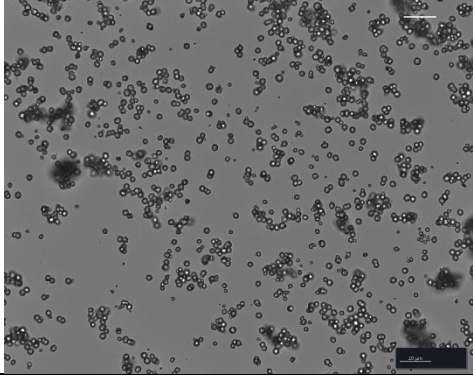
Sample	Image (each scale bar represents 20 μm)	Average particle size ± SD (μm) (n=5)
Enc-Hex _{4x}		5.92 ± 0.64
Enc-TA		7.20 ± 1.46
Enc-TA-No Thr		9.47 ± 1.56
Abs-TA _{Enc}		7.60 ± 1.2

Table 4. Cont.

Sample	Image (each scale bar represents 20 μm)	Average particle size $\pm$ SD (μm) (n=5)
Abs-No TA		4.13 ± 0.55
Abs-TA		4.27 ± 1.19
Abs-TA _{FD}		3.73 ± 0.998

3.2. Gel electrophoresis

Gel electrophoresis was used to detect fibrinogen and thrombin, respectively. As depicted in Figure 1, the initial sample of fibrinogen showed the typical triplet of bands corresponding to α -, β - and γ -chains of fibrinogen (Fib lane) at a respective molecular weight of approximately 64 kDa, 56 kDa, and 47 kDa [39]. It should be noted that the fibrinogen product contained human albumin and its band (Alb lane) might overlap with the α -chain band of fibrinogen given their similar molecular weights (66 vs. 64 kDa) [40].

The CaCO_3 -encapsulated fibrinogen particles of Enc-Initial, Enc-Tre showed three distinct bands (strong band of α -chain around 64 kDa, faint bands indicative of the β and γ chains around 56 and 47 kDa), suggesting the presence of fibrinogen. The intensity of each band from particle samples was less than that from their corresponding supernatant samples and the particle prepared by the precipitation method (Pre), suggesting that most fibrinogen was present in the supernatant and not much incorporated into the particle, compared to the precipitated fibrinogen- CaCO_3 particle. Furthermore, apart from the

band around the 70 kDa mark, there was no other bands indicative of the β and γ chains distinguishable in the particle samples of Enc-Fib_{high}, Enc-AC_{low}, Enc-Fib_{high}-AC_{low}, implying the presence of small amount of fibrinogen. Alternatively, it could be the presence of albumin in the fibrinogen product that caused a false positive indicator. Overall, this test highlights that the majority of fibrinogen was not encapsulated into the particles and remained in the supernatant. Given its qualitative nature, the gel electrophoresis was not able to show the amount of encapsulated fibrinogen significantly affected by the encapsulation conditions. It should be noted that the estimated amount of fibrinogen per mg particle prepared by the precipitation method was 0.03 mg [19].

