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IN-VITRO COMPARATIVE COAGULATION STUDIES OF NOVEL BIODEGRADABLE N, O-CARBOXYMETHYLCHITOSAN (NO-CMC) AND OLIGO-CHITOSAN (O-C)

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Keywords:

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ABSTRACT: Naturally obtained chitosan biopolymers are increasingly being formulated as bioadhesives due to their hemostatic properties. Comparative studies have been conducted to measure the hemostatic capacity of biodegradable 7% N,O-carboxymetylchitosan (NO-CMC) (with 0.45 mL collagen), 8% NO-CMC, oligo-chitosan (O-C) and oligo-chitosan 53 (O-C 53). Biodegradability (loss of mass) of these chitosans in phosphate-buffered saline (PBS) (0-30 days) and lysozyme (0-14 days) was measured. Blood coagulation ability was observed (0-15 minutes). Coagulation profiles were measured using Coagulation Analyzer. Blood morphologies were assessed through scanning electron microscopy (SEM) and light microscopy (Nikon Eclipse E200). Rapid weight loss was observed in PBS for all the chitosans until the 5th day, after which the weight remained constant. NO-CMCs were completely degraded by lysozyme by day 12 ($p<0.05$). Highly deacetylated chitosans (O-C) were observed to degrade slowly. Upon macroscopic observations, NO-CMC promoted erythrocyte aggregation within 7 min. Microscopic analysis showed 7% NO-CMC promoted the most platelet aggregation on its surface, approaching 90% coverage. The current findings contribute substantially to our understanding of all the tested biomaterials that can enzymatically degrade, swell and aggregate erythrocytes and platelets to accelerate hemostasis. Evidence suggests that 7% NO-CMC is the best biodegradable hemostatic agent tested, followed by O-C.

INTRODUCTION: Chitosan is a natural biopolymer extracted from marine arthropods such as crab, shrimp, and lobster contain amino and hydroxyl groups that are useful for chemical modification.

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Chitosan was introduced as an antihemorrhagic biomaterial because it is cationic and insoluble at higher pH, but when it is reversibly sulfated, it becomes anionic and water-soluble properties and initiates blood coagulation[1]. Previously, it has been reported that an increase in chitosan concentration, molecular weight, and degree of deacetylation and that a decrease in temperature will increase chitosan biomaterial viscosity [2]. High viscosity could regulate biodegradation and biological properties in medical interventions in angiogenesis [3].

Thus, chitosan possesses various favorable physiochemical and biological properties that are useful in many fields such as medicine, food processing, agriculture, nutrition, cosmetics, and pharmaceuticals. In recent years, there has been an increasing interest in chitosan biomaterial research in diverse areas due to its non-toxicity, biocompatibility, biodegradability, hypoallergenicity and absorbability [4-6]. Most hemostatic agents approved by the U.S. Food and Drug Administration (FDA) still suffer drawbacks such as being expensive, causing allergic reactions, not being able to stop heavy bleeding, not being biodegradable and requiring specific handling [7].

Hence, developing novel bioadhesives and hemostatic agents has been a continued priority for reducing hemorrhage morbidity and mortality. In our present work, we tested *N, O*-carboxymethyl chitosan (NO-CMC) and oligo-chitosan (O-C), chitosans produced by Standard and Industrial Research Institute of Malaysia (SIRIM Berhad) with a degree of deacetylation of 75-98%. To date, the controversy regarding scientific evidence on chitosan derivatives' being biodegradable, biocompatible and antihemorrhagic has stormed unabated, and it is still under investigation.

These conflicting results have been reported because each chitosan preparation's properties depend on its grade, form, concentration, molecular weight, degree of deacetylation, pH, temperature, incubation period, and viscosity. Therefore, we developed our study to explore the biodegradability, coagulation ability, coagulation factors and morphology of blood cells upon the coagulation of distinguishable forms of chitosan derivatives. One of the conclusions that emerged from our current study is that different formulations of chitosan promote hemostasis to different levels. Our present results produce several notable contributions to enhance our understanding of chitosan derivatives as biodegradable hemostatic agents.

MATERIALS & METHODS:

Materials: Chitosan sponges with variable chitosan formulations (7% NO-CMC with 0.45 mL collagen, 8% NO-CMC, O-C and a powdered variety of chitosan termed O-C 53) were used. Lyostypt® was used as positive control.

Methods:

Degradation Studies:

Degradation in PBS: Chitosan samples weighing 100 mg were immersed in 20 mL of PBS (pH 7.4, 37 ºC) (Gibco, Life Technologies, USA) for the predetermined time (0-30 days) in small petri dishes. Every five days chitosan biomaterials were removed from the solution and left to dry for 10 min. The exact mass of each dried biomaterial was measured using an analytic balance every five days. After being weighed, the chitosan samples were returned to the petri dishes. Medium in each dish was changed once every two days. Weight loss of the chitosan biomaterials was recorded, and the tests were performed in eight replicates [8, 9].

