Trends in Biomaterials and Artificial Organs

journal homepage: www.biomaterials.org.in/tibao

ISSN 0971-1198

Original Research Article

# *In Vitro* Evaluation of the Coagulation Activity of a Chitosan-Based Emergency Hemostatic Sponge: A Domestically Developed Product in Vietnam

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**Received:** *19 January 2026*
**Accepted:** *4 February 2026*
**Published online:** *24 February 2026*

**Keywords:** *chitosan, hemostatic sponge, in vitro, clotting kinetics, plasma coagulation*

Rapid control of hemorrhage is essential in emergency and surgical settings. Chitosan-based hemostats act primarily through electrostatic interactions with blood components and can function even when the intrinsic coagulation pathway is impaired. This study aims to evaluate the in vitro coagulation performance of a domestically developed chitosan-based emergency hemostatic sponge (EHS) compared with a commercial chitosan dressing (Axiostat®). Red blood cell (RBC) adhesion was assessed by scanning electron microscopy. Whole-blood interaction and absorption were quantified by gravimetric uptake and by residual free-RBC absorbance at 540 nm. Whole-blood clotting kinetics (5–15 min) and clotting blood time (CBT) were measured using citrated rabbit blood. Plasma coagulation parameters (PT, APTT, fibrinogen) were assessed in rabbit platelet-poor plasma after 10, 20 and 30 min incubation with the test materials. EHS promoted dense RBC adhesion on the sponge surface. Blood uptake was 712.6 ± 58.0 mg for EHS and 704.8 ± 37.2 mg for Axiostat® ($p > 0.05$). In the clotting-kinetics assay, residual free-RBC absorbance decreased markedly for both materials (e.g., at 10 min: 0.031 ± 0.005 for EHS vs 0.737 ± 0.009 in the control; $p < 0.001$). CBT was significantly shorter with EHS (213.2 ± 5.4 s) and Axiostat® (214.9 ± 6.0 s) than with no material (535.3 ± 8.1 s; $p < 0.001$). PT and fibrinogen did not differ significantly among groups, whereas APTT was significantly reduced in both material groups compared with the control ($p < 0.01$). EHS demonstrates effective in vitro hemostatic activity comparable to a commercial chitosan dressing, mainly through cellular interactions and acceleration of clot formation, supporting further evaluation in clinically relevant bleeding models.

## Introduction

Topical hemostatic agents play a critical role in surgical settings as well as in emergency treatment by controlling blood loss and minimizing complications that could lead to morbidity or mortality [1,2]. Over the past few decades, various hemostatic dressings - such as sponges and gauzes - have been developed to replace traditional methods like direct compression or suturing [3].

A wide range of biomaterials, including collagen, gelatin, alginate, oxidized cellulose, and chitosan (CS), have been employed in the development of modern hemostatic devices [4]. Among these, chitosan—a positively charged polysaccharide derived from the controlled deacetylation of chitin (commonly found in the shells of crustaceans such as shrimp and crabs) - has demonstrated strong hemostatic potential. Its cationic nature promotes red blood cell aggregation, fibrinogen adsorption, platelet adhesion, and activation, while also inhibiting contact system activation [5]. Several studies have shown that chitosan can activate or enhance various coagulation factors, thereby accelerating the hemostatic process [6].

Animal models are not ethically permitted for hemostatic product testing without prior in vitro evaluation. In vitro models help simulate the hemostatic response under controlled conditions and are increasingly recognized as essential tools for predicting clinical efficacy. These models aim to assess interactions between biomaterials and blood components (RBCs, platelets, and plasma) and are typically categorized based on the stages of hemostasis: vascular, platelet, and coagulation phases.

Among these, the coagulation phase involves a complex network of plasma coagulation factors and offers multiple in vitro approaches for assessment. Models simulating coagulation disorders, often using anticoagulants, have become more common due to their clinical rel-

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https://doi.org/10.65795/a5nsce32

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evance [7].

In this study, we evaluate an emergency hemostatic sponge (EHS) developed by the Vietnam–Russia Tropical Center, based on chitosan, intended for rapid bleeding control at the site of injury. This is the first in vitro investigation in Vietnam focusing specifically on the coagulation phase using this material.

## Material and Methods

### Emergency hemostatic sponge (EHS)

The investigational product is an emergency hemostatic sponge (EHS), with deacetylated chitosan as its main component. It is designed for local bleeding control in emergency situations. Each sponge measures 8 cm × 8 cm and weighs between 2.8 and 3.4 grams. The sponge is individually packaged in a sealed aluminum pouch (12 cm × 11 cm), with 10 pouches placed in a cardboard box (15 cm × 12 cm × 11 cm) (figure 1). The product meets the institutional quality standard (Code: CSQP 2024/NDVN) and is manufactured by the Vietnam–Russia Tropical Center under the Ministry of National Defense of Vietnam.

### Reference material

The reference product used in this study is Axiostat, a hemostatic sponge from India composed entirely of 100% chitosan. It is designed to seal open wounds and prevent uncontrolled bleeding. Axiostat has been approved by the U.S. Food and Drug Administration (FDA) and is currently used by the Indian Armed Forces. The sponge dimensions are the same as EHS (8 cm × 8 cm) (figure 1). EHS is being developed with the goal of achieving comparable quality to Axiostat.

### Animal blood used in the study

Blood samples were collected from white rabbits and laboratory rats provided by the Laboratory Animal Supply Unit of the Military Medical Academy. All animals were acclimatized in the laboratory for at least one week before experimentation, fed with standard laboratory diet, and given access to boiled and cooled water ad libitum. The types of blood components used for each test are summarized in table 1.