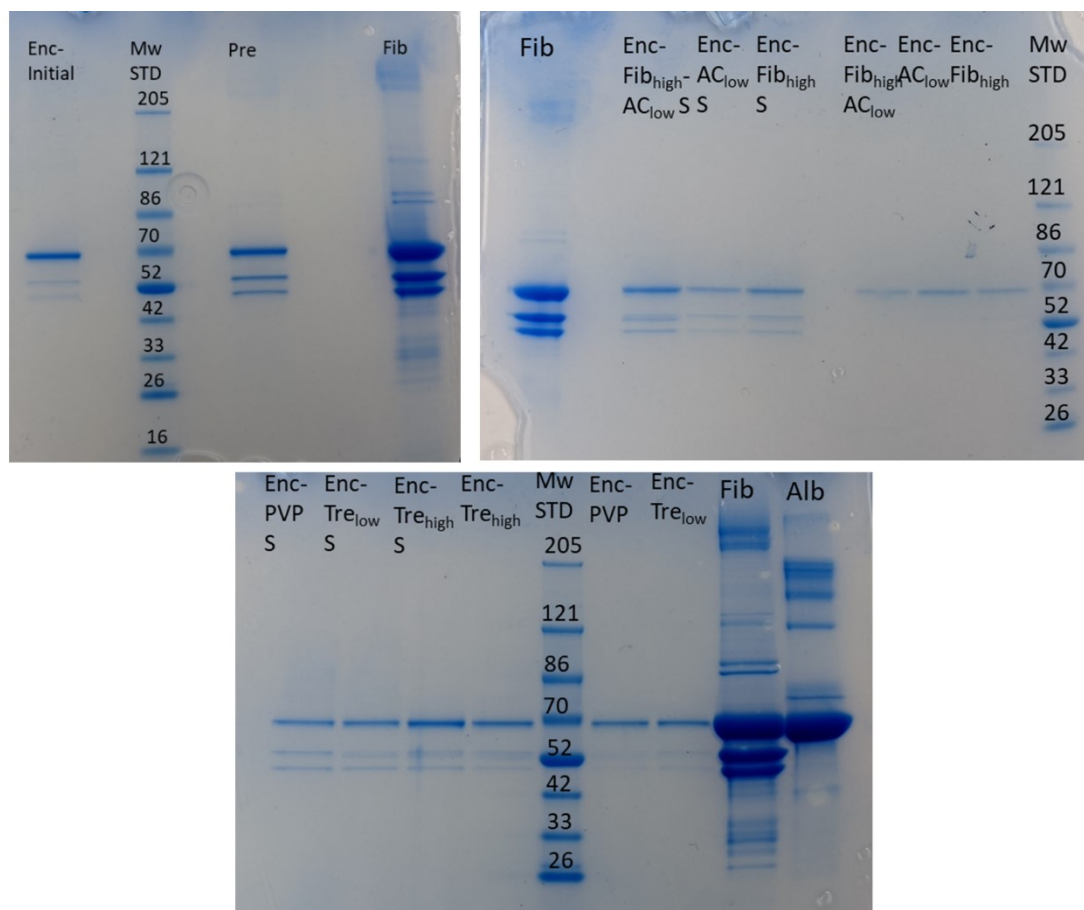


Figure 1. Coomassie-stained SDS-polyacrylamide gel electrophoresis analysis of particle samples dissolved in TXA⁺. Each sample was reduced with 5% dithiothreitol and analyzed by continuous SDS-polyacrylamide gel electrophoresis. Indicated molecular weights were estimated by standard proteins (Mw STD) with known molecular weights from 16 to 205 kDa. Alb, Fib, and S represent fibrinogen, albumin and supernatant. See Table 1 for details of each batch of samples.

Similarly, gel electrophoresis was done on the CaCO₃-encapsulated thrombin samples to confirm the presence of thrombin. As seen in Figure 2, all particles prepared using the encapsulation method showed a distinct band at around 52 kDa, identical to one of the thrombin bands. However, the particles prepared under various encapsulation conditions do not show remarkable differences in band intensity and any other strong bands in particular around 37 kDa, being the molecular weight of the bovine thrombin sample used in these experiments (though very faint bands can be observed around those regions) [41]. Alternatively, the thrombin control showed some important bands around 20, 52, 140, 205

kDa, as they were also visible in some of the thrombin-containing CaCO_3 samples. No bands were observed as expected for the sample of Enc-No Thr: the CaCO_3 particle prepared by the encapsulation method without thrombin, which acts as a negative control for the gel electrophoresis. Furthermore, the sample (Enc-Initial) consistently showcased the same band around 52 kDa in two separate runs. The results do confirm that there is a presence of thrombin.

However, the CaCO_3 -thrombin particle made using the initial absorption method (Abs) showed no band, while its supernatant (Abs S) showed very strong and visible bands similar to thrombin control. This indicates that the thrombin likely remained in the supernatant and did not absorb into CaCO_3 . In contrast, the particle prepared by the absorption method using TA (Abs-TA) showed several thrombin-like bands at around 20, 37, 52, 140 kDa, but this is not the case for the encapsulation method (Enc-TA and Abs-TA_{Enc}).

Overall, strong bands were observed in all CaCO_3 -encapsulated thrombin samples around 52 kDa. While faint bands at other molecular weights could suggest lower activity, these particles did show improved hemostatic properties (ranging from a CT of 52–356 sec) as measured by ROTEM in section 0, providing further evidence for the presence of functional thrombin.

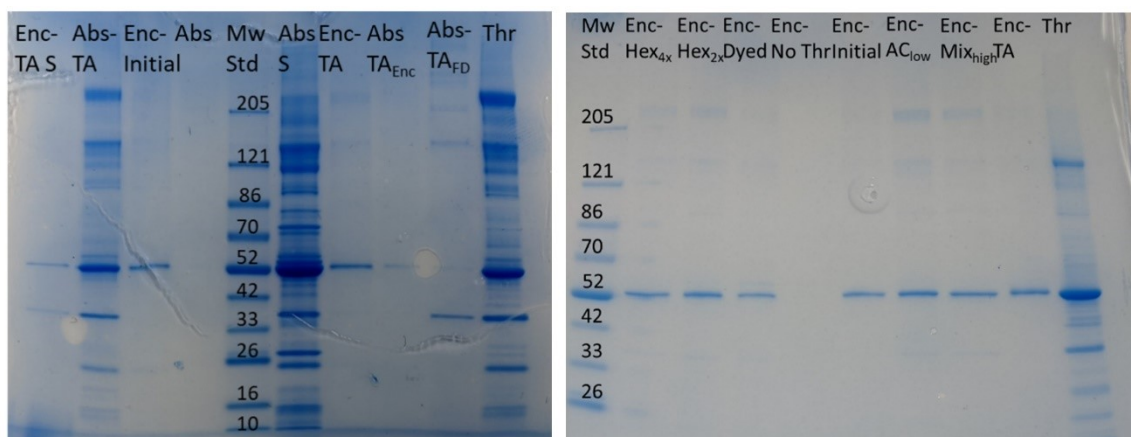


Figure 2. Coomassie-stained SDS-polyacrylamide gel electrophoresis analysis of particle samples dissolved in TXA^+ . Each sample was analyzed by continuous SDS-polyacrylamide gel electrophoresis. Indicated molecular weights were estimated by standard proteins (Mw STD) with known molecular weights from 10 to 205 kDa. Thr and S represent thrombin and supernatant. See Table 2 for details of each batch of samples.

3.3. Hemostatic properties

To further evaluate whether fibrinogen or thrombin was present within the particles and their hemostatic functions, ROTEM tests were performed with human plasma containing low or normal levels of fibrinogen to observe any changes in the hemodynamic viscoelastic properties of the plasma, as measured by CT and MCF when exposed to the particles.

Figure 3 shows that the CaCO_3 -encapsulated fibrinogen particles shortened CT and increased MCF of low-fibrinogen plasma compared to the blank and control. However, the effects were mild. The preparation conditions did not seem to have any significant influence of the hemostatic effects. It appears that high fibrinogen and low ammonium carbonate concentrations (Enc-Fib_{high}-AC_{low}) led to shortest CT

and largest MCF, and thus most hemostatic effects. In contrast, the precipitated fibrinogen- CaCO_3 particle (Pre) showed larger hemostatic effects as indicated by lower CT and larger MCF.