Degradation in Lysozyme: Chitosan samples weighing 100 mg each were immersed in 10 mL of Lysozyme (Chicken Egg White, Calbiochem, Germany), at the concentration of 1 mg/mL in small petri dishes at 37 ºC for 0-14 days. The chitosan biomaterials were taken out of the enzyme solution and left to dry for 10 minutes, and the exact weights of each dried sample were measured using an analytic balance once every two days. After being weighed, the chitosan samples were returned to the petri dishes. The medium in each dish was changed once every two days. Weight loss of the chitosan samples was recorded, and the tests were performed in eight replicates [8].

Blood Coagulation Study: Two milliliters of blood was drawn from the antecubital vein of each of the six healthy donors for a total of 12mL, which was stored in six vials of BD Vacutainer [K2 Ethylenediaminetetraacetic acid (EDTA) 3.6 mg (REF 367842)] tubes. We recruited 6 healthy male and female donor altogether aged 18 to 40 years with written informed consent. Before commencing the study, ethical clearance was obtained from the Human Ethics Committee of Universiti Sains Malaysia (USM).

To prepare Platelet-rich Plasma (PRP) blood was centrifuged at 1200 g for 3 min. Platelet-Poor Plasma (PPP) was withdrawn from the top of the centrifuged tube using a sterile needle, leaving 1 mL of PRP in each tube [10]. Each type of chitosan tested duplicate with blood samples from each donor weighing 100 mg were dissolved in 100 µL of PBS (pH 7.4) and incubated at 37 ºC for 30 min

[11, 12]. After 30 min, 1 mL of whole blood (WB) or PRP was added to each 1.5 mL Eppendorf tube. The time at which blood coagulated was recorded for each chitosan sample, and after 15 minutes, the tests were stopped. The blood clot formed on each sample was photographed by a digital camera (Fujifilm Finepix J150W, Japan).

Coagulation Factors Analysis: We enrolled 10 healthy donors in this analysis upon consent. Ten milliliters of blood was collected in BD Vacutainer 0.109 M (3.2%) trisodium citrate anticoagulant tubes (REF 363083). Blood was kept warm and centrifuged at 3000 *g* for 10 min at 22 °C to obtain Platelet Poor Plasma (PPP). PPP was prepared in accordance with the approved guidelines of the Clinical and Laboratory Standard Institute [13]. PPP was briefly agitated on a vortex mixer. Five hundred milliliters of isolated PPP was combined with 10 mg of prepared chitosans that had pre-absorbed 50 µL PBS and was incubated for 10 min [11, 12]. We measured Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT), Thrombin Time (TT) and Fibrinogen (Fib) on an STA Compact Coagulation Analyzer (Diagnostica Stago, France) according to the manufacturer's instructions. Quality control measurements were set in the proper range according to standard clinical laboratory protocol prior to testing [13-15].

Morphological Analysis: Chitosan samples were pre-moistened with PBS as previously described [11, 12]. Platelets were isolated by differential centrifugation [11, 16]. Five hundred milliliters of isolated platelets was combined with each chitosan sample and incubated for 30 min in 12-well tissue culture plates. Each well was then washed with penicillin-infused PBS for 2 h, fixed in 100 µL of glutaraldehyde for 1 h, and then washed with distilled water. Different concentrations of ethanol (30%, 70%, and 100%) were added to dehydrate the chitosan. Finally, the samples were dried at room temperature and sputter-coated with gold for SEM, in which their surface and cross-section morphology were examined [11, 14, 15]. A drop of whole blood was placed on each slide, and peripheral blood films were prepared. Slides were stained with Geimsa stain for 5 min and washed with PBS (pH 6.8) for 15 min. Slides were mounted, adhered cells were observed using light microscopy (Nikon Eclipse E200), and images were captured using Mirax Desk Zeiss[16, 17].

RESULTS:

Degradation in PBS:

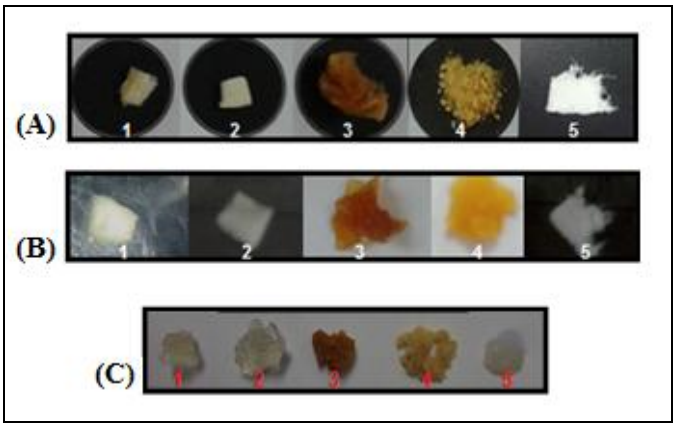

FIG. 1: DEGRADATION TEST IN PBS AT DAY 0 AND DAY 30. (A) DAY 0, BEFORE IMMERSION IN PBS. (B) DAY 0, AFTER IMMERSION IN PBS. (C) DAY 30. [(1) 7% NO-CMC. (2) 8% NO-CMC. (3) O-C. (4) O-C 53. (5) LYOSTYPT]