### Equipment and reagents

*Laboratory supplies*
Sterile gauze, medical adhesive tape, test tubes, blood collection tubes pre-filled with EDTA or sodium citrate, blood collection needles, test tube racks, surgical razors, medical scissors, Eppendorf tubes, and micropipette sets.

*Equipment*
Centrifuge; Platelet aggregometer (CHRONO-LOG 530 VS, USA); Automated coagulation analyzer (ACL TOP 500, Instrumentation Laboratory); UV–Vis spectrophotometer (Evolution 3000, Biochemical Systems International, Italy) operating at 540 nm; Hematology analyzer (Beckman Coulter LH 750/LH 755); Laboratory incubator and refrigerated storage units.

Table 1: Types of blood components used for each test

| Test | Blood component used |
|---|---|
| Red blood cell adhesion test | 10% RBC suspension prepared from rabbit blood anticoagulated with 3.2% sodium citrate, centrifuged at 3000 rpm for 15 minutes, and diluted 1:10 in phosphate-buffered saline (PBS). |
| Blood interaction and absorption test | Whole rabbit blood anticoagulated with 3.2% sodium citrate. |
| Clot formation kinetics and CBT test | Whole rat blood anticoagulated with EDTA (for clot kinetics test).<br>Whole rat blood anticoagulated with 3.2% sodium citrate (for clotting time test). |
| Plasma coagulation analysis | Platelet-poor plasma (PPP) obtained by centrifuging whole rabbit blood anticoagulated with 3.2% sodium citrate at 3000 rpm for 10 minutes. |

Figure 1: Images of EHS and Axiostat

*Reagents and chemicals*
Double-distilled water; Glutaraldehyde 2.5%; Ethanol solutions (70%, 80%, 90%, and 100%); Calcium chloride ($CaCl_2$) solution, 25 mmol/L; Other standard reagents and consumables required for the experiments.

### Red blood cell adhesion (aggregation) assay

This experiment was conducted based on the method described by Yu Cheng et al [8]. EHS samples (1 cm × 1 cm) were placed in 12-well tissue culture plates and incubated at 37°C for 5 minutes. Then, 2000 μL of a 10% red blood cell (RBC) suspension (prepared from rabbit blood in phosphate-buffered saline, PBS) was added to each well and incubated at 37°C for 30 minutes. After incubation, the samples were gently removed and rinsed with 4000 μL PBS to eliminate unbound RBCs from the surface. The samples were then fixed in 2.5% glutaraldehyde solution for 30 minutes, followed by dehydration using a graded ethanol series (70%, 80%, 90%, and 100%). Finally, the samples were air-dried at room temperature and examined using scanning electron microscopy (SEM) to evaluate RBC adhesion and aggregation on the material surface. The same procedure was performed for the reference group using Axiostat sponges. Each condition was tested in triplicate.

### Blood interaction and absorption assay

This experiment was conducted based on the method described by Pogorielov et al [9], to evaluate the blood absorption capacity and the stability of the clot formed upon contact with the test material. The procedure included three main steps:

*Blood absorption assessment:*
EHS samples (1 cm × 1 cm) were accurately weighed before the experiment and then immersed in test tubes containing 2 mL of rabbit whole blood anticoagulated with 3.2% sodium citrate. The tubes were incubated at 37°C for 10 minutes. After incubation, the samples were removed, gently blotted, and weighed again to calculate the amount of absorbed blood.

*Clot stability assessment:*
The blood-absorbed samples were then transferred to a new test tube and rinsed with normal saline. Clot stability was evaluated based on the degree of red coloration in the saline solution. A stable clot was indicated by no or minimal red color released into the solution.

*Complete blood count (CBC):*
The remaining blood in the tube, after removing the sample, was subjected to a complete blood count to evaluate the residual red blood cells and platelets.

The experiment included a reference group using Axiostat hemostatic sponge (India) and a control group with blood only (no material). All procedures for the reference and control groups were conducted under identical conditions and repeated three times.

### Clotting kinetics and clotting blood time (CBT) test

The study was conducted following Meng Zhang [10] and Leilei Sun et al [11].

## Clotting kinetics test

Polypropylene tubes were prepared containing 1 mL of whole blood from white rats anticoagulated with EDTA, a 0.5 cm × 0.5 cm piece of EHS, and 500 mL of 0.1 M $CaCl_2$ solution. The tubes were incubated at 37°C for three time points: 5, 10, and 15 minutes.

The tubes with EHS samples were then removed from the incubator, and 20 mL of deionized water (double-distilled water) was gently added along the tube wall over 5 minutes without disturbing the clot. This process lysed the red blood cells that were not trapped in the clot at 5, 10, and 15 minutes. The lysate containing free red blood cells was collected, and the absorbance was measured at 540 nm at 10, 15, and 20 minutes.

## Clotting blood time (CBT) test

In a 12-well cell culture plate, 0.5 cm × 0.5 cm pieces of EHS were placed and pre-incubated at 37°C for 5 minutes. Then, 1 mL of whole blood from white rats anticoagulated with 3.2% sodium citrate was added and incubated for another 3 minutes at 37°C. Finally, 500 μL of $CaCl_2$ solution (25 mmol/L) was added. The time from adding $CaCl_2$ to the formation of a stable clot was recorded as the CBT.

The tests were performed in triplicate with the reference group (Axiostat sponge) and the control group (blood without any sponge). The same procedures were followed for all groups.

