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Black hemostatic sponge based on facile prepared cross-linked graphene

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ABSTRACT

In this study, we demonstrate for the first time the remarkable hemostatic performance of a cross-linked graphene sponge (CGS) as a superb hemostat. The CGS can absorb plasma immediately (<40 ms) to form a blood cell layer and promotes subsequent clotting. The interaction between the interface of the CGS and blood cells reveals that the fast blood coagulation is primarily attributed to the enrichment of hemocytes and platelets on the wound surface. An *in vitro* dynamic whole-blood clotting test further highlights the effectiveness of the CGS. Considering the facile preparation, low cost, nontoxicity, and long shelf life of the portable black sponge, the CGS has great potential for trauma treatment.

1. Introduction

Graphene, a two-dimensional (2D) monolayer of carbon atoms, has been studied in recent years as one of the most useful nanomaterials [1–5]. Recently, applications of graphene-based materials in biomedical and pharmaceutical fields have attracted much attention due to the materials' biocompatibility, good water dispersity, potential for functional modification, and unique optical and electronic properties. As reported, graphene-based materials have been widely utilized as platforms for drug delivery [6,7], biosensors [8–10], cellular imaging [11–13], and cancer therapy [14–16], demonstrating their promising prospects for use in biomaterials. Furthermore, for safe applications *in vivo*, significant efforts have been undertaken to improve the biocompatibility of graphene-based materials including amine modification and heteroatom doping [6,8,17,18]. However, graphene and its derivatives are xenobiotic substances. Thus, utilizing their superb characteristics in *in vitro* biomedical applications should be actively explored and developed. To the best of our knowledge, there are few studies that have focused on graphene-based materials for use in the field of hemostasis, although hemostatic materials are vital in both civilian and military medicine because rapid hemostasis is essential for optimal recovery and even survival [19,20]. Traditional hemostatic agents, such as zeolite [20,21] and mesoporous silica [22–24], mainly depend on their quick plasma absorbability to accelerate the aggregation of blood cells, while chitosan-based hemostatic materials [25,26] promote blood coagulation by charge effect – positively charged chitosan attracts negatively charged red blood cells to form a layer of blood clot. These studies especially on the hemostatic mechanisms have a great significance for the development of new generation of hemostatic materials.

Among graphene-based materials, cross-linked graphene, generally prepared from a suspension of graphene oxide (GO) nanosheets with suitable chemical linkers, has exhibited prominent advantages, including large pore volumes, high surface areas, low density, and ideal structural stability [27–31], which make it suitable for hemostatic applications. Cross-linked graphene possesses a remarkable capability for rapid liquid absorption [32,33], which inspired us to develop a novel black hemostatic sponge based on its three-dimensional (3D) structure for rapidly absorbing plasma and thus increasing the concentration of blood cells to accelerate blood clotting [21,34]. In this study, we demonstrate for the first time a cross-linked graphene sponge (CGS) as an exemplary hemostat and its outstanding hemostatic performance.

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2. Experiments

2.1. Preparation and characterization of the CGS

A 200 μl volume of ethylenediamine (EDA) used as a cross-linking agent was added to 20 ml GO dispersion (3 mg ml^{-1}, prepared *via* a modified Hummers' method [35]). The mixture was sealed in a hydrothermal synthesis reaction kettle (inside diameter, 4 cm) and heated to 96 °C for 6 h to obtain a GO hydrogel. After freeze-drying for 48 h, the GO aerogel (GOA) was embathed with ethyl alcohol in a Soxhlet extractor for 48 h to remove the unreacted EDA. Next, the purified GOA was dried at ambient temperature for 24 h, and under air conditions, it was treated with microwave radiation (800 W) for 5 s to obtain the CGS (3 cm diameter, 1.2 cm thickness).

Scanning electron microscopy (SEM) was performed using an S-4700 Hitachi microscope. The cross sections of the CGS and GOA samples were observed at different resolutions to compare the internal structure before and after microwave treatment. Brunauer–Emmet–Teller (BET) surface area measurements were determined by the nitrogen gas adsorption method by using a Micromeritics ASAP 2020 analyzer at liquid nitrogen temperature. Methylene blue (MB) dye adsorption test was further performed to evaluate the surface area according to the reported method [36]. An elemental analyzer Vario ELcube was used to conduct elemental analysis. The CGS and GOA samples were processed into powder, and the contents of C, H, O, and N were determined.

2.2. Evaluation of the hemostatic performance

A droplet of blood was dropped onto the CGS surface, and a high-speed camera (40 ms per frame) was used to record the entire process of blood absorption. The rate of blood absorption was calculated based on the frame rate and alignment. Next, the CGS was dipped in a blood pool to absorb blood adequately. The CGS was weighed twice before and after dipping to determine the weight of absorbed blood. The blood absorption capability of the CGS was presented as the mass ratio of blood and CGS. As a control, the water absorption rate and capability were tested using the same method mentioned above.