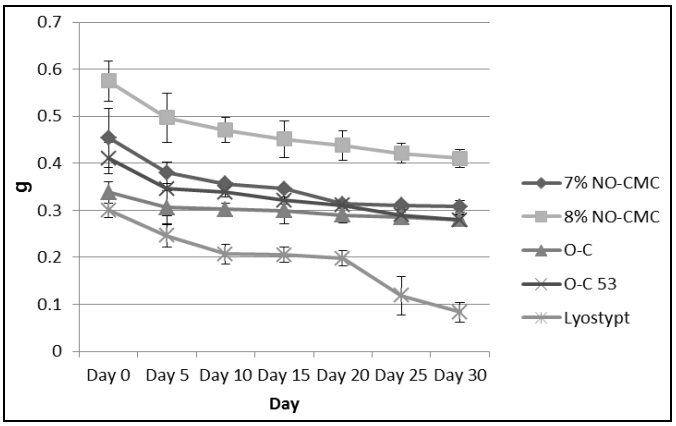

FIG. 2: DEGRADATION LEVELS (MASS) OF EACH BIOMATERIAL TESTED IN PBS AT DAY 0, 5, 15, 20, 25 AND 30. THE DATA ARE PRESENTED AS THE MEAN ± STANDARD DEVIATION.

Drastic weight changes were observed in all tested biomaterials **Fig. 1**. When PBS was added to the plate, chitosan samples swelled to more than triple their original size; the greatest increase observed for 8% NO-CMC, which increased from 0.100 g to 0.575 g. All the examined chitosan samples decreased in weight continuously until day 5 **Fig. 2**. Lyostypt samples were observed to decrease to exactly 72.3% of its original weight from day 0 to 30, followed by 7% NO-CMC with 32.16%, O-C with 32.11%, 8% NO-CMC with 28.52% and O-C 53 with 17.19%. All the NO-CMC and O-C samples remained to their original shapes over the

course of 30 days, meaning that they did not shatter into pieces. NO-CMC samples changed color from light yellow to opaque cream-colored substance. Because the O-C 53 samples were loose particles, we filtered the medium, dried the powdery sponges and returned them to fresh medium for the next evaluation. The weight of the O-C biomaterial decreased considerably more than that of the NO-CMC samples.

Degradation in Lysozyme: The enzymatic degradation of NO-CMC and O-C were compared *in-vitro*. Weight changes of the tested biomaterials are shown in **Fig. 3A-3E**. The 7% and 8% NO-CMC samples were completely degraded by day 12 **Fig. 3A, 3B**. On day 6, all NO-CMC samples appeared to be jelly-like, and no longer retained the original shape (at day 0). For all samples, the lysozyme solution appeared turbid white at day 14, and the volume of the lysozyme solutions had decreased. Media on the NO-CMC samples became turbid once the lysozyme solution was added **Fig. 3**.

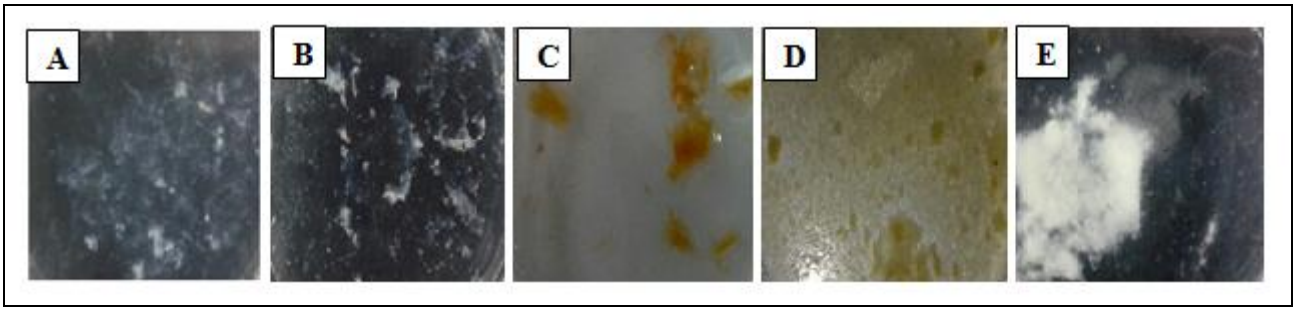

FIG. 3: DEGRADATION TEST IN LYSOZYME AT DAY 14. [(A) 7% NO-CMC. (B) 8% NO-CMC. (C) O-C. (D) O-C 53. (E) LYOSTYPT]

Weight changes in degradation are presented in **Fig. 4**, and these changes elucidate the differences in physical changes among the samples. Drastic weight changes were observed in all NO-CMC samples, as previously discussed. In contrast, the O-C chitosan samples were degraded only 37.8% from their original weight. The O-C samples shattered into pieces lost their hardness and thickness and eventually became delicate. Although the O-C 53 samples were in a powdered form, the rate of degradation of these samples was slow, with only a 17.8% decrease from the initial weight **Fig. 4**.