## Plasma coagulation analysis

This experiment was conducted following the method described by Meng Zhang et al [10]. Each polypropylene tube was filled with 1 mL of platelet-poor plasma (PPP) obtained from rabbit blood anticoagulated with 3.2% sodium citrate, along with a 1 × 1 cm piece of EHS. The tubes were incubated at 37°C for 10, 20, and 30 minutes with continuous shaking at a constant speed. After incubation, the EHS samples were removed, and the plasma was collected for analysis. The following coagulation parameters were measured using the ACL TOP 500 automated coagulation analyzer: Activated Partial Thromboplastin Time (APTT); Prothrombin Time (PT); Fibrinogen concentration

The tests were performed in triplicate with the reference group (Axiostat sponge) and the control group (blood without any sponge). The same procedures were followed for all groups.

## Evaluation criteria

*Red blood cell aggregation (adhesion) test*
The morphology and adhesion (aggregation/clumping) of red blood cells on the EHS samples and the reference Axiostat sponges were observed using scanning electron microscopy (SEM).

*Evaluation parameters for blood interaction and absorption test*
Blood absorption capacity: The amount of blood absorbed by the samples (EHS/Axiostat) was calculated based on weight measurements before (W1) and after (W2) exposure to whole blood, using the formula: S = W2 - W1

Where S = blood absorption (mg); W1 = initial weight of the sample (mg); W2 = weight after blood exposure (mg). Results were compared between the EHS and the reference Axiostat sponge.

Table 2: Blood absorption capacity of test samples after 10 minutes of incubation in the warming chamber (n = 3 per group).

| Weight | EHS (mg) (1) | Axiostat (mg) (2) |
|---|---|---|
| Sample weight before testing (mg ± SD) | 88.4 ± 1.8 | 74.9 ± 2.0 |
| Sample weight after testing (mg ± SD) | 767.7 ± 11.7 | 768.6 ± 20.4 |
| Amount of absorbed blood (mg ± SD) | 712.6 ± 58.0 | 704.8 ± 37.2 |
| p-value (comparison 1 vs. 2) | p > 0.05 | |

Clot stability assessment: After clot formation, physiological saline (0.9% NaCl) was added to the sample to evaluate clot integrity. A stable clot was indicated by no or minimal red discoloration (hemolysis). EHS was compared to both the Axiostat group and the negative control.
Complete blood count (CBC): The remaining blood after removal of the sample was tested for red blood cell (RBC) and platelet counts, both before and after the experiment. Comparisons were made between the EHS and Axiostat groups, as well as pre- and post-exposure values.

## Clotting kinetics and clotting blood time (CBT)

*Clotting kinetics*

The clotting kinetics were assessed by quantifying the residual red blood cells (RBCs) in plasma. This was done by measuring the absorbance of the lysed blood at 540 nm using a spectrophotometer. Samples were collected at 10, 15, and 20 minutes after the removal of the test materials (EHS, Axiostat, and a negative control with no material).

Comparative results were analyzed between the EHS group, the Axiostat reference group, and the control group at each time point (10, 15, and 20 minutes).

*Clotting Blood Time (CBT)*
CBT was defined as the total time required for blood to form a stable clot and lose its fluidity. The clotting time of the EHS group was compared with that of the control and the Axiostat reference group.

## Evaluation parameters in plasma coagulation assay

Platelet-poor plasma (PPP) was collected after removing the EHS or Axiostat samples from the test tubes. The collected plasma was analyzed using the ACL TOP 500 automated coagulation analyzer to determine the following parameters: Activated Partial Thromboplastin Time (APTT); Prothrombin Time (PT); Fibrinogen levels.

Measurements were taken at 10, 20, and 30 minutes after incubation with the test materials. The results were compared: Before and after exposure to the materials; Among the EHS group, the reference Axiostat group, and the control group (without material).

## Data analysis and statistical processing

Each experiment was performed in triplicate (n=3) for each group unless otherwise stated. All quantitative results are presented as mean ±

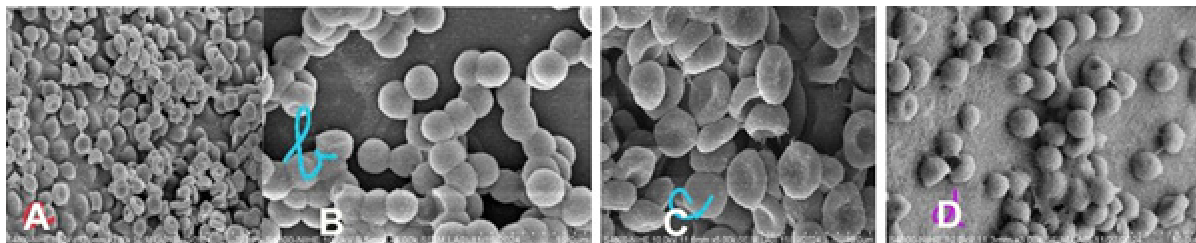


Figure 2: SEM images showing erythrocyte aggregation and adhesion on the surface of hemostatic sponges (A, B on EHS; C, D on Axiostat)

Table 3: Red blood cell (RBC) and platelet (PLT) counts before and after sample application (n = 3 per group)

| Sample exposure time | EHS (1) | | Axiostat (2) | |
|---|---|---|---|---|
| | RBC ×$10^{12}$/L | PLT ×$10^9$/L | RBC ×$10^{12}$/L | PLT ×$10^9$/L |
| Before Application (3) | 5.3 ± 0.09 | 430.33 ± 19.4 | 5.3 ± 0.09 | 430.33 ± 19.4 |
| After Application (4) | 1.11 ± 0.22 | 99.33 ± 1.73 | 1.09 ± 0.3 | 97.78 ± 1.26 |
| Reduction Compared to Baseline (%) | 79.1 | 76.9 | 79.4 | 77.2 |
| p | P(3,4) < 0.01<br>P(1,2) > 0.05 | | | |

standard deviation (SD). Group comparisons were performed using one-way analysis of variance (ANOVA). When ANOVA was significant, Tukey's honestly significant difference (HSD) post-hoc test was applied for pairwise comparisons (EHS vs. Axiostat vs. Control). A p value <0.05 was considered statistically significant.