A rat-tail cutting experiment was used to evaluate the hemostatic performance of the CGS. Healthy male Sprague-Dawley (SD) rats (250 ± 20 g, 7 weeks of age) were used as animal models. They were purchased from the Vital (Charles) River Laboratory, Beijing, China. Animals were treated and cared for in accordance with the National Research Council's Guide for the care and use of laboratory animals. Each rat was raised in a single cage. The rats were raised for 3 days in a standard environment to ensure a healthy physiological state. All rats were anesthetized with 1.25 ml 10% chloral hydrate (0.5 ml per 100 g) before surgery. Each rat tail, measuring 16 cm in length, was cut at 6 cm from the tip by surgical scissors. The wound section was covered with the CGS directly to control bleeding with slight pressing. The hemostatic time was recorded. The CGS was weighed before and after hemostasis to determine the weight of the blood loss. Gauze sponge served as a control in this study.

2.3. Interaction between the interface of CGS and blood cells

2.3.1. Morphology study of blood cells

A total of 1 ml volume of phosphate buffered saline (PBS; pH 7.4) with 50 μl ACD-whole blood was added onto the CGS (3 cm diameter, 1.2 cm thickness) surface. The material containing whole blood was incubated for 3 min at 37 °C. The sample was then gently rinsed three times with PBS and immobilized with 2.5% glutaraldehyde for 2 h at 4 °C. Blood cells were dehydrated with 50, 60, 70, 80, 90 and 100% ethanol for 10 min. The samples were freeze-dried for 12 h prior to SEM observation [25].

2.3.2. Blood cell adhesion

A small piece of the CGS (1 × 1 cm^2, 0.25 cm thickness) was immersed in 10 ml PBS for 2 h at 37 °C. Next, 0.5 ml ACD-whole blood was added. The material containing whole blood was incubated for 1 h at 37 °C. The sample was then treated according to the above-mentioned method prior to SEM observation.

2.3.3. Platelet adhesion

The ACD-whole blood was centrifuged at 300 × *g* for 20 min at 4 °C to obtain platelet-rich plasma (PRP). A portion of the PRP was further centrifuged at 2000 × *g* for 20 min at 4 °C to obtain platelet-poor plasma (PPP). The platelet concentration was adjusted to 1×10^5 platelets μl^{-1} by adding PPP to PRP, and the number of platelets was counted by a hemocytometer. The CGS (1 × 1 cm^2, 0.25 cm thickness) was then placed on a Petri dish covered with PRP and incubated for 1 h at 37 °C. The sample was treated according to the above-mentioned method prior to SEM observation [22,37].

2.4. In Vitro *dynamic whole-blood clotting*

Total 50 μl volume of fresh blood drawn from SD rats was directly dropped onto four test groups, including a blank, Ca^{2+} (4 μl $CaCl_2$ solution, 0.2 mol L^{-1}), gauze sponge, and the CGS in 50 ml glass beakers, respectively. The volume of the gauze sponge was same as that of the CGS (1 × 1 × 0.25 cm^3). Each group was allowed to interact for 30, 60, 120, 180, and 240 s. Then, 10 ml distilled water was added slowly down the inside wall of the beaker without disturbing the clotted blood. The beaker was shaken slightly to dissolve free RBCs. The absorbance of each of the resulting samples was measured by an ultraviolet spectrophotometer at 542 nm. As a reference value, the absorbance of 50 μl fresh blood in 10 ml distilled water was measured. The content of hemoglobin was quantified by the following equation: Hemoglobin absorbance = $I_s/I_r \times 100\%$, where I_s is the absorbance of the resulting sample, and I_r is the absorbance of the reference value. This experiment was repeated three times with different healthy SD rats under the same conditions [38,39].

2.5. Cytotoxicity evaluation

L929 mouse fibroblast cells (purchased from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) were adjusted to 8×10^5 cells ml^{-1} in complete medium (CM), which consisted of 90% RPMI-1640 medium, 10% fetal bovine serum (FBS), and 1% antibiotics (100 units ml^{-1} penicillin and 100 units ml^{-1} streptomycin). The cell suspension was added into 96-well plates (100 μl per well) and incubated for 24 h at 37 °C in an air environment of 5% CO_2. Meanwhile, 5 ml RPMI-1640 medium was added to the CGS as the test group, and an equal volume of RPMI-1640 medium was added to an empty bottle as the negative control group. After 24 h, the impregnating solution was removed from the test group and added to the corresponding CM. After 48 h of incubation, cell viability was determined by a MTT assay kit based on the manufacturer's instructions. The relative growth rate (RGR) of the cells was calculated according to the following formula: RGR = $Abs_{490\ test}/Abs_{490\ control} \times 100\%$. Finally, the toxicity grade was assessed based on the RGR. This experiment was repeated three times [40,41].