Chitosan–Kaolin Infused Foam Dressing for Rapid Hemostasis and Enhanced Blood Absorption

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Abstract

Uncontrolled hemorrhage is a leading cause of early fatalities after traumatic injuries. Hemostats have been developed to control bleeding effectively by various mechanisms like activation of intrinsic pathways, positive charge-mediated platelet activation, fibrinogen interaction, coagulation cascade acceleration, clotting factor initiation, and providing a surface for platelet adhesion. It was proposed that a foam-based hemostat involving actives working on multiple mechanisms would yield better hemostatic activity. Chitosan and kaolin incorporated in polyurethane foam (CKD) was evaluated for *in vitro* and *in vivo* hemostatic activity. Initially, various levels of chitosan and kaolin were screened for parameters like foam degradation and *in vivo* hemostasis. The levels were further optimized for hemostatic activity using *in vitro* and *in vivo* models. CKD showed approximately 12 times more blood absorption as compared to control. Invitro blood clotting time for CKD (2.39-2.54 mins) was found to be much lesser as compared to control (7.26 mins). Scanning electron microscopy studies confirmed clot formation inside the CKD. Dynamic coagulation test showed rapid

coagulation of blood under the influence of CKD as revealed by absorbance measured at 540nm. *In vivo* hemostasis was evaluated by rat femoral artery hemorrhage model and showed completed stoppage of blood within 1.43 to 2.40 mins.

Keywords: hemostats; chitosan; kaolin; polyurethane foam; blood absorption; dynamic blood coagulation

1. Introduction

Uncontrolled hemorrhage forms a major cause of early mortalities after traumatic injuries[1]. In cases of severe trauma, the body's innate hemostatic mechanisms may be overwhelmed, leading to uncontrolled bleeding and increased mortality rates. Hemostats have evolved in the last few decades to enhance the hemostatic process. Hemostats are substances or devices that promote and accelerate clot formation, thereby aiding in the control of bleeding[2,3]. In situations like warfronts and accidents where immediate medical care may be limited, the use of hemostats becomes crucial. They can provide temporary measures to control bleeding and provide precious time until subsequent medical treatment becomes available. Hemostats are designed to be rapidly deployable, easy to use, and effective in a variety of wound types and locations[4,5]. Topical hemostats are directly applied to the site of bleeding and work locally to promote clotting. These agents may include hemostatic dressings, sponges, powders, or gels that contain substances such as thrombin[6,7], fibrinogen, collagen, or chitosan[8]. Thrombin converts fibrinogen to fibrin, which forms the structural framework of a blood clot. Earlier reported works include a fibrin glue[9] and fibrin sealant patches like Evicel, TachoSil and Tissel[10–13]. Chitosan promotes clot formation by interacting with blood components like platelets and erythrocytes. It activates the intrinsic pathway of the clotting cascade, leading to the production of fibrin, which helps in the formation of stable blood clots[14,15]. Chitosan's positive charge attracts negatively charged red blood cells and

platelets, facilitating their aggregation and clot formation at the wound site[16,17]. It is reported that its efficacy in controlling bleeding and initiating hemostasis is not consistently reliable. Therefore, there is a crucial need to refine chitosan-based dressings to enhance their effectiveness in managing hemorrhage and combat-related emergency situations[18]. Another class of hemostatic agents activates the intrinsic pathway of coagulation and concentrates the clotting factors at the site of bleeding by rapidly absorbing the water content of the blood. Kaolin is an example of this class[19,20].The combination of chitosan with kaolin in a hemostatic dressing system can improve the overall hemostatic efficiency compared to a chitosan-based dressing system alone. This combination can promote hemostasis by enhancing absorption, promoting clot formation, offering better stability and can offer versatility of usage. Incorporating chitosan and kaolin into foam-type dressing systems can increase efficiency and offer an effective means of application on the site of hemorrhage. This work was based on a hypothesis that a foam based dressing system infused with chitosan and kaolin can work as an effective hemostat.

2. Materials and methods

2.1 Materials

Chitosan was purchased from Analab Fine Chemicals (Mumbai, India). Kaolin, calcium chloride, sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). All solvents and chemicals used were of analytical grade. Blood samples were obtained from Sasoon blood bank, Pune.