## Results

### Erythrocyte aggregation (adhesion) assay

SEM images were used to observe erythrocyte adhesion on the material surface. The SEM images in figure 2 demonstrate a significant aggregation and adhesion of numerous erythrocytes on the surfaces of both EHS and Axiostat, with good dispersion and characteristic biconcave shapes.

Table 4: Clotting kinetics of test samples at 5, 10, and 15 minutes (absorbance values of free RBCS not trapped in clots) (n=3 per group)

| Sampling time | Absorbance value | | | p-values |
|---|---|---|---|---|
| 5 min (4) | 0.622 ± 0.006 | 0.604 ± 0.002 | 0.973 ± 0.006 | p1,2 > 0.05<br>p3,2 < 0.001<br>p1,3 < 0.001 |
| 10 min (5) | 0.031 ± 0.005 | 0.029 ± 0.002 | 0.737 ± 0.009 | |
| 15 min (6) | 0.026 ± 0.001 | 0.024 ± 0.003 | 0.654 ± 0.033 | |
| p | p4,5 < 0.05 | p4,5 < 0.05 | p4,5 < 0.05 | |
| | p4,6 < 0.05 | p4,6 < 0.05 | p4,6 < 0.05 | |
| | p5,6 > 0.05 | p5,6 > 0.05 | p5,6 < 0.05 | |

Table 5: Whole blood clotting time (CBT) of the test samples (total time from the addition of CaCl to the formation of a stable clot and loss of blood flowability) (n=3 per group)

| | Clotting time (CBT) (seconds, s) | p-value |
|---|---|---|
| EHS (1) | 213.2 ± 5.4 | p1,2 > 0.05 |
| Axiostat (2) | 214.9 ± 6.0 | p1,3 < 0.001 |
| Control (3) | 535.3 ± 8.1 | p3,2 < 0.001 |

Table 6: Prothrombin Time (PT) in rabbit platelet-poor plasma (PPP) after sample removal at 10, 20, and 30 minutes (n=3 per group)

| Time point | PT (s) | | | p |
|---|---|---|---|---|
| | EHS (1) | Axiostat (2) | Control (no sample) (3) | |
| 10 min (4) | 13.83 ± 0.25 | 13.87 ± 0.29 | 14.97 ± 0.57 | p1,2,3 > 0.05<br>p4,5,6 > 0.05 |
| 20 min (5) | 13.80 ± 0.35 | 13.83 ± 0.49 | 14.93 ± 0.29 | |
| 30 min (6) | 13.80 ± 0.26 | 13.80 ± 0.69 | 14.93 ± 0.25 | |

### Blood interaction and blood absorption testing

*Blood absorption of the test samples*

Whole rabbit blood was incubated with the test materials (EHS/Axiostat) in an incubator for 10 minutes. The materials were then removed and weighed to evaluate blood absorption (by comparing the sample weight before and after the experiment, in milligrams). The results are presented in table 2 below.

As shown in table 2, the amount of blood absorbed by EHS (712.6 mg) was slightly higher than that of Axiostat (704.8 mg), indicating a better blood absorption capacity. However, the difference was not statistically significant (p>0.05).

*Clot stability*

After incubating the test samples (EHS/Axiostat) in blood tubes at 37°C for 10 minutes, the samples were removed and placed into small test tubes. Physiological saline was then added to observe whether any red color leached out, in order to evaluate the stability of the blood clot formed through interaction with the test materials. The results are shown in figure 3.

Both test materials -EHS and Axiostat- did not show any red color leaching when physiological saline was added, indicating the formation of stable blood clots. In contrast, the control sample without any test material dissolved and turned red upon saline addition. This test is an important step in evaluating the effectiveness and stability of hemostatic materials, ensuring safety and efficiency in medical applications.

*Complete blood count (CBC) test*

After removing the test samples, the remaining whole blood was used to measure red blood cells (RBC) and platelets (PLT). The results are presented in table 3.

The results from table 3 indicate that, after exposure to the EHS sample, both red blood cell (RBC) and platelet (PLT) counts signifi-

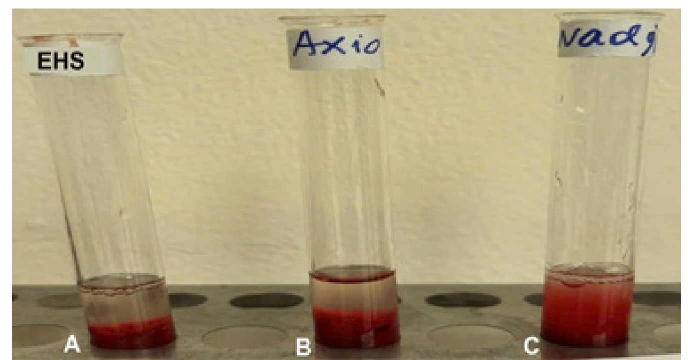


Figure 4: Images showing the stability of blood clots (A, samples of EHS; B, Axiostat sponge; and C, blood without test material after being rinsed with physiological saline, following incubation in blood samples)

Table 7: Activated partial thromboplastin time (APTT) in rabbit platelet-poor plasma (PPP) after sample removal at 10, 20, and 30 minutes (n = 3 per group)

| Time point | APTT (s) | | | p |
|---|---|---|---|---|
| | EHS | Axiostat | Control (no sample) | |
| 10 minutes | 24.1 ± 0.66 | 23.1 ± 0.79 | 33.57 ± 1.1 | < 0.01 |
| 20 minutes | 23.03 ± 0.61 | 22.33 ± 0.40 | 33.33 ± 0.55 | < 0.01 |
| 30 minutes | 22.95 ± 0.31 | 22.21 ± 0.42 | 33.3 ± 0.46 | < 0.01 |
| p (within group) | > 0.05 | > 0.05 | > 0.05 | |