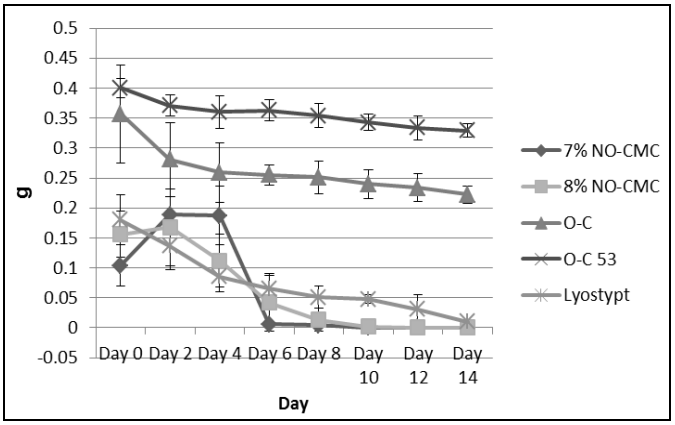

FIG. 4: MASS OF EACH BIOMATERIAL TESTED IN LYSOZYME AT DAY 0-14. THE DATA ARE PRESENTED AS THE MEAN ± STANDARD DEVIATION

Blood Coagulation Study: Fig. 5 shows blood coagulation in the presence of NO-CMC and O-C. We chose the Westergren method in which blood samples were allowed to settle, and blood coagulation was tested by inverting the tubes and observing erythrocyte and platelet agglutinations. All the NO-CMC samples were able to agglutinate fully within 7 minutes **Fig. 5A, 5B**.

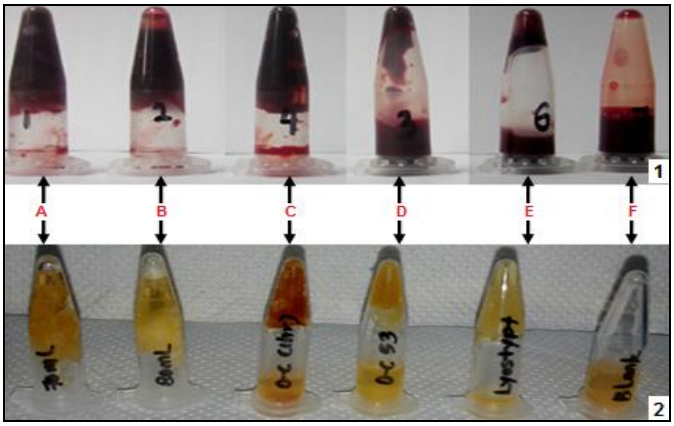

FIG. 5: BLOOD COAGULATION TEST. (1) WB COAGULATION. (2) PRP COAGULATION. [(A) 7% NO-CMC. (B) 8% NO-CMC. (C) O-C. (D) O-C 53. (E) LYOSTYPT. (F) BLOOD OR PRP ALONE.]

The O-C samples fully agglutinated whole blood samples within 10 min, but for PRP, O-C was unable to fully utilize the platelets for coagulation.

Within 15 min, only 75% of the PRP had been utilized, and contrary to our expectations, only O-C 53 was unable to coagulate completely with the whole blood samples **Fig. 5D**. Our findings suggest that the ability of each type of chitosan to coagulate blood varies depending on its weight, thickness, hardness and tensile strength, but overall, NO-CMC and O-C promoted blood coagulation.

Coagulation Profiles: The normal ranges of PT, APTT, TT and Fib in human serum are a 10-21 sec, 30-45.8 sec, 11-21 sec and 14-21 g/dL. In this experiment, only blood alone without any biomaterial adherence showed faster coagulation.

All the chitosan-adhered samples prolonged clotting time but did not show any abnormal coagulation profile ratios. O-C 53, followed by 7% NO-CMC, was the chitosan that extended clotting time. One-way ANOVA followed by posthoc comparisons using Scheffe's test showed that PT differed significantly among the tested groups (p<0.05). Otherwise, no significant differences were found between the tested groups for APTT (p=0.399), TT (p=0.598) and Fib (p=0.848) **Fig. 6**.