2.2 Foam degradation test

To assess the degree of degradation, the initial dry samples of the dressing were weighed and recorded as W_0. The samples were then fully suspended in 7 ml of phosphate-buffered saline (pH 7.4) in a 6-well plate. The plates were placed in an incubator set at a temperature of 37°C. The buffer solution was changed daily, ensuring a fresh environment for the samples. After a 48-hour incubation period, the samples were carefully removed from the solution and washed with distilled water to remove any residual buffer solution. The washed samples were dried at a temperature of 40°C for an additional 48 hours to remove any moisture. This ensured that the samples reached their final dry state. Finally, the dry samples were weighed

again and recorded as W_1, representing the final dry weight after degradation. By comparing the initial dry weight (W_0) with the final dry weight (W_1), the degree of degradation of the dressing can be quantified as follows[21–24].

$$\text{Percent degradation} = \frac{(W0 - W1)100}{W0}$$

2.3 Preparation of chitosan-kaolin dispersion and loading on PUF

Polyurethane foam pieces of size 1 square inch were initially sterilized using ethylene oxide and further processes were carried out in aseptic conditions. Kaolin powder was gradually added to purified water while being heated and stirred at 65°C to form a milky white solution. Chitosan was dissolved in a 0.1M acetic acid solution under continuous heating and stirring. Kaolin dispersion was gradually added to the chitosan solution. 0.1M sodium hydroxide solution was added to neutralize the excess acid. This process resulted in the formation of a milky white dispersion with water-like fluidity and a minor sticky texture. Pieces of PUF were then immersed in the mixture and allowed to dry for 24-48 hours in a desiccator[25]. The composition of trial batches is shown in Table 1 and the hemostatic dressings are shown in Fig 1.

Based on physical examination and organoleptic properties 3 batches were selected for the next studies. During the assessment, specific problems related to flattening, distortion and other visual irregularities were taken into consideration. Foam dressings that exhibited issues such as non-uniform coating, powder shading and twisting of the corners were excluded from further studies. Three batches were selected and evaluated for *in vivo* hemostasis activity as described below.

2.4 *In vivo* hemostatic activity

Twenty-four wistar rats weighing in the range of 180–200 gm were used in this study. The experimental protocol was approved by the Institutional Animal Ethics Committee of AISSMS College of Pharmacy (Approval no. CPCSEA/IAEC/PT-18/02-2K22). The rats were divided into four groups, with twelve animals in each group. Ketamine (10 mg per 1 kg of weight) was administered to anesthetize the animals. The medial surface of the thigh was shaved and the superficial femoral artery was chosen as the access point for each

animal[26–30]. Using a surgical scalpel, the incision was done halfway across its diameter (Fig.2). In all groups, standard manual compression (SMC) was applied to control bleeding[31,32]. The second, third and fourth groups were treated with CKD along with SMC, while the first group served as the control. In all groups, SMC was provided for 60 seconds and if bleeding continued, an additional 10 seconds of SMC was applied and continued if hemorrhage persisted. Hemorrhage was considered as uncontrolled if hemostasis was not achieved at the end of 10 minutes of treatment. Once hemostasis occurred, the time required for the standard PUF and the CKD to demonstrate their activity was noted[33–36].

2.5 Experimental Design

From the outcomes of the evaluation of preliminary batches, one batch was selected with minimum hemostatic time. Considering this batch as reference point 9 experimental batches were prepared using 3^2 full factorial design. The composition of the batches as per the design is mentioned in Table 2

2.6 Evaluation of absorption rate

To evaluate the rate of absorption, drops of a standard solution containing 2.298g sodium chloride and 0.368g calcium chloride dihydrate dissolved in 1 liter of de-ionized water were poured using a dropper onto the wound contact layer surface of each dressing. The absorption process was allowed to reach completion and the duration of absorption was measured in seconds. A total of twenty drops were instilled onto each dressing and the mean absorption time was calculated[37,38].

2.7 Whole blood absorption

The PUF and CKD were immersed in whole blood for 10 minutes. The blood absorption per unit surface area was calculated by determining the weight differences before and after blood absorption.

$$\text{Blood Absorption} = \frac{(W2 - W1)}{A}$$

Where A= Area of dressing system in square cm, W1= initial weight, W2= final weight (after blood absorption) and repeated 10 times and the mean value was considered[39–42].