Table 8: Fibrinogen levels in rabbit platelet-poor plasma (PPP) after sample removal at 10, 20, and 30 minutes (n = 3 per group)

| Time point | Fibrinogen (g/L) | | | p |
|---|---|---|---|---|
| | EHS | Axiostat | Control (no sample) | |
| 10 minutes | 3.69 ± 0.10 | 3.70 ± 0.15 | 3.83 ± 0.15 | > 0.05 |
| 20 minutes | 3.65 ± 0.06 | 3.63 ± 0.19 | 3.80 ± 0.10 | > 0.05 |
| 30 minutes | 3.63 ± 0.05 | 3.61 ± 0.06 | 3.70 ± 0.17 | > 0.05 |
| p (within group) | > 0.05 | > 0.05 | > 0.05 | |

cantly decreased compared to baseline ($1.11\times10^{12}$/L vs. $5.3\times10^{12}$/L for RBC and $99.33\times10^{9}$/L vs. $430.00\times10^{9}$/L for PLT), corresponding to a reduction efficiency of 79.1% and 76.9%, respectively. These differences were statistically significant (p<0.01).

In the reference group using Axiostat, RBC and PLT counts also significantly decreased after sample contact (p < 0.01), from $5.3\times10^{12}$/L to $1.09\times10^{12}$/L and from $430.00\times10^{9}$/L to $97.78\times10^{9}$/L, with reduction efficiencies of 79.4% and 77.2%, respectively. The reductions observed in the EHS and Axiostat groups were statistically comparable (p>0.05).

### Clotting kinetics and clotting blood time (CBT)

The results in table 4 show that the absorbance values of free red blood cells (RBCs) not trapped in the clot for EHS gradually decreased at 10 and 15 minutes compared to the 5-minute time point (0.026 and 0.031 vs. 0.622), and the differences were statistically significant (p<0.05). Although the value at 15 minutes was lower than at 10 minutes, the difference was not statistically significant (p>0.05).

Compared to the control group (no sponge), the absorbance values at all three time points (5, 10, and 15 minutes) were significantly lower (0.622; 0.031; and 0.026 vs. 0.973; 0.737; and 0.654, respectively), with statistically significant differences (p<0.001). When compared to Axiostat, the RBC absorbance values at 5, 10, and 15 minutes were similar (0.622; 0.031; and 0.026 vs. 0.604; 0.029; and 0.024), and the differences were not statistically significant (p>0.05).

Clotting time is defined as the point at which uniform and stable blood clots are formed in test tubes. The results in table 5 show that the clotting time for EHS was significantly shorter than that of the control group (blood sample without test material) - 213.2 seconds vs. 535.3 seconds, with a statistically significant difference (p< 0.001). Compared to Axiostat, the clotting time of EHS was statistically similar (213.2 seconds vs. 214.9 seconds, p>0.05).

### Study on the evaluation of plasma coagulation analysis

After removing the EHS sample, the PT in rabbit PPP at 30 minutes was slightly lower than at 10 minutes and comparable to 20 minutes (13.8 s vs. 13.83 s and 13.80 s). However, these differences were not statistically significant (p > 0.05) (table 6). At all time points, the PT values of the samples exposed to EHS were lower than the control group (13.83 s; 13.80 s; and 13.8 s vs. 14.97 s; 14.93 s; and 14.93 s), but again, the differences were not statistically significant (p>0.05). These values were also comparable to those of the reference group, Axiostat (13.83 s; 13.80 s; and 13.8 s vs. 13.87 s; 13.83 s; and 13.80 s), with no statistically significant differences observed (p>0.05).

After removing the EHS sample, the APTT in rabbit PPP slightly decreased at 30 minutes compared to 20 and 10 minutes (22.95 s vs. 23.03 s and 24.1 s), though this difference was not statistically significant (p > 0.05) (table 7). At all time points, APTT values in the EHS group were significantly lower than in the control group (24.1 s, 23.03 s, and 22.95 s vs. 33.57 s, 33.33 s, and 33.1 s), with statistically significant differences (p < 0.01). The values for EHS were comparable to those of the reference material, Axiostat (24.1 s, 23.03 s, and 22.95 s vs. 23.1 s, 22.33 s, and 22.21 s), with no statistically significant differences (p > 0.05).

After removal of the EHS sample, fibrinogen levels in rabbit PPP slightly decreased at 30 minutes compared to 20 and 10 minutes (3.63 g/L vs. 3.65 g/L and 3.69 g/L), but this difference was not statistically significant (p > 0.05) (table 8). At all time points, fibrinogen levels in samples with EHS were slightly lower than those in the control group (3.69 g/L, 3.65 g/L, and 3.63 g/L vs. 3.83 g/L, 3.80 g/L, and 3.70 g/L), though the difference was not statistically significant (p > 0.05). Fibrinogen levels in the EHS group were comparable to the reference Axiostat group (3.69 g/L, 3.65 g/L, and 3.63 g/L vs. 3.70 g/L, 3.63 g/L, and 3.61 g/L), with no significant differences (p > 0.05).

## Discussion

### Red blood cell aggregation (adhesion/aggregation) test

Red blood cells (RBCs) are the most abundant blood cells and are increasingly recognized as active contributors to hemostasis and thrombosis. Beyond serving as passive fillers within the fibrin network, RBCs can enhance platelet margination, promote thrombin generation, and increase clot density and stability [12]. Electrostatic interactions between positively charged chitosan and negatively charged RBC membranes may further accelerate clot formation [13].