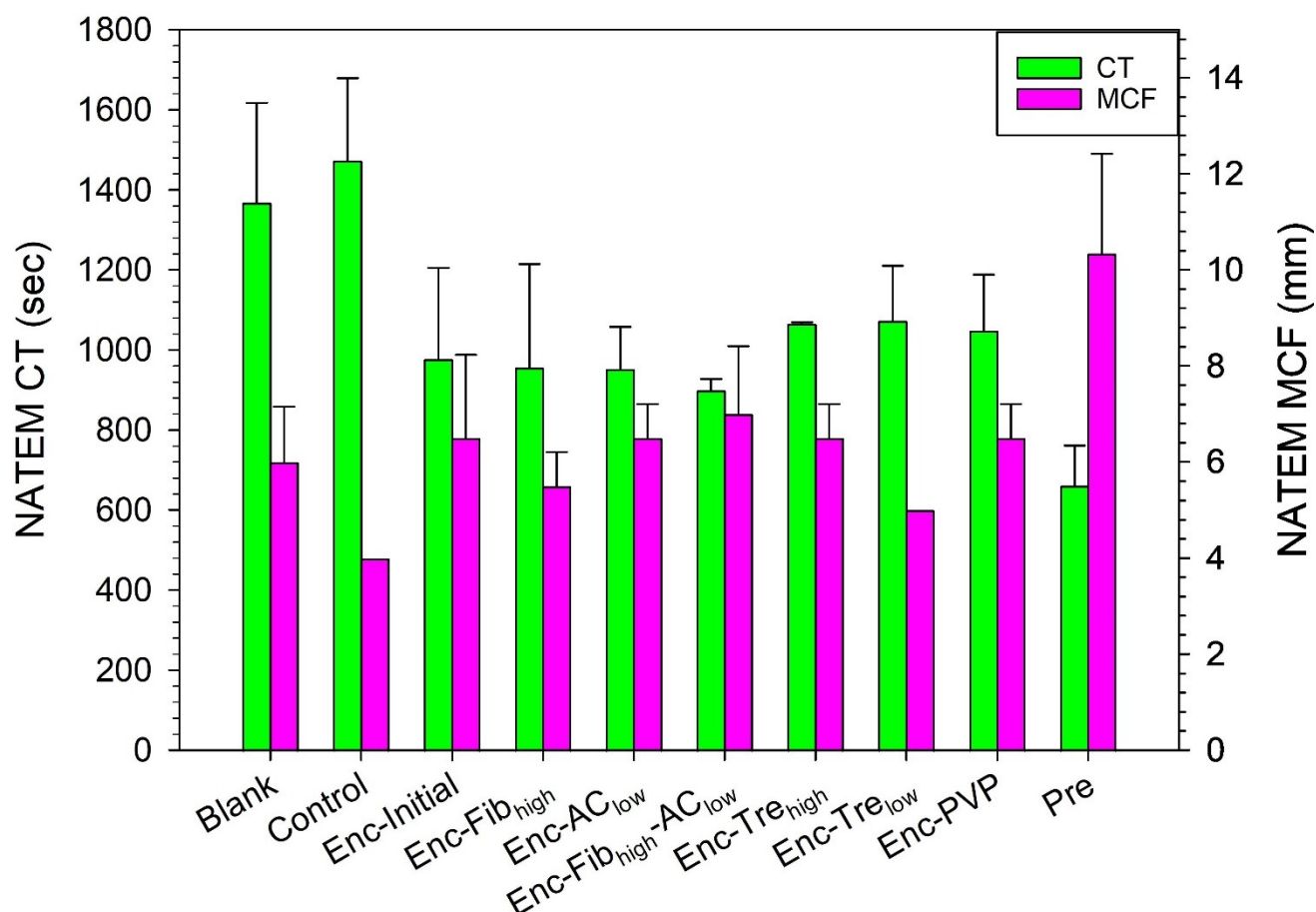


Figure 3. Effects of CaCO_3 -encapsulated fibrinogen particles on ROTEM coagulation time (CT) and maximum clot firmness (MCF). ROTEM NATEM tests were performed with low-fibrinogen plasma in the absence (blank) or presence of the particles. The particles were prepared without (control) and with fibrinogen under different conditions (see Table 1 for details). Error bars represent mean $\pm$ SD.

Figure 4 shows significantly lower CT of low-fibrinogen plasma in the presence of thrombin-encapsulated particles compared to the blank and control particle. Changes in thrombin concentrations (Enc-Initial, Enc-Thr_{low} and Enc-Thr_{high}) had most effects on CT. No further reduction in CT was observed for the particle that was prepared using TA (Enc-TA). Overall, the preparation conditions had a marginal effect on MCF. It should be noted that the thrombin- CaCO_3 particle synthesized with the absorption method in the presence of TA (Abs-TA) showed a longer CT compared to its counterpart prepared by the encapsulation method (Enc-TA), albeit the former showed more thrombin-like bands in the gel electrophoresis (Figure 2). On the other hand, the thrombin- CaCO_3 particle prepared by the absorption method without TA (Abs) resulted in no coagulation. Compared to the mild hemostatic effects of the fibrinogen- CaCO_3 particles, the hemostatic effects of the thrombin- CaCO_3 particles appear stronger as indicated by remarkably decreased CT.