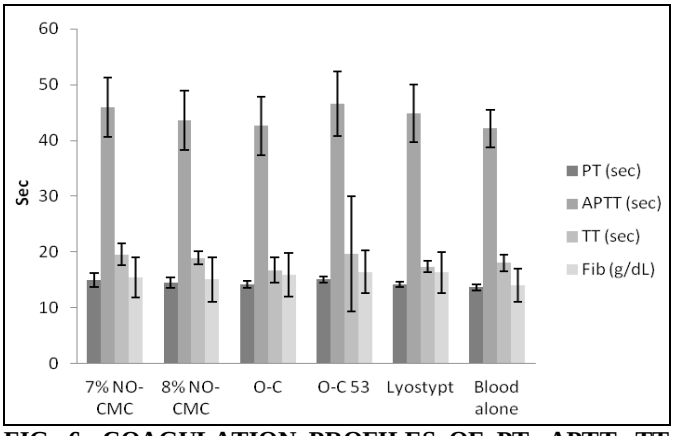

FIG. 6: COAGULATION PROFILES OF PT, APTT, TT AND FIB SHOWING THE MEANS, WITH ERROR BARS REPRESENTING STANDARD DEVIATION. DATA ARE PRESENTED AS THE MEAN ± STANDARD DEVIATION

Morphological Analysis by SEM:

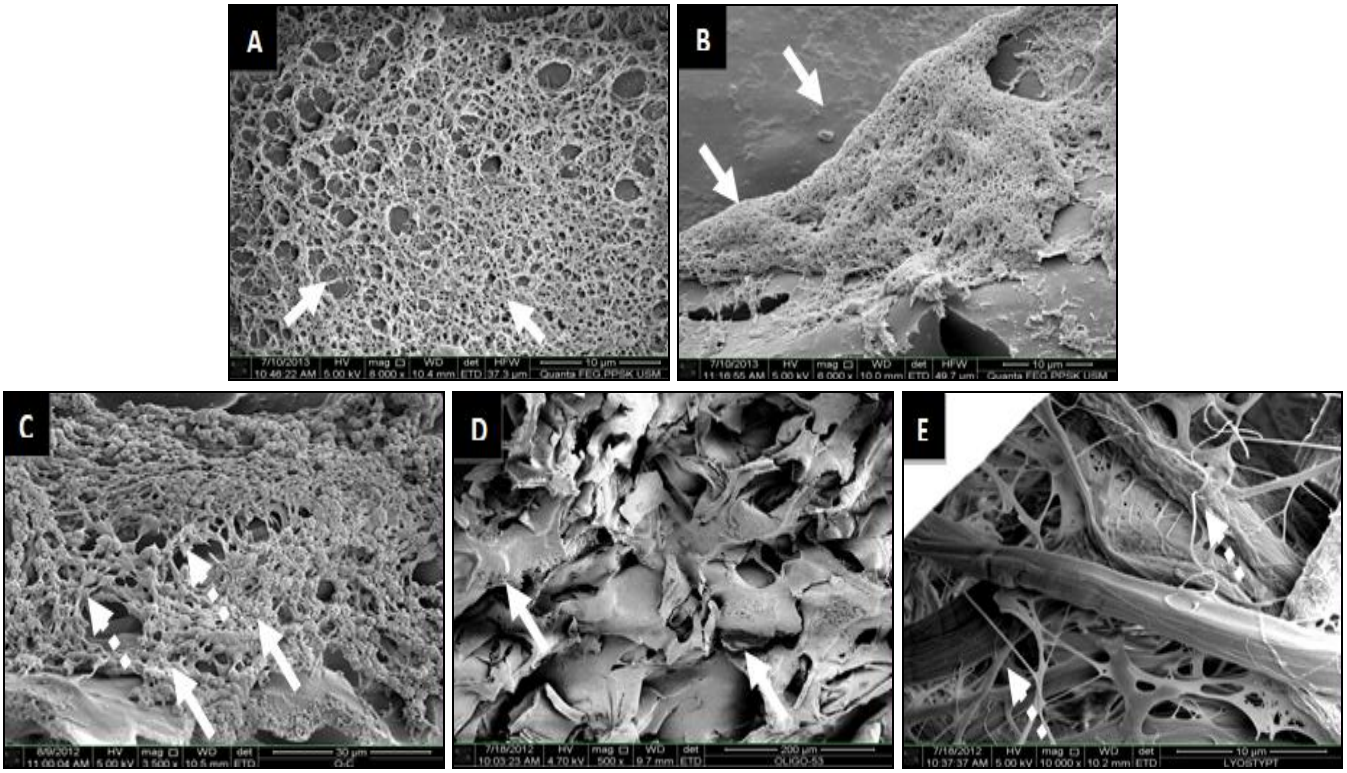

FIG. 7: PLATELET MORPHOLOGY UPON THE ADHERENCES OF NO-CMCS, O-CS, AND LYOSTYPT. [(A) 7% NO-CMC. (B) 8% NO-CMC. (C) O-C. (D) O-C 53. (E) LYOSTYPT], PLATELET SHAPES CHANGED INTO IRREGULAR PSEUDOPODAL UPON AGGREGATION (SOLID WHITE ARROW). FIBRIN MESHES FORMED UPON BLOOD COAGULATION (DASHED WHITE ARROW).