The cationic charge density of chitosan is governed by its degree of deacetylation (DD) and, to a lesser extent, molecular weight and protonation (pH). Higher DD increases the number of free amine groups, strengthening electrostatic interactions with negatively charged cell membranes and plasma proteins, which may enhance RBC/platelet adhesion and accelerate clot formation. Therefore, reporting DD (and molecular weight) is important for mechanistic interpretation and batch-to-batch reproducibility. In this study, DD was not analytically determined; future product characterization should include DD measurement (e.g., FTIR or 1H-NMR) and relate these parameters to coagulation outcomes.

SEM observations in figure 2 show that a large number of RBCs adhere to the surface of the battlefield hemostatic sponge (EHS), comparable to the Axiostat sponge. RBCs adhere to the EHS in clusters because its main component, chitosan, carries a positive charge that attracts negatively charged RBCs, facilitating their aggregation on the surface. This promotes faster coagulation reactions. These results are consistent with previous studies [14], where RBCs adhered to sponges containing chitosan such as CTS and SHCF.

Moreover, the RBCs observed on EHS exhibit a typical biconcave shape with good dispersion, showing no deformation or pressure-induced changes. This indicates that the material's surface is compatible with blood cells and does not harm their morphology. The ability of EHS to induce RBC aggregation suggests good interaction with blood cells, supporting hemostasis by slowing blood flow locally and promoting clot formation. This aligns with findings from [13].

Since RBCs are the main component of thrombi, an effective hemostatic material must be able to induce significant RBC adhesion and stable aggregation. Additionally, clustering of RBCs into polyhedral aggregates helps form a tight seal, effectively preventing bleeding [13].

In summary, EHS does not affect the normal morphology or function of RBCs, demonstrating good biocompatibility. The high number of well-dispersed RBCs maintaining their biconcave shape on the sponge surface in vitro is a positive indicator that EHS could be an effective and safe hemostatic material.

## Blood interaction and blood absorption tests

This test aims to evaluate the interaction between rabbit blood cells and chitosan-based materials, a crucial property for effective hemostatic agents. Materials that rapidly absorb plasma and concentrate blood cells, especially red blood cells (RBCs) and platelets, can promote faster clot formation at the injury site.

*Blood absorption of the samples*
Hemostatic sponges typically have a porous structure, providing a large surface area to contact blood and enhance absorption capacity. The absorption ability determines how much blood the sponge can hold. Blood absorption was measured by the weight difference of the samples before and after immersion in blood for 10 minutes at 37°C. Our results (table 2) show that EHS absorbed blood amount similar to the reference sponge Axiostat (712.6 mg vs. 704.8 mg). Hemostatic effectiveness mainly depends on the sponge's ability to absorb and retain blood at the wound site. High absorption removes excess blood and facilitates clotting. These findings align with Pogorielov [9], who reported blood absorption ranging from 650 to 850 mg using chitosan gauze with different molecular weights on human blood, compared to 913.3 mg for medical cotton gauze. Therefore, EHS shows potential for bleeding control. However, natural coagulation factors and blood cell interactions are not fully activated in vitro, so further animal studies are necessary.

Physical structure (porosity and pore size) influences capillary-driven uptake, cell entrapment, and clot consolidation. In the present work, we assessed functional uptake (gravimetric absorption) and cellular entrapment (RBC absorbance at 540 nm) as indirect indicators of an interconnected porous network. However, quantitative structural parameters such as mean pore size, pore-size distribution, and percent porosity were not measured and should be characterized in future work using image analysis with calibrated SEM/micro-CT, mercury intrusion porosimetry, or liquid displacement methods to better link architecture to hemostatic performance.

*Clot stability*
After incubating samples (EHS/Axiostat) in blood at 37°C for 10 minutes, they were transferred to small tubes and saline was added to check for red color release, indicating clot stability. Both samples showed no red color leaching, whereas blood without any material dissolved and released red color (figure 3). In practice, the ability of hemostatic materials to form and maintain stable clots is essential for effective bleeding control, preventing excessive blood loss, and promoting wound healing. Unstable clots can break down, causing rebleeding, delayed healing, and complications. This indicates that EHS effectively forms stable clots resistant to saline washing and is suitable for practical hemostasis.

*Complete blood count (CBC) test*
After adding samples to 2 ml of blood and incubating for 10 minutes, the samples were removed, and RBC and platelet counts were measured (table 3). Results showed significant reductions in RBC and platelet counts in blood interacting with EHS compared to before treatment and the control group ($p < 0.01$). RBC count decreased by 79.1% vs. before treatment and 78.8% vs. control; platelet count decreased by 76.9% and 76.3%, respectively. Compared to the reference Axiostat sponge, RBC and platelet counts were similar ($1.11 \times 10^{12}$/L vs. $1.09 \times 10^{12}$/L and $99.33 \times 10^{9}$/L vs. $97.78 \times 10^{9}$/L).

The observed reduction in platelet count likely reflects platelet sequestration within the forming clot and/or adhesion to the chitosan surface. Because platelet activation was not directly measured, we cannot distinguish activated platelets from mechanically trapped platelets in this assay. Future studies should include platelet activation endpoints such as surface P-selectin (CD62P) expression by flow cytometry/ELISA, â-thromboglobulin or PF4 release, or platelet morphology/pseudopodia formation on SEM to clarify whether EHS triggers the biochemical platelet phase of hemostasis.

Pogorielov found no significant differences in RBC and platelet concentrations between control blood and blood exposed to chitosan-based gauze [9]. Li J et al reported 30% RBC and 8.5% platelet absorption using chitosan microspheres in rat plasma, lower than our results, possibly due to differences in samples and product types [15]. An ideal hemostatic sponge should rapidly absorb plasma while concentrating RBCs at the wound to promote clot formation. The high porosity of EHS enables quick absorption of large amounts of RBCs, facilitating aggregation and platelet activation, making it a promising hemostatic material.