2.8 *In vitro* blood clotting

A series of 3 test tubes were set up, each containing 10 ml of anticoagulant containing whole blood obtained from blood bank and the sample material with dimension of 0.25 cm^2. The test tubes were then placed in a water bath at 37°C for 10 minutes to allow for equilibration. After the equilibration period, 2 ml of 0.2M Calcium chloride solution was added to each test tube for recalcification of the blood. This step was repeated for all the test tubes. The test tubes containing the recalcified whole blood were then placed back into the 37°C water bath. At 10-second intervals, the first test tube of each material was removed and the flow of blood at an angle was observed. The remaining test tubes were kept in the water bath for observation. This process continued removing test tubes at 10-second intervals until the blood in all test tubes was completely coagulated. At that point, the timing was stopped and the overall coagulation time for each material was recorded. If the coagulation time exceeded 30 minutes, the material was recorded as non-coagulated[43–45].

2.9 Dynamic blood coagulation

The CKD and PUF were cut into pieces with an area of 1 cm^2. Seven pieces of each material were placed in individual clean petri dishes. Seven-time points were established, with duration of 5 minutes between each neighbouring point. 200 µl of whole blood was carefully added onto the surface of each material in every petri dish. After the blood was applied, the timer was started and 100 ml of distilled water was added to a designated petri dish every 5 minutes. This step was repeated at each time point. Following each addition of purified water, the sample was allowed to stand for 5 minutes. Subsequently, a spectrophotometer set to a wavelength of 540 nm was used to measure the optical density values of free hemoglobin present in the leaching solution at various time points[46–49].

2.10 *In vivo* hemostatic activity

The *in vivo* hemostatic activity for experimental batches was done by the same procedure as described earlier for *in vivo* study of trial batches. Fifty-four Wistar rats divided into nine groups were used in this study. The time required for the chitosan-kaolin dressing (CKD) to completely stop the bleeding was recorded[50].

2.11 Scanning Electron Microscopy (SEM)

The CKD was prepared for SEM analysis. The FEI Nova NanoSEM 450 instrument was used. SEM images of the dressing's coated surface were captured at a magnification of 250 times to assess the coating and effective adhesion of the hemostatic agents. The second image was captured after the dressing's *in vivo* hemostatic activity to evaluate the formation of blood clots on the dressing's surface under real-life conditions. The SEM analysis procedure allowed for the investigation of the dressing's efficacy as a hemostatic agent[51–54].

3. RESULT AND DISCUSSION

3.1 Foam degradation studies

The purpose of the test was to evaluate how a foam dressing material holds up when exposed to a phosphate buffer solution at a pH of 7.4 for 48 hours, simulating body fluid conditions. The results, shown in Table 3, indicate that the PUF exhibited only a very slight weight loss of approximately 0.56% during the degradation test. This minor degradation suggests that the foam material displayed a high level of resistance to deterioration under the specified test conditions. The significance of these findings lies in the foam's ability to maintain its structural integrity and physical properties even after exposure to potentially degrading factors. This resilience makes PUF a promising choice for practical applications, where stability and durability are critical factors. In practical terms, this means that the dressing remains structurally intact and retains its properties even when exposed to conditions simulating body fluids over a 48-hour incubation period. Such data is crucial in assessing the performance and reliability of the dressing for its intended medical application, such as wound management[55,56].

3.2 *In vivo* hemostatic activity

For the pre-formulation *in vivo* studies, three batches, namely B1, B2 and B4, were chosen based on a visual inspection of the dressing system (Fig.3). Among the selected batches, batch B4, having the composition of 3gm chitosan and 1.5gm kaolin, required the lowest time for hemostatic activity during the *in vivo* study. The time required for hemostatic activity is mentioned in table no. 4.