Platelet morphology was observed by SEM. The greatest number of platelet attachments were observed on the tested chitosan biomaterials, as nearly 90% of platelets adhered to the chitosan surface. Most platelets were irregular and pseudopodal in shape. **Fig. 7A-E** shows

observations of these shapes under different magnifications (8000 x, 6000 x, 3500 x, 500 x, 10000 x). These varying shapes are due to the different surface properties of the biomaterials. The contact angle of O-C 53 **Fig. 7D** under 500 x was 200 µM in diameter. Because O-C 53 is a powder, observation at a higher magnification was ineffective because the beam could not readily penetrate the sample to detect platelet contact with the desired crossover diameter and failed to form an acceptable image. Platelets were defective and did not display any granulation. All the aggregated platelets had already discharged their granules, shown in **Fig. 7A-7C**. No single platelets were observed. All the platelets coagulated to form a large group, forming a fibrin clot that reinforced platelet aggregation. Our results were positive compared to those of the Lyostypt, which was made up of highly flexible strands that allowed platelets to form fibrin networks **Fig. 7E**.

Morphological Analysis by Light Microscopy: Erythrocyte morphology was assessed by peripheral blood smear **Fig. 8**. The clearest finding from this study was that erythrocyte agglutinations were observed in all the chitosan-adhered biomaterials. Irregular agglutinations of erythrocytes into grape-like clusters and aggregation of platelet cells were observed in both NO-CMC samples **Fig. 8A, 8B**. Fibrin meshes also formed around the erythrocyte agglutinations **Fig. 8A, 8E**. However, in the presence of both O-Cs, erythrocytes swelled and aggregated vigorously. Platelet aggregation was difficult to observe because erythrocytes covered 80% of the chitosan surface **Fig. 8C, 8D**. Similarly, in the presence of Lyostypt, erythrocytes overlaid maximally, but in this case, the fibrin mesh was clearly visible **Fig. 8E**. In conclusion, our results show that aggregation was stimulated and induced by NO-CMC and O-C.