## Whole blood clotting kinetics and clotting blood time (CBT)

*Clotting kinetics (measured via red blood cell absorbance)*
Red blood cells (RBCs) are a major plasma component and play a central role in blood clot formation. Whole blood clotting kinetics refers to the rate and stages of clot formation, maturation, and dissolution in whole blood - key to evaluating the blood cell aggregation capacity of a hemostatic material. In this assay, absorbance values reflect the concentration of free RBCs in whole blood; thus, an inverse correlation exists between absorbance and clotting rate. Lower absorbance at given time points indicates higher RBC entrapment by the material and faster clotting [16].

In our study, hemostatic activity of EHS was assessed by measuring absorbance of lysed free RBCs at 540/nm at 5, 10, and 15 minutes after clot initiation, comparing EHS to the reference Axiostat and to blood-only controls. The results (table 3) demonstrate a significant decline in absorbance for EHS: at 10 and 15 minutes, absorbance values dropped to 0.031 and 0.026 from 0.622 at 5 minutes, substantially lower than the control's 0.973, 0.737, and 0.654 at corresponding times. Differences were statistically significant. EHS's reduction pattern closely mirrors that of Axiostat (0.622; 0.031; 0.024). Between 10 and 15 minutes, both sponges showed stable low absorbance, implying that RBCs were efficiently captured and clot continued to grow again, like Axiostat. Overall, EHS produced larger clots compared to control at all time points ($p<0.001$). The residual RBC absorbance is a quantitative indicator of whole blood hemostatic potential [15].

In comparison, absorbance values in other studies were higher: TOCN/gelatin sponge reduced absorbance from 0.96 (5/min) to 0.21 (50/min) (Li et al., 2017); another chitosan microsphere system (CSMS) exhibited similar trends (Wu et al., 2020); a chitosan-based hydrogel (CS/SS/Ag@MOF GO) recorded absorbance decline over 5, 10, 15 minutes (2.6; 0.4; 0.1) [10]. Our EHS achieved much lower absorbance values, likely due to its porous sponge form providing more effective RBC entrapment and faster clot formation.

*Clotting blood time (CBT)*
Clotting Blood Time (CBT) is defined as the moment when stable, non-flowing clots form in the test tubes - an effective measure of hemostatic material performance. EHS significantly shortened CBT compared to control (213.2/s vs. 535.3/s; p/</0.001) and matched Axiostat (214.9/s). For comparison, other materials showed varying CBTs: CS/SS/Ag@MOF GO hydrogel formed clots in 160/s [10]; chitosan-based foam SHCF in rat blood clotted in 1200/s [14]; commercial Celox in ~347/s [17]; CSMS in 118/s [16]. These results indicate that EHS promotes rapid clotting and has hemostatic efficacy comparable to commercial Axiostat.

## Evaluation of plasma coagulation parameters

Plasma coagulation represents a critical phase in hemostasis. We investigated the effects of EHS on plasma clotting to better understand its hemostatic mechanism. The cascade includes intrinsic, extrinsic, and common pathways - with a shared activation point of factor X to Xa, leading to prothrombin conversion to thrombin [18]. We measured the following key parameters in rabbit platelet-poor plasma (PPP) after contact with EHS (at 10, 20, and 30 minutes): Prothrombin Time (PT), assessing the extrinsic coagulation pathway; Activated Partial Thromboplastin Time (APTT), evaluating intrinsic pathway functionality [10]; Fibrinogen concentration, critical in the final common pathway of clot formation. We compared results for EHS against a reference sponge (Axiostat) and a control group with no material.

PT: EHS samples exhibited slightly lower PT than controls at all time points (13.83/s; 13.80/s; 13.80/s vs. 14.97/s; 14.93/s; 14.93/s), and were comparable to Axiostat (13.87/s; 13.83/s; 13.80/s); differences were not statistically significant (p/>/0.05).

APTT: EHS significantly shortened APTT compared to controls across all time points (24.1/ s; 23.03/s; 22.95/s vs. 33.57/s; 33.33/s; 33.1/s); values were similar to Axiostat (23.1/s; 22.33/s; 22.21/s); differences between time points were not significant ($p>0.05$), but overall, APTT reduction was significant ($p<0.01$).

Fibrinogen: Levels were slightly lower in EHS samples compared to controls and statistically similar to Axiostat at all time points ($p>0.05$).

EHS modestly reduced PT and fibrinogen levels compared to controls (though not significantly), while significantly lowering APTT. This indicates that EHS primarily enhances coagulation through the intrinsic pathway rather than the conventional extrinsic or common pathways.

This aligns with prior findings: Chitosan–CaCO sponges accelerated APTT more than PT when compared to control, indicating enhanced intrinsic pathway activation [19]; Chitosan fibers in PPP prolonged APTT significantly, suggesting a modulating effect on intrinsic factors [20]; Modified chitosan-containing materials (e.g., CTS–NF, CS/SS) demonstrated significant reductions across PT, APTT, and fibrinogen-suggesting activation of both intrinsic and extrinsic pathways [8,10]; yet, other formulations (e.g., PCS65, CS/SS) showed no significant effect on these indicators [13]; In some composites (e.g., PEC10, CMS), intrinsic and extrinsic pathways were both accelerated—though chitosan incorporation did not further alter the results [21]. These discrepancies highlight the influence of material formulation, chitosan concentration, and incubation time on coagulation outcomes. Such, EHS effectively activates the intrinsic coagulation pathway (as evidenced by reduced APTT), while also promoting RBC aggregation and rapid clot formation in vitro—demonstrating strong hemostatic potential.

