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Full Length Article

Synergy in thrombin-graphene sponge for improved hemostatic efficacy and facile utilization

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ABSTRACT

Composites are attractive for its potential synergistic effects that can result in high-performance, but the synergy depends on subtle design. In this study, a hemostatic composite, a thrombin/cross-linked graphene sponge (TCGS), was developed through a facile gradient composite strategy. The porous structure of the CGS assures that the thrombin is stably embedded in the TCGS, avoiding a burst release but maintaining its bioactivity. In the synergy between proper thrombin stimulation and the fast absorption of the sponge, TCGS exhibits outstanding hemostatic performance, ultrafast bleeding cessation, within 100 s, which is superior to both CGS and equal amounts of native thrombin. Lower or excessive thrombin dosages prolong the bleeding time. The study revealed that the balance between plasma absorption and thrombin stimulation at the interface is critical for improving hemostatic efficacy. TCGS is also highlighted for its biosafety and stability, even after 6 months of storage in environment. This potentially ultra-long shelf life is conducive to its practical applications. Therefore, TCGS not only provides a new strategy for developing a hemostatic composite but also provides a new method and understanding for the design of hemostatic materials.

1. Introduction

The use of hemostatic agents is critical for saving lives from rapid blood loss from a variety of causes, such as surgical treatments or traumatic accidents, because massive hemorrhage leads to a great threat of death [1–4]. Currently, the commonly used hemostatic agents are generally classified as either inorganic clay (such as zeolite, mesoporous silica and kaolinite) [5], or organic materials (such as chitosan, gelatin and fibrin) [6,7] and their composites [8–10]. Compared with the former two kinds of hemostats, composites of these materials have advantages for improvement of hemostatic performance, while minimizing their shortcomings [11]. For example, silicate-gelatin hydrogel inherits the separate, excellent properties of injectability, rapid mechanical recovery, physiological stability, and an ability to promote coagulation [12]. Chitosan wound dressing is refined by incorporating polyphosphate to increase platelet adhesion and plasma absorption that accelerates blood clotting [13]. Therefore, suitable hemostatic composites are advantageous in their hemostatic performance and biosafety.

Cross-linked graphene sponge (CGS) is a new hemostat with great potential for treatment of trauma [14]. It rapidly absorbs plasma, accelerating coagulation. But, the shortcoming of CGS is that, when in contact with blood, it cannot stimulate hemocytes to promote bleeding control. To enhance the hemostatic ability of CGS, increasing the charge density [15] and incorporating clay [16] have been attempted to enhance its performance effectively. Further, those studies demonstrated that CGS can work as an alternative platform to carry different hemostatic mechanisms. Thus, creating a novel composite with a new active mechanism is important to achieve a high-performance of hemostasis.

Thrombin, a serine proteinase known as the activated coagulation factor II, is widely used for hemostasis [17–19]. It can directly convert fibrinogen into fibrin, polymerizing strong fibrin clots in the coagulation cascade [20,21]. However, thrombin may also cause hypersensitivity reactions, and severe bleeding and transmit infections or thrombosis, when improperly used [22–25]. To meet the demands of security and stability, thrombin-based composites were developed, such as the loading of thrombin into a polymeric microsphere [26–28], or immobilizing it in a gelatin matrix [29–32]. However, those composites mainly target in-body

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hemostasis. Although the side effects of thrombin are reduced for this composite, there still exists the risk of a high internal dosage of thrombin that would be completely absorbed by the patient [33–35]. Therefore, utilizing thrombin's function while avoiding the risks of excess release and dwelling should be highlighted. Based on the aforementioned CGS used as a suitable supporter, the combination of thrombin as a bio-safe and effective trauma hemostat is thus desired and valuable.

In this study, we developed a facile spray method to prepare thrombin-coated CGS (TCGS) composites. Thrombin is absorbed into the CGS in a gradient manner due to the porous sponge structure. The inside surfaces of CGSs are known as reduced graphene oxide [14], which is effective in immobilizing thrombin-like protein [36]. This property helps the TCGS to prevent a burst release of thrombin and maintain its bioactivity within the small cavities of the sponge structure. Therefore, a suitable amount of thrombin on TCGS not only ensures the fast absorbability by the fundamental CGS supporter but also triggers the coagulation pathway as soon as thrombin comes in contact with blood. In a potential synergistic effect of the above-mentioned two mechanisms, this TCGS composite is expected to be a new thrombin based trauma hemostat.

2. Experimental

2.1. Materials

Thrombin was obtained from Peking University Third Hospital. Coomassie Brilliant Blue G-250 (CBB) was purchased from Tokyo Chemical Industry (TCI). Chloral hydrate was purchased from Sigma. Sprague-Dawley (SD) rats were purchased from Vital (Charles) River Laboratory, Beijing, China. Fresh blood was obtained from the SD rat, and the anticoagulant (ACD) whole blood was prepared by combining fresh blood and citrate dextrose in the ratio of 9:1 [37]. Other commonly used chemical reagents were purchased commercially. All of the SD rats used for animal experiment in this research were treated and cared for in accordance with the National Research Council's Guide.