Figure 5 further elucidates the hemostatic effects of different batches of thrombin- CaCO_3 particles on normal plasma as measured by reduced CT in comparison with the blank and control particle. Overall, all thrombin encapsulated particles significantly shortened CT to different extents. It appears that low ammonium carbonate concentration ($\text{Enc-AC}_{\text{low}}$) resulted in less hemostatic effects as measured by a longer CT relative to other encapsulation conditions and that high thrombin concentration ($\text{Enc-Thr}_{\text{high}}$)

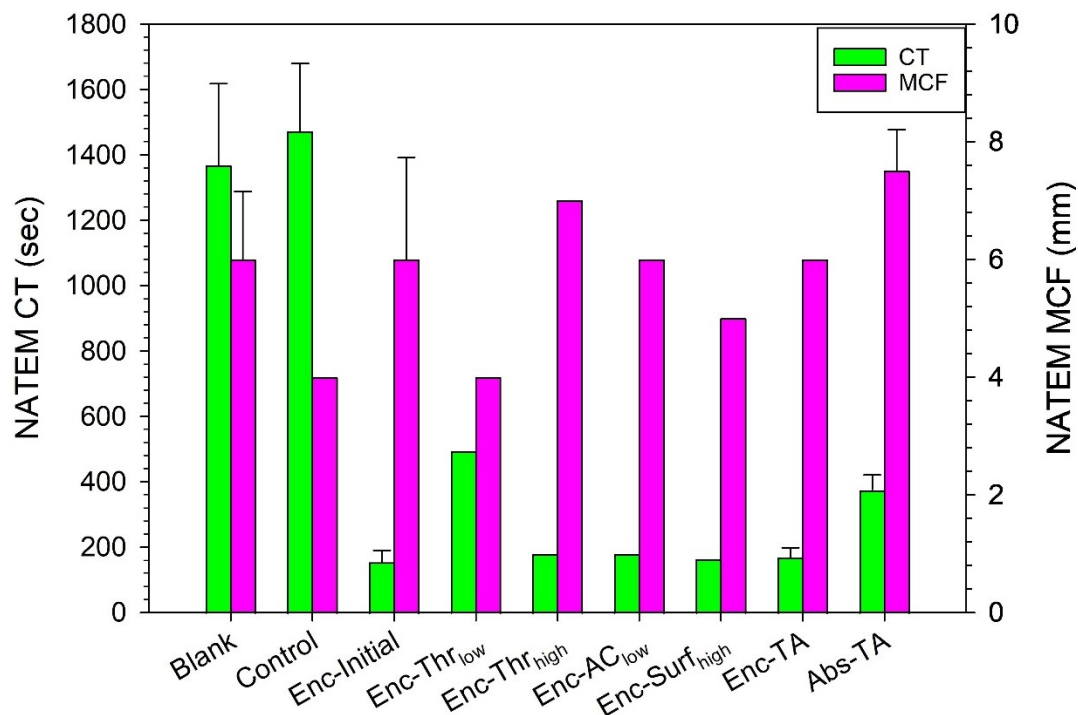


Figure 4. Effects of CaCO_3 -encapsulated thrombin particles on ROTEM coagulation time (CT) and maximum clot firmness (MCF). ROTEM NATEM tests were performed with low-fibrinogen plasma without (blank) or with the particles. The particles were prepared without (control) and with thrombin under different conditions (see Table 2 for details). Error bars represent mean $\pm$ SD.

did not lead to increased hemostatic effects. In contrast with the hemostatic effects on low-fibrinogen plasma, the encapsulated thrombin could also reduce MCF compared to the blank likely due to the detrimental effect of residuals from the encapsulation process on fibrin polymerization, as the ROTEM test performed with the normal plasma in the presence of freeze-dried supernatants from various encapsulation solutions showed no coagulation.

Figure 6 depicts the hemostatic effects of the CaCO_3 -encapsulated thrombin increased with the amount of particles (from 6 to 18 mg). At 18 mg, MCF was slightly decreased likely due to interference of fibrin polymerization by the residuals from the encapsulation as seen in Figure 5. Interestingly, the hemostatic effect was enhanced when combined with 2 mg TXA^+ as measured by CT, but not augmented when combined with TXA. The synergistic hemostatic effects are perhaps mainly due to the dissolution of the particle other than anti-fibrinolytic of TXA. It should be noted that there was no coagulation in the combination with 4 mg TXA^+ , likely due to significantly reduced plasma pH.