## Conclusion

EHS demonstrated good hemostatic potential under in vitro conditions. The material promoted red blood cell adhesion (as observed via SEM imaging), facilitated rapid clot formation (with the absorbance of free red blood cells significantly decreasing from 0.622 to 0.031 after 15 minutes), and shortened whole blood clotting time (213.2 seconds compared to 535.3 seconds in the control group). Moreover, EHS significantly reduced APTT values at 10, 20, and 30 minutes, indicating activation of the intrinsic coagulation pathway (24.1s; 23.03s; 22.95s compared to 33.57s; 33.33s; 33.1s in the control group). Its mechanism of action may involve physical adsorption, electrostatic interactions, and biological activation, suggesting strong potential for widespread application in hemostatic medicine and surgical procedures.

## References


1. Brown KGM, Solomon MJ. Topical haemostatic agents in surgery. Br J Surg, 111(1), (2024).
2. Berry C, Gallagher JM, Goodloe JM, Dorlac WC, Dodd J, Fischer PE. Prehospital Hemorrhage Control and Treatment by Clinicians: A Joint Position Statement. Prehosp Emerg Care, 27(5), 544-551, (2023).
3. Nepal A, Tran HDN, Nguyen NT, Ta HT. Advances in haemostatic sponges: Characteristics and the underlying mechanisms for rapid haemostasis. Bioact Mater, 27, 231-256, (2023).
4. Guo Y, Wang M, Liu Q, Liu G, Wang S, Li J. Recent advances in the medical applications of hemostatic materials. Theranostics, 13(1), 161-196, (2023).
5. Cassano R, Perri P, Scarcello E, Piro P, Sole R, Curcio F, et al. Chitosan Hemostatic Dressings: Properties and Surgical Applications. Polymers (Basel), 16(13), (2024).
6. Hu Z, Lu S, Cheng Y, Kong S, Li S, Li C, et al. Investigation of the Effects of Molecular Parameters on the Hemostatic Properties of Chitosan. Molecules, 23(12), (2018).
7. Hartmann J, Hermelin D, Levy JH. Viscoelastic testing: an illustrated review of technology and clinical applications. Res Pract Thromb Haemost, 7(1), 100031, (2023).
8. Cheng Y, Yang Q, Wang J, Hu Z, Li C, Zhong S, et al. Optimization Preparation and Evaluation of Chitosan Grafted Norfloxacin as a Hemostatic Sponge. Polymers (Basel), 15(3), (2023).
9. Pogorielov M, Kalinkevich O, Deineka V, Garbuzova V, Solodovnik A, Kalinkevich A, et al. Haemostatic chitosan coated gauze: in vitro interaction with human blood and in-vivo effectiveness. Biomater Res, 19, 22, (2015).
10. Zhang M, Wang D, Ji N, Lee S, Wang G, Zheng Y, et al. Bioinspired Design of Sericin/Chitosan/Ag@MOF/GO Hydrogels for Efficiently Combating Resistant Bacteria, Rapid Hemostasis, and Wound Healing. Polymers (Basel), 13(16), (2021).
11. Sun L, Li B, Song W, Zhang K, Fan Y, Hou H. Comprehensive assessment of Nile tilapia skin collagen sponges as hemostatic dressings. Mater Sci Eng C Mater Biol Appl, 109, 110532, (2020).
12. Weisel JW, Litvinov RI. Red blood cells: the forgotten player in hemostasis and thrombosis. J Thromb Haemost, 17(2), 271-282, (2019).
13. Huang Z, Zhang D, Tong L, Gao F, Zhang S, Wang X, et al. Protonated-chitosan sponge with procoagulation activity for hemostasis in coagulopathy. Bioact Mater, 41, 174-192, (2024).
14. Chen R, Du F, Yuan Q. Multifunctional Sodium Hyaluronate/Chitosan Foam Used as an Absorbable Hemostatic Material. Bioengineering (Basel), 10(7), (2023).
15. Li J, Wu X, Wu Y, Tang Z, Sun X, Pan M, et al. Porous chitosan microspheres for application as quick in vitro and in vivo hemostat. Mater Sci Eng C Mater Biol Appl, 77, 411-419, (2017).
16. Pan M, Tang Z, Tu J, Wang Z, Chen Q, Xiao R, et al. Porous chitosan microspheres containing zinc ion for enhanced thrombosis and hemostasis. Materials Science and Engineering: C, 85, 27-36, (2018).
17. Zhang J, Chen Z, Zeng D, Xia Y, Fan Y, Zhang X, et al. Antibacterial and rapidly absorbable hemostatic sponge by aldehyde modification of natural polysaccharide. Communications Materials, 5(1), 129, (2024).
18. Chen Z, Yao X, Liu L, Guan J, Liu M, Li Z, et al. Blood coagulation evaluation of N-alkylated chitosan. Carbohydr Polym, 173, 259-268, (2017).
19. He W, Huang X, Zhang J, Zhu Y, Liu Y, Liu B, et al. CaCO3–Chitosan Composites Granules for Instant Hemostasis and Wound Healing. 14(12), 3350, (2021).
20. Wang YW, Liu CC, Cherng JH, Lin CS, Chang SJ, Hong ZJ, et al. Biological Effects of Chitosan-Based Dressing on Hemostasis Mechanism. Polymers (Basel), 11(11), (2019).
21. Chen X, Yan Y, Li H, Wang X, Tang S, Li Q, et al. Evaluation of absorbable hemostatic agents of polyelectrolyte complexes using carboxymethyl starch and chitosan oligosaccharide both in vitro and in vivo. Biomater Sci, 6, 3332–44, (2018).