2.2. Preparation of TCGS

First, CGS was prepared according to the procedures in the literature procedures [14]. Briefly, 200 μL of ethylenediamine (EDA) was mixed with 20 mL of graphene oxide (GO) dispersion (3 mg mL^{-1}). The mixture was heated to 96 °C for 6 h in a reaction kettle to obtain a GO hydrogel. After freeze-drying and Soxhlet extraction by alcohol, the purified GO aerogel was dried and treated with microwave radiation (800 W, 5 s) to obtain the CGS. Then, thrombin solution (1 mL, 25 U mL^{-1}, where U is unit of activity) was sprayed uniformly onto the surface of the CGS. The composite material was kept at room temperature for 5 min and then underwent freeze-drying for 24 h to immobilize the thrombin, forming the named TCGS.

2.3. Thrombin distribution

Fluorescence image analysis. Water-soluble fluorescent labels (strontium aluminate and polyacrylic acid suspension, purchased from Shanghai Future Industrial Co., Ltd.) were diluted with water (1/4, v/v) and dissolved with thrombin. The mixed thrombin solution (1 mL, 25 U mL^{-1}) was sprayed on the surface of the CGS. The final material was obtained after freeze-drying. After UV (365 nm) irradiation for 5 min, fluorescent of the labelled TCGS could be observed in a darkroom.

Quantitative analysis. TCGS was equally divided into three layers: the top layer, the middle layer and the bottom layer (as shown in Fig. 1c inset). Each layer was stirred at a high velocity for 1 h in 20 mL of deionized water to elute the immobilized thrombin. One mL eluent was mixed with 4 mL CBB standard liquid. The mixture was measured by an ultraviolet spectrophotometer (MAPADA UV-1100) at 595 nm. The thrombin content of each layer was calculated by the thrombin-CBB standard curve [38].

2.4. Stability of the modified thrombin in TCGS

The TCGS was immersed in 20 mL of deionized water and extracted or slowly stirred (300 rpm) for 5 min. Then, 1 mL of the obtained extract was mixed with 4 mL of CBB standard liquid. The mixture was measured by a spectrophotometer (MAPADA UV-1100) at 595 nm. The untreated deionized water was served as a negative control, and the equivalent concentration of thrombin (2.5 U mL^{-1}) served as a positive control. The thrombin content of each group was calculated using the thrombin-CBB standard curve [38].

2.5. Blood cell evaluations

Morphology Study of Blood Cells. The ACD-whole blood was directly applied onto the top layer of the TCGS. After 3 min, a layer of blood scab was formed, and excess phosphate buffer solution (PBS) was added 3 times to rinse the free blood cells. Then, the material was immobilized with 2.5% glutaraldehyde for 2 h. To dehydrate, the immobilized blood cells were immersed in a graded ethanol series (50%, 60%, 70%, 80%, 90% and 100%) for 10 min. The sample was freeze-dried and metal-sprayed in preparation for scanning electron microscopy (SEM, S-4700 Hitachi) observation [39].

Blood Cell Select Adhesion. The top layer of the TCGS was equilibrated in 20 mL of PBS for 2 h at 37 °C. Then 1 mL of ACD-whole blood was added and allowed to fully contact for 1 h at 37 °C. PBS was added 3 times to remove the blood cells that did not adhere on the TCGSs. Then, the material was immobilized with 2.5% glutaraldehyde for 2 h. To dehydrate, the immobilized blood cells were immersed in a graded ethanol series (50%, 60%, 70%, 80%, 90% and 100%) for 10 min. The sample was freeze-dried and metal-sprayed before SEM observation.

Platelet Adhesion. For the platelet selective adhesion test, the top layer of the TCGS was equilibrated in 20 mL of PBS for 2 h at 37 °C. Then, 2 mL of platelet-rich plasma (PRP), which was separated from the ACD-whole blood by centrifuge at 2000 × g for 20 min at 4 °C, was added to allow fully contact for 1 h at 37 °C. PBS was added 3 times to remove the blood cells that did not adhere to the TCGS. Then, the material was immobilized with 2.5% glutaraldehyde for 2 h. To dehydrate, the immobilized blood cells were immersed in a graded ethanol series (50%, 60%, 70%, 80%, 90% and 100%) for 10 min. The sample was freeze-dried and metal-sprayed before SEM observation [40,41].

2.6. In vitro clotting tests

Approximately 50 μL of fresh blood was added into test samples to react for 30, 60, 120, 180 and 240 s. The tested samples included the CGS, the TCGS and native thrombin. The CGS and TCGS were prepared into equal volumes. The amount of native thrombin was equal to that in the TCGS, and the native thrombin was dissolved in 10 μL of deionized water. Blood without a reaction was served as a blank control. After each reaction time, 10 mL of deionized water was added to dissolve free red blood cells, with a slightly shaking. Then, the solutions were measured by UV at 542 nm and the content of the hemoglobin for each sample at each time point was calculated by the following equation: Hemoglobin absorbance = $Is/Ir \times 100\%$, where Is is the absorbance of the resulting sample, and Ir is the absorbance of the blank control [42,43].