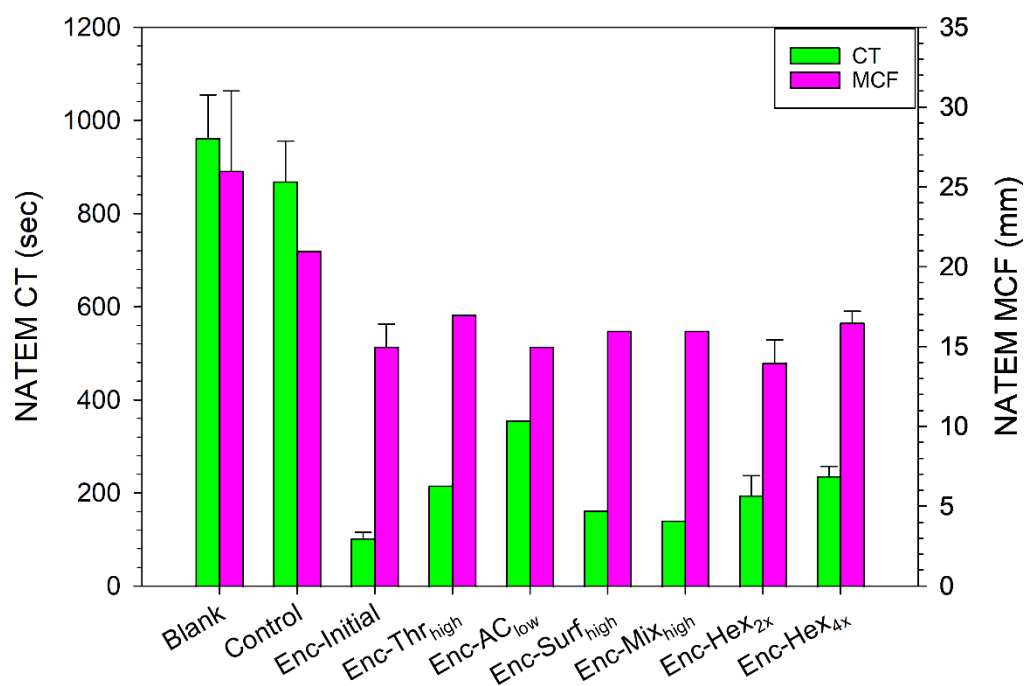


Figure 5. Effects of different batches of CaCO_3 -encapsulated thrombin (Thr) particles on ROTEM coagulation time (CT) and maximum clot firmness (MCF). ROTEM NATEM tests were performed with normal plasma in the absence (blank) or presence of the particles unloaded (control particle) and loaded with thrombin, respectively. Error bars represent mean $\pm$ SD.

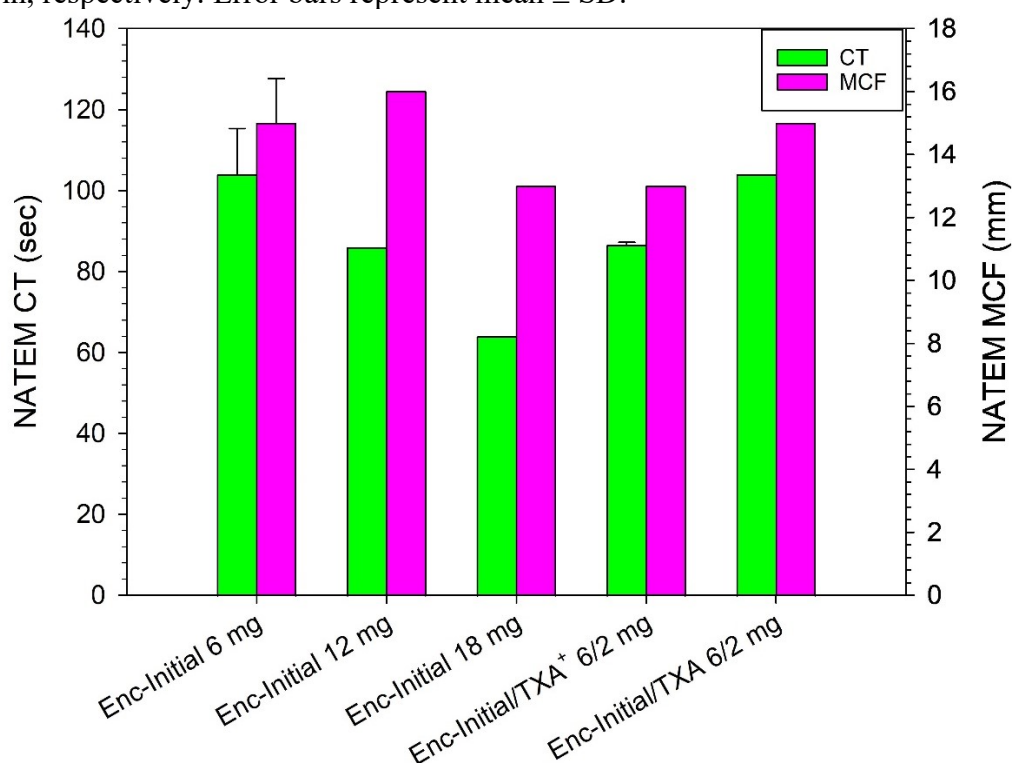


Figure 6. Effects of the amount of CaCO_3 -encapsulated thrombin particles and combination with TXA^+/TXA on ROTEM coagulation time (CT) and maximum clot firmness (MCF). ROTEM NATEM tests were performed with normal plasma in the presence of the particles alone or in combination with TXA^+/TXA . Error bars represent mean $\pm$ SD.

3.4. Self-propelling properties

The self-propelling properties were measured by the self-propulsion tests on mixed CaCO_3 particles and TXA^+ at the optimal mass ratio of 12:10 as previously determined [19]. The self-propulsion test is a direct measure of the capability that the particle has to deliver fibrinogen and thrombin against blood flow to a bleeding site. The movement was quantified by the video analysis as described in section 0. The shorter lag time and faster moving speed indicate better self-propelling ability.

Initially, the self-propulsion test was performed with control CaCO_3 particle fabricated using the emulsion process without loading of either fibrinogen or thrombin (Enc- Control CaCO_3 , Enc- Control CaCO_3 -TA) to evaluate whether these particles display self-propulsive properties. Most of the material travelled to the top of the liquid, although some particles were in larger clumps compared with others. As summarized in

Table 4, the measured lag time of these particles was 3.636 and 3.191 sec, and the average particle speed was 1.725 and 1.673 cm/sec. Furthermore, self-propulsion tests using the CaCO_3 -encapsulated fibrinogen and thrombin particles were conducted. Overall, the fibrinogen and thrombin encapsulated particles exhibited shorter lag time than the control particles, but the encapsulation conditions did not significantly affect the lag time, except for the one using TA (Enc-TA) showing the shortest lag time of 1.69 sec on average, likely because of increased particle wettability by TA. In contrast, both the particle composition and size could affect the self-propelling speed. The smallest thrombin encapsulated particle (Enc-Hex_{2x}) with a diameter of 4 μm showed the fastest speed of 3.611 cm/sec, while the large particle (Enc-No Thr) with a diameter of 12.41 μm and encapsulated fibrinogen particles (Enc-Initial, Enc-Fib_{high}) showed a slow speed in the range of 1.414 - 1.725 cm/sec. The particle prepared by the absorption method (Abs-Control CaCO_3 -TA) had a longer lag time and a similar speed compared to its counterpart made by the encapsulation method (Enc- Control CaCO_3 -TA). In addition,

Table 4. Self-propulsion of CaCO_3 -encapsulated fibrinogen and thrombin particles under various conditions. Data represent mean $\pm$ SD. Particle: TXA^+ mass ratio = 12:10. Refer to Table 1 and Table 2 for sample details.