3.3 Rate of absorption

The observed time required for absorption of standard solution of sodium chloride and calcium chloride ranged between 37 to 56 seconds, indicating variations in the dressing material's absorption rate within this time frame. The results for nine different batches (F1 to F9) are summarized in Table 5. Certain batches (F1, F4, and F5) exhibited longer mean absorption times with relatively smaller variations, suggesting higher consistency and precision in their absorption rates. Conversely, some batches (F6, F8, and F9) showed shorter mean absorption times with low variation, indicating consistent and precise absorption rates. One batch (F2) displayed a mean absorption time of 38 seconds. The observed variations in absorption rates among the batches could be attributed to factors such as dressing material thickness, porosity, and surface characteristics. The presence of different additives or processing methods might also influence the dressing's ability to absorb fluids effectively. Understanding the rate of absorption is crucial in wound management. Dressings with faster absorption rates may be preferred for injuries with higher hemorrhage. Conversely, dressings with slower absorption rates may be more suitable for injuries with minimal hemorrhage[57–60].

3.4 Whole blood absorption

The results showed that all formulated CKD dressings (F1 to F9) demonstrated higher blood absorption compared to the PU foam dressing (Control) (Fig.4). The CKD dressings exhibited a blood absorption range of approximately 0.78 gm/cm^2 to 2.04 gm/cm^2, while the PUF dressing had a significantly lower blood absorption rate of 0.16 gm/cm^2 (Table 6). Batch F3 of the CKD dressing stood out as it absorbed approximately 12 times more blood than the PU foam dressing after 30 minutes of immersion in the blood (Fig.5). This significant difference suggests that the CKD formulation possesses superior absorbent properties, making it a promising candidate for advanced hemostatic applications where higher blood absorption is required. The enhanced blood absorption capacity of CKD can be attributed to its specific formulation and material properties. The structure and surface properties of CKD may facilitate rapid and efficient blood uptake, thus making it a more effective option for managing injuries with significant hemorrhage[61–63].

3.5 *In vitro* blood clotting

The results indicated that CKD consistently induced faster blood clotting across all tested variations. The coagulation times for CKD ranged from 2 minutes 39 seconds to 2 minutes 54 seconds. In contrast, the PUF exhibited a significantly longer coagulation time of approximately 7 minutes and 26 seconds. The relatively short coagulation time observed for CKD indicates its potential for hemostatic dressings, where rapid clot formation is essential in controlling bleeding and promoting wound healing (Fig.6). Table no.7 presents the coagulation times for each batch of CKD and the control (PUF dressing). The control's significantly longer coagulation time serves as a reference to highlight CKD's superior coagulation-promoting properties.

3.6 Dynamic blood coagulation

The resulting absorbance values were recorded for both the control dressing and the CKD at each time point and are presented in Table 8. The results of the experiment provided valuable insights into the coagulation effects of the two dressings. At the 5-minute mark, the CKD demonstrated a significantly lower absorbance value (0.832 ± 0.05) compared to the control dressing (1.212 ± 0.05). This indicates that the CKD exhibited a more efficient and rapid initiation of the coagulation process within the initial 5 minutes. This finding is particularly noteworthy as it suggests that CKD possesses inherent properties that actively promote clotting, possibly attributed to the presence of chitosan and kaolin components.

As the experiment progressed, at the 10-minute mark, the CKD (0.648 ± 0.05) and the PUF (1.081 ± 0.05) showed a significant decrease in absorbance compared to the initial time point. This observation indicates that both dressings successfully initiated coagulation and triggered the natural clotting processes within the first 10 minutes (Fig.7). The decrease in absorbance over time suggests that the clotting mechanisms inherent to whole blood were engaged after the initial coagulation induced by the dressings. A crucial aspect of the study was to evaluate the coagulation rates of the materials. Comparative analysis revealed that CKD displayed rapid rate of coagulation compared to PUF. Throughout the dynamic coagulation process, the absorbance values for the CKD declined more rapidly, indicating a more efficient clotting performance than the PUF which served as control.

The dynamic coagulation study of the CKD further highlighted its superior coagulation performance. The CKD demonstrated the most substantial decline in absorbance and the steepest slope compared to the PUF, suggesting that the CKD dressing maintained a higher rate of clotting over time. In contrast, the PUF dressing displayed a relatively smaller decline in absorbance, indicating comparatively weaker coagulation properties. While the PUF may still facilitate clotting, its performance appears to be less effective compared to CKD, especially during the initial stages of the coagulation process. The results of this experiment underscore the favorable coagulation properties of the CKD compared to the PUF. The CKD exhibited a more efficient and rapid initiation of the coagulation process, a higher coagulation rate, and sustained effective clotting over time. These findings suggest that the CKD dressing holds significant promise for wound healing applications where swift and efficient clotting is of paramount importance for patient recovery.