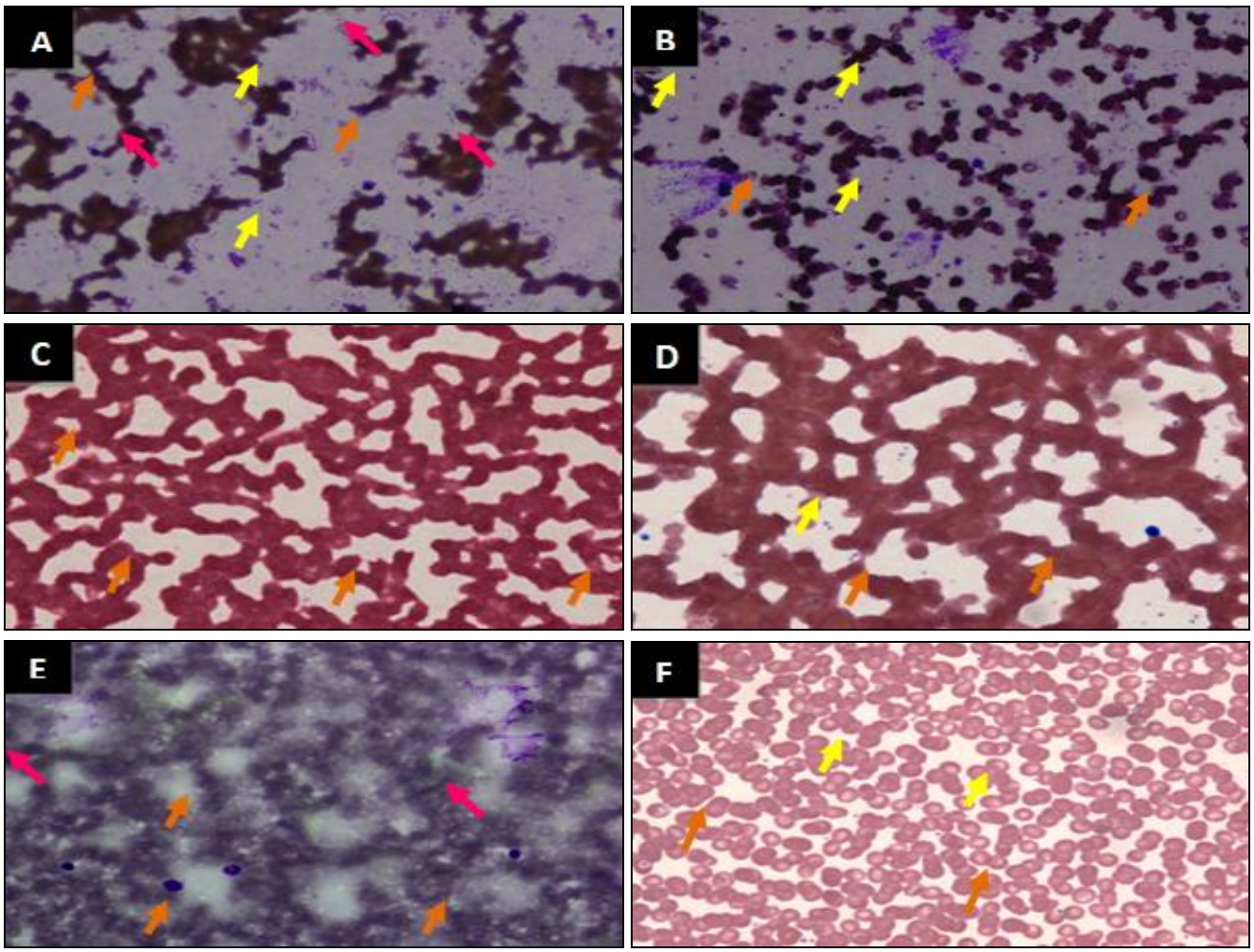

FIG. 8: ERYTHROCYTE MORPHOLOGY. 400X X0.45 MM [(A) 7% NO-CMC. (B) 8% NO-CMC. (C) O-C. (D) O-C 53. (E) LYOSTYPT. (F) BLOOD ALONE]. THE ORANGE ARROW INDICATES ERYTHROCYTE AGGREGATIONS, THE YELLOW ARROW INDICATES PLATELETS AGGREGATIONS AND THE PINK ARROW INDICATES FIBRIN MESHES

Erythrocyte morphology was assessed by peripheral blood smear **Fig. 8**. The clearest finding from this study was that erythrocyte agglutinations were observed in all the chitosan-adhered biomaterials. Irregular agglutinations of erythrocytes into grape-like clusters and aggregation of platelet cells were observed in both NO-CMC samples **Fig. 8A, 8B**. Fibrin meshes also formed around the erythrocyte agglutinations **Fig. 8A, 8E**. However, in the presence of both O-Cs, erythrocytes swelled and aggregated vigorously. Platelet aggregation was difficult to observe because erythrocytes covered 80% of the chitosan surface **Fig. 8C, 8D**. Similarly, in the presence of Lyostypt, erythrocytes overlaid maximally, but in this case, the fibrin mesh was clearly visible **Fig. 8E**. In conclusion, our results show that aggregation was stimulated and induced by NO-CMC and O-C.

DISCUSSION: Prompt action in avoiding morbidity and mortality in response to hemorrhage remains a substantial problem in modern medicine. Therefore, alternative or naturally derived wound dressings have been intensely researched to identify and resolve challenges in treating hemorrhage. Chitosan a natural polysaccharide, biomaterials have been widely studied as a biodegradable hemostatic agent. With the drawbacks and innovations of novel hemostatic agents, we may continue to witness dynamic changes in the future [8]. The aim of our present study was to measure the biodegradability of NO-CMC and O-C and to determine the coagulability, coagulation factor profile, and morphology of blood cells upon adherence of NO-CMC and O-C. We tested biodegradability by measuring the gradual breakdown of biomaterials mediated by the biological activity of PBS and lysozymes. We used PBS because it is a water-based salt solution as the concentrations of this solution, as it is isotonic to that of the human body.

Lysozyme is an enzyme that hydrolyzes *N*-acetylglucosamine-containing polysaccharides on the cell wall. Hydrolysis begins when the polymer loses its crystalline features displays amorphous properties [18]. We discovered that all chitosan samples swell to nearly triple their initial weight upon PBS addition, most likely because chitosan is highly deacetylated and protonated by water molecules. O-C biomaterials failed to degrade but only became brittle and shattered into pieces, suggesting that ionic interactions marginally prevent degradation in PBS. All the examined chitosan biomaterials retained their original shape even after 30 days in PBS. Both NO-CMCs fully degraded in the lysozyme, and the solutions were utilized fully by NO-CMCs for degradation **Fig. 4**. Lysozyme actively degraded the scaffolds rapidly within 12 days because it is capable of substantially reducing chitosan's tensile strength [18]. O-C only shattered into small pieces and, no longer retaining its initial stiffness and eventually becoming soft due to its higher molecular weight (<40,000 Da), degree of deacetylation (95%) and thickness of the prepared samples. We can assure that lysozyme was totally removed before the subsequent weight measurement by drying the biomaterials for two hours in room temperature.

We found that the Westergren method is practical for assessing blood coagulation. Chitosan is a biopolymer known to act on negatively charged, low-molecular-weight plasma proteins to promote aggregation [19]. All the screened chitosan biomaterials in this study accelerated erythrocyte and PRP agglutination. We also found that NO-CMC activated platelets and erythrocytes faster than O-C. O-C 53 in particular only partially aggregated erythrocytes and platelets by extending the coagulation time. The optimal biomaterials weight of 100 mg was what we found, and 7% and 8% NO-CMC was each able to agglutinate 1 mL of erythrocyte or PRP within 7 minutes, consistent with a recent study by Ina Maria in which within 10 minutes of incubation with chitosan-coated films, blood in an inverted test tube became gel-like due to an increased viscosity and the presence of blood clots.