Sample batch	Preparation method	Loading	Lag time (sec)	Moving speed (cm/sec)
Enc- Control CaCO_3	Encapsulation method	None	3.636 $\pm$ 0.334	1.725 $\pm$ 0.527
Enc-Initial		Fibrinogen	2.626 $\pm$ 0.284	1.414 $\pm$ 0.644
Enc-Fib _{high}		Fibrinogen (higher concentration)	2.380 $\pm$ 0.197	1.427 $\pm$ 0.568
Enc-Thr _{high}		Thrombin (higher concentration)	2.035 $\pm$ 0.811	1.75 $\pm$ 0.498
Enc-AC _{low}		Thrombin	2.469 $\pm$ 0.234	3.250 $\pm$ 0.570
Enc-Suf _{high}			2.769 $\pm$ 1.16	3.039 $\pm$ 0.943
Enc-Mix _{high}			2.74 $\pm$ 0.248	2.725 $\pm$ 0.348
Enc-Hex _{2x}			2.206 $\pm$ 0.885	3.611 $\pm$ 0.840
Enc-Hex _{4x}			2.39 $\pm$ 1.08	2.45 $\pm$ 0.511
Enc-TA			1.690 $\pm$ 0.113	2.809 $\pm$ 0.717
Enc- Control CaCO_3 -TA		None	3.191 $\pm$ 0.042	1.673 $\pm$ 1.871
Abs-Control CaCO_3 -TA	Absorption method	None	5.553 $\pm$ 1.551	1.761 $\pm$ 0.834

the thrombin- CaCO_3 particles exhibited faster moving speed than the fibrinogen- CaCO_3 particles when mixed with TXA^+ . This was likely due to the smaller molecular size of thrombin.

In summary, hemostatic and self-propelling CaCO_3 -encapsulated fibrinogen and thrombin particles can be prepared by an interfacial reaction method using water-oil-water emulsion. The amount of fibrinogen and thrombin was low compared to the precipitation and absorption methods when CaCO_3 particles were lyophilized with fibrinogen or thrombin solution as previously reported [19], showing only mild to moderate hemostatic effects, but enhanced when combined with TXA^+ . The self-propelling properties of the resultant fibrinogen- and thrombin- CaCO_3 particles were not compromised and even better compared to their CaCO_3 control particles. The hemostatic and self-propelling properties could be optimized by adjusting the concentrations and composition of fibrinogen and thrombin solutions, the aqueous solution to hexane volume ratio, the amount of surfactants and mixing speed.

3.5. Release profiles

Figure 7 showed the release of thrombin from three particle formulations over time as measured by its effects on ROTEM CT. A dramatic reduction in CT was observed at 1–2 h and continued over time up to 90% at 24 h in TXA^+ solution, implying immediate release of thrombin followed by continuous release. A less reduction in CT was seen in PBS solution and plateaued after about 5 h, implying less release of thrombin. The release profiles are consistent with more particle dissolution in acidic TXA^+ solution than in PBS, and increased amount of thrombin with the loading capacity of each type of particles ($\text{Enc-Thr}_{\text{high}} > \text{Enc-Initial} > \text{Enc-Thr}_{\text{low}}$).

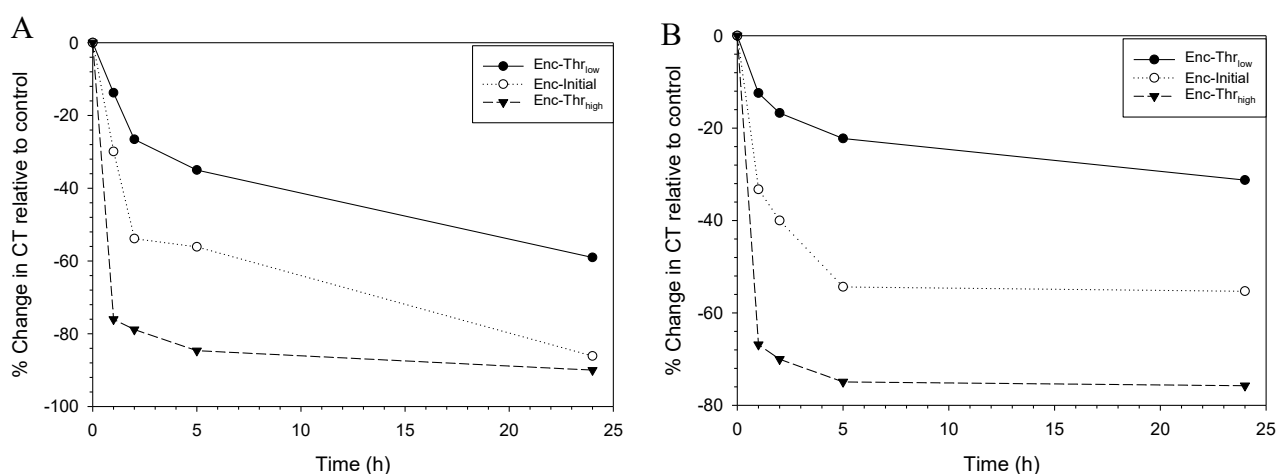


Figure 7. The release of CaCO_3 -encapsulated thrombin in TXA^+ (A) and PBS solution (B) as measured by the percentage change in ROTEM CT relative to controls (see section 2.2.6 for experimental details).