3.7 *In vivo* hemostatic activity

The CKD dressing system demonstrated significant effectiveness in reducing bleeding time compared to the control group without any dressing (Table 9). These findings confirm CKD's hemostatic properties and its ability to promote clot formation and control bleeding effectively. Batch F7, among the CKD batches tested exhibited the shortest hemostatic time, indicating superior hemostatic activity compared to others (Fig 8, 9 and 10). This suggests that specific modifications or changes in the CKD formulation, possibly including an increased concentration of kaolin, contributed to the enhanced hemostatic effectiveness of batch F7. The positive outcomes observed with CKD dressings, along with SMC, support the clinical use of Chitosan-based and Polyurethane foam dressings in scenarios where prompt bleeding control is crucial. The observed variations in hemostatic activity among CKD batches may be linked to differences in chitosan properties and changes in kaolin concentration, which can influence chitosan's interactions with blood components and clotting mechanisms, affecting the dressing's hemostatic efficacy. Although the study successfully demonstrated the effectiveness of CKD in the controlled bleeding model in rats, further studies and clinical trials are required to validate these results and explore the applicability of these dressings in managing bleeding in human patients. The controlled bleeding model in rats provided a valuable preclinical evaluation, but human physiology and

wound characteristics may introduce additional complexities that need consideration in clinical settings[64].

3.8 Scanning electron microscopy (SEM)

The FEI Nova NanoSEM 450 instrument was used to analyze a CKD. SEM images of the dressing's coated surface were captured at a magnification of 250 times. Fig.11 (A) shows the SEM image of the CKD before *in vivo* hemostatic activity, while Fig.11 (B) displays the SEM image of the CKD after *in vivo* hemostatic activity, showcasing clot formation. The SEM analysis results revealed proper coating and effective adhesion of the hemostatic agents, chitosan, and kaolin, on the foam dressing's surface (Fig.11 A). The images displayed a well-distributed and tightly adhered layer of chitosan and kaolin, indicating uniform coverage across the dressing. This suggests that the dressing's surface was adequately coated with the hemostatic agents, which is crucial for achieving efficient hemostasis and controlling bleeding effectively. Fig.11 (B) demonstrates the *in vivo* SEM image, which showed the formation of blood clots on the CKD's surface. This provides concrete evidence of the dressing's effective hemostatic activity under real-life conditions. The presence of blood clots indicates that the chitosan and kaolin in the dressing successfully initiated and supported the clotting process, which is essential for controlling bleeding and promoting wound healing.

4. Conclusion

This research work was focused on the development and evaluation of a novel hemostatic chitosan-kaolin dressing (CKD), designed to address the critical issue of uncontrolled hemorrhage after traumatic injuries. Uncontrolled bleeding remains a leading cause of early mortalities in such cases, necessitating effective hemostatic solutions to improve survival rates. The CKD, demonstrated remarkable hemostatic efficacy through various in vitro and in vivo studies. Its ability to rapidly absorb blood, accelerate in vitro blood clotting, and sustain clotting over time surpassed that of the control (PUF) dressing. Scanning electron microscopy (SEM) analysis confirmed the successful coating and adhesion of chitosan and kaolin on the dressing's surface, providing visual evidence of the CKD's effectiveness in initiating clot formation in real-life conditions. The CKD's potential in emergency medical scenarios, such as combat-related injuries and remote locations with limited access to

immediate medical care, is significant. By offering a rapid and efficient means of controlling bleeding, CKD can provide essential time for subsequent medical interventions, potentially saving lives in critical situations. While the present studies demonstrated promising results, further research and clinical trials can validate CKD's efficacy and safety in managing bleeding in human patients. Additionally, exploring its applicability in various clinical settings will provide valuable insights into its potential impact on reducing mortality rates in traumatic injury cases. The chitosan-kaolin dressing represents a promising advancement in hemostatic solutions, with the potential to revolutionize the management of hemorrhage. Its superior clotting capabilities and ease of use make it a valuable addition to emergency medical care, offering new hope for improved patient outcomes and decreased fatalities in traumatic injuries. Continued research and development of CKD hold the promise of transforming emergency medicine and enhancing the chances of survival for those affected by severe bleeding incidents.

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