Chitosan likely triggers hemostasis through its ionic affinity [20]. Arand *et al*. reported that erythrocytes attach to one another and capture cells to build artificial clots upon chitosan adherence [21]. Rao and Sharma also reported that platelets exposed to chitosan displayed distinct adhesion to the chitosan surface within 30 to 60 sec [18]. This aggregation response results from the fact that chitosans are made of glucosaminoglycan, which induces cells to adhere and form a hemostatic plug. Chitosan has also been shown to induce platelet

adhesion and aggregation in a time- and concentration-dependent manner [22]. Therefore, we discovered that it is possible for O-C, O-C 53 and Lyostypt to coagulate to the desired degree if the weight of the biomaterials were adjusted depending on their type and thickness.

To complement our blood coagulation test outcomes, we have measured the coagulation profiles of PT, APTT, TT, and Fib, which are commonly measured in blood coagulation profiles to diagnose certain disorders. These tests are sensitive to quantitative and qualitative abnormalities of any of the factors involved in the intrinsic, extrinsic and common pathways of the coagulation system. PT, APTT, TT, and Fib were measured to test the response of intrinsic, extrinsic and fibrin formation pathways in plasma to the presence of chitosan. All of the tested biomaterials promoted coagulation compared to blood alone **Fig. 5**.

However, the findings of our study do not support the erroneous prolongation of PT and APTT levels because we measured whole blood counts before the blood was subjected to further testing. Normal values of TT suggested that no anticoagulants were present in the blood samples. Coagulation profiles were not significantly different among the tested biomaterials, with the exception of PT. Sometimes, PT and APTT do erroneously prolong if the blood samples contain high levels of hematocrit (55%) or are collected in under-filled collection tubes [23], which is why we have considered the hematocrit level in our analysis. The hemostasis induced by chitosan, therefore, did not involve the normal blood coagulation cascade that leads to fibrin formation. In recent research, sulfated chitosan supplied sufficient hemostatic effects to accelerate coagulation [24].

Our present results are consistent with those of other studies such as that by Romani *et al*., who showed that, as measured by coagulation profiles, sugar-modified chitosan did not affect coagulation pathways [25], supporting the idea of chitosan-modified biomaterials' functioning independently without intervening with existing coagulation mechanisms. Moreover, chitosan also sustains cell proliferation and endothelial adhesions [25, 26], which was attributed to the physical interaction between chitosan and the cell membranes of red blood cells. Hemostatic capacity conveyed by chitosan biomaterials enhanced only the agglutination of erythrocytes but did not accelerate the activation of the clotting time in normal coagulation pathways [18]. Future Fib measurement studies on chitosan could lead to prevention of abnormal fibrinolysis in coagulopathy patients. However, our data do not rule out the possible role of higher Fib levels contributing to a hypercoagulable state. The impact of chitosan biomaterials on the coagulation profiles depended on their physical and chemical structure and properties, particularly the amino residues [16]. Malette and Klokkevold also found that chitosan initiated hemostasis independently from platelets and coagulation factors [27-29]. However, introducing carboxyl groups into the *N*- and *O*- positions prolonged APTT and TT more than introducing amino groups did [30]. This fact is very encouraging for our results as well because the biocompatible chitosans we tested contained *N*- and *O*- positions in their structure.

Upon morphological analysis, platelet attachments were detected in most of the chitosan surface. Platelets adhered to one another and extended into pseudopodal forms to aggregate depending on their surface roughness, thickness and absorbability [11]. We observed agglutination of erythrocytes and platelets and formation of a fibrin network in the peripheral blood films study. The hydrophilic surface of NO-CMC promoted platelet adhesion and activation, achieving hemostasis. Recent evidence suggests that NO-CMC significantly accelerates hemostasis *in-vitro* by decreasing the plasma recalcification time (PRT) and also by accelerating fibrin formation compared to controls. Platelets adherence increased and activated on the NO-CMC membrane [31]. Chitosan formed coagulum when adhered to whole blood samples and was capable of agglutinating without lysing the erythrocytes [18, 27, 32]. The data from several sources confirm the finding that chito-oligomers stimulate hyaluronan synthesis, which stimulates cell adherence and proliferation towards morphogenesis, inflammation and wound healing.

CONCLUSION: Novel O-C and NO-CMC were produced with varying molecular weights, degrees of deacetylation, and physical and chemical properties in response to different temperatures and pH levels. The current findings add substantially to

our understanding of the tested biomaterials, which are able to enzymatically degrade, swell and aggregate erythrocytes and platelets to accelerate hemostasis. However, the evidence suggests that 7% NO-CMC is the best biodegradable hemostatic agent tested, followed by O-C. Chitosans that were altered chemically to have different molecular weight and degree of deacetylation promoted hemostasis of blood cells to different degrees. Further investigation and experimentation need to be performed to determine the thickness, tensile strength, morphology and mechanical properties of the chitosan scaffolds. In the future, scaffold improvements may result in chitosan's becoming the most effective naturally obtained biodegradable hemostatic adhesive yet.

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COMPETING INTERESTS: The authors have declared that no competing interest exists.

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