4. Discussion

Many self-propelling particle systems have been developed for the delivery of various therapeutics [42]. In the previous reports, we have shown that fibrinogen could be added to CaCO_3 particle with hemostatic effects and self-propelling properties using absorption and precipitation methods, respectively [19], and the fibrinogen- and thrombin- CaCO_3 particles can be loaded together with TXA^+ onto medical gauze

[43]. This report presents a new method to prepare self-propelling particles by encapsulation of fibrinogen and thrombin inside CaCO_3 particle, which may provide better delivery against blood flow and release of the coagulation factors at bleeding sites when combined with TXA^+ . By combining fibrinogen and thrombin encapsulated particles with TXA^+ , hemostatic efficacy would be further increased due to their synergistic effects on coagulation and fibrinolysis, and self-propelling delivery as supported by the results from ROTEM and self-propulsion tests. In addition, other procoagulants could be used, including factor XIII, and solid acids with antifibrinolytic effect, such as aminocaproic acid [44].

Thrombin has been well incorporated into a variety of hemostatic materials to further promote hemostasis including CaCO_3 particles [33], alginate-calcium microspheres [45], calcium alginate and silk peptide composite microspheres [46], microporous starch particles [47], oxidized nanocellulose-silk fibroin based scaffolds [48], graphene sponge [49], and porous silica materials [50]. Generally, thrombin was incorporated by soaking the matrix materials in an aqueous solution containing different amount of thrombin. Hemostatic gauze could be produced based on biomimetic mineralized thrombin [51]. In contrast, topical use of fibrinogen for hemorrhage control is limited to its combination with thrombin as fibrin sealants [52]. Alternatively, Jun et al. reported a temperature-sensitive triblock copolymer (poly ethylene oxide-poly propylene oxide-poly ethylene oxide) containing fibrinogen to promote local blood coagulation through gel formation at body temperature and hemostatic function of fibrinogen [53].

Calcium carbonate may be an ideal carrier for delivery of procoagulants against blood flow in combination with TXA^+ [12,23]. The absorption into pre-formed CaCO_3 and precipitation of CaCO_3 alone with biomacromolecules are two most used loading methods that lead to different properties of resultant particles [54]. We have used and compared the two methods [19], but did not see any increase in fibrinogen loading by the precipitation method as reported [55], perhaps because the fibrinogen molecule is large and does not co-precipitate well with CaCO_3 .

Methods have been reported for encapsulation of bioactive compounds inside CaCO_3 particle using water-oil-water emulsion [34], and polymers as templating agents [56]. For example, biomacromolecules such as bovine serum albumin and papain, could be encapsulated into CaCO_3 microcapsules by an interfacial reaction method using carbonate and calcium salts in a water-oil-water emulsion, when the biomacromolecule were dissolved in the aqueous solution of the inner phase [34]. The CaCO_3 particle formed using the method showed an interior hollow structure [57].

We adopted the process for synthesizing CaCO_3 -encapsulated fibrinogen and thrombin particles. Although the encapsulation of various biomacromolecules in CaCO_3 has successfully been exhibited in previous studies [34,58], the largest one is bovine serum albumin with a molecular weight of 66 kDa and high encapsulation efficiency of 92.6%, much smaller than the molecular weight of fibrinogen and thrombin (340 and 37 kDa, respectively). Our process involved using W/O emulsion of $(\text{NH}_4)_2\text{CO}_3$, fibrinogen, and HEPES in water, emulsified in an oil mixture of n-hexane, with Tween 80 and Span 80 as emulsifying agents. This mixture was then poured into a CaCl_2 solution, resulting in a W/O/W emulsion that synthesized the CaCO_3 microcapsules.

The type of carbonate salts plays an important role in the protein encapsulation, secondary structure, and bioactivity in agreement with the literature [34]. These CaCO_3 microcapsules likely underwent encapsulation of proteins and phase transition from vaterite to calcite in various aqueous solutions [59]. The reaction rate of CaCO_3 with TXA^+ , which affects self-propelling and release of procoagulants can be adjusted by controlling the crystal phase of CaCO_3 [60].

In the previous study [18], thrombin was successfully combined with CaCO_3 by the absorption method. Fibrinogen is much larger than thrombin (Mw 340 versus 37 kD) and difficult to incorporate [19]. In addition, a larger therapeutic dose of fibrinogen than of thrombin is expected.

In this study, we have shown two primary findings. First, fibrinogen and thrombin could be encapsulated into CaCO_3 particles with hemostatic activities. Second, it appeared that the proteins thus encapsulated were hardly liberated into aqueous solution unless mixed with TXA^+ , which could also generate gas bubbles resulting in self-propulsion even better than control CaCO_3 particles with no protein and thus efficient delivery against blood flow to bleeding sites. Therefore, we are able to confirm our primary hypothesis that hemostatic and self-propelling CaCO_3 -encapsulated fibrinogen and thrombin particles can be prepared to enhance hemorrhage control. It should be noted that fibrinogen encapsulated particles showed less hemostatic effects compared to thrombin encapsulated particles (Figure 3 versus Figure 4), and both effects were less than those obtained by the absorption and precipitation methods as previously reported [19]. However, there is no consensus between fibrinogen and thrombin-encapsulated particles from the current study. Both types of particles should be used for further investigation to provide clarity and insight into which would be the best candidate going forward and whether their combination would provide synergistic blood coagulation effects.

TA is a natural plant polyphenol consisting of pyrogallol (i.e. catechol group) in the structure and approved by the Food and Drug Administration [16]. TA can be an effective hemostatic material due to its immediate binding with proteins in the blood, leading to instant blood coagulation. In our study, TA increased thrombin loading in the absorption method as reported [33], but not in the encapsulation method as suggested by the gel electrophoresis (Figure 2).

Both low-fibrinogen and normal plasma were used to mimic conditions where blood clotting mechanisms are coagulopathic and normal, respectively. CT and MCF are two most used ROTEM parameters, mainly affected by plasma clotting factors and anticoagulants and direct function of the maximum dynamic properties of fibrin and platelet number and function, respectively [61]. The mild to moderate hemostatic effects are consistent with the small amount of encapsulated fibrinogen and thrombin relatively to precipitation and absorption method as indicated by the gel electrophoresis (Figure 1 and Figure 2). No significant increase in the hemostatic effects was observed with the use of TA in either method as opposed to that reported [62]. On the other hand, as encapsulated fibrinogen and thrombin might not release completely to affect coagulation during the ROTEM measurement, larger hemostatic effects were achieved when combined with TXA^+ resulting from increased release of the coagulation factors.

It is known that particle size has a significant impact on hemostasis activation [63]. However, given its narrow range (4–10 μm) the particle size might have no remarkable impact on hemostatic effects in our study.

ROTEM performed with plasma in the presence of 6 mg of freeze-dried supernatants from various encapsulation particle preparations showed no coagulation regardless of the presence of the coagulation factors, suggesting impairment of residuals from the preparation e.g., surfactants, hexane, on the coagulation, which should be considered when analyzing the hemostatic functions of encapsulated fibrinogen and thrombin.

Different methods have been used to measure self-propelling properties, such as static and dynamic self-propulsion behavior as measured by the maximum propulsion distance of the particle in glass tubes

with stagnant or flow fluids (buffers or blood) fixed at different angles [64]. In our study, rapid reaction between the CaCO_3 particles and TXA^+ has been confirmed under light microscopy and quantified by the self-propulsion test.

The self-propelling properties as determined by the self-propulsion test were not compromised when CaCO_3 particles were loaded inside with either fibrinogen or thrombin. Instead, the lag time was even shorter than the control CaCO_3 particles with no encapsulated proteins. This was in contrast with the fibrinogen- and thrombin- CaCO_3 particles prepared by the absorption and precipitation methods, showing a longer lag time (4.011 - 15.218 sec) and slower speed (1.286 - 3.067 cm/sec), especially true for those lyophilized with the fibrinogen or thrombin solution as previously reported [19]. This is likely due to higher crystallinity and thus reduced wettability in these CaCO_3 particles compared to the encapsulation particles. As a result, the particles aggregated and did not interact well with water and TXA^+ , compromising reactivity. However, since trends in the self-propulsion tests did not exactly follow the increasing/decreasing amounts of fibrinogen and thrombin loaded per particle, other properties such as particle size, porosity, and composition, which depend on the preparation conditions, may be playing a role in the reactivity. Overall, the encapsulation method led to better self-propelling properties (shorter lag times and faster movement).

Although encapsulation was confirmed by light microscopy and a qualitative analysis of encapsulated proteins was performed using gel electrophoresis, further confirmation of encapsulation is needed using fluorescent-labelled proteins and fluorescence microscopy as well as scanning electron microscopy [34,65,66]. More quantitative analysis of encapsulated proteins could be performed using enzyme-linked immunosorbent assay [67] and coagulation functional assay [68]. Another limitation of the study is that we did not investigate the cytocompatibility of these particles which is to be addressed in our future study, but expected to be biocompatible as reported for similar self-propelled hemostats in the literature [69].

5. Conclusion

As hypothesized, it is feasible to encapsulate fibrinogen and thrombin inside CaCO_3 particles that exhibit hemostatic effects and self-propelling properties when combined with TXA^+ . These properties can be optimized by varying preparation methods and conditions. The hemostatic effects of these particles were mild to moderate likely due to small amount of encapsulated coagulation factors while the self-propelling capability was better compared to CaCO_3 control particles. The self-propelling particles composed of thrombin- CaCO_3 and TXA^+ showed synergistic promotion of hemostasis. Specifically, high fibrinogen and low carbonate solution concentrations may be recommended to produce CaCO_3 -encapsulated fibrinogen particle for future studies, while the use of TA should be further investigated for CaCO_3 -encapsulated thrombin particle.

More importantly, a number of encapsulation methods will be investigated to increase the protein loading efficiency and achieve large hemostatic effects, such as coacervate-directed encapsulation of the coagulation factors with CaCO_3 microcarriers via the combination of complex coacervation and mineralization of calcium carbonate [70], liquid–solid core-shell microcapsules of CaCO_3 coated liposomes based on a biomimetic polymer-induced liquid-precursor mineralization process [71]. The loading efficacy may be also increased by doping CaCO_3 with heparin [72]. We also need to investigate

the hemostatic effects on whole blood by the CaCO₃-encapsulated fibrinogen and thrombin particles, and TXA⁺ alone and in combination.

Additionally, *in vivo* testing is being explored for future development of the self-propelling particle, both as a standalone hemostatic agent and loaded onto gauze. *In vitro* tests are helpful for refining the particle formulation but do not provide an accurate representation of the behavior of these compounds in the body, since there are literally and figuratively more factors to consider. Small animal tests in mice are being discussed, potentially followed by rabbit or swine models for severe hemorrhage.

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Conflicts of Interests

The authors have no conflicts to declare.

Authors' contribution

HTP. designed and performed experiments, analyzed data, and wrote the manuscript. MMS. performed experiments, analyzed data, and edited the manuscript.

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