Hemostatic Evaluation of Chitosan Derivatives: Effects on Platelets In Vitro

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1 Introduction

Since the middle of World War II, half of all recorded combat deaths have occurred as a result of exsanguinating hemorrhage. A military post-mortem study of casualties in Operation Iraqi Freedom suggested that up to 24% of all battlefield mortalities could be eliminated with improved anti-hemorrhaging methods and that 85% of deaths were caused by uncontrolled hemorrhage (Kelly et al., 2008). The development of new methods or devices for hemorrhage control may contribute to a future reduction in hemorrhage morbidity and mortality (Pusateri et al., 2003). In many hospital settings, maintaining a good hemostatic balance in bleeding patients remains a major challenge (Shander, 2007). Successful approaches in hemostasis research may contribute to a significant reduction in hemorrhage related fatalities. Among the novel hemostatic agents approved by the U.S. Food and Drug Administration (FDA), chitosan-based agents have shown great promise in preventing major hemorrhaging in pre-hospital settings and in animal models of major blood loss (Kozen et al., 2008). Recently, a chitosan-derived an-
hemorrhage biomaterial, which contains \(N\)-acetyl glucosamine (found abundantly as a major component in the shells of arthropods such as crabs, shrimps, lobsters and insects) (Koide et al., 1998), was identified as having potential clinical utility. Chitosan is well known for its potential as a non-toxic, biocompatible and biodegradable product (Jayakumar et al., 2008; Muzzarelli R.A.A; Muzzarelli C., 2009). Chitosan has the ability to expedite the wound healing process and arrest bleeding by facilitating platelet recruitment and promoting coagulation by forming a pseudo-clot. The chitosan structure can be chemically modified and has been widely employed both as a biomaterial scaffold for the controlled release of pharmaceuticals and as a component of successful wound dressing (Khor & Lim, 2001). The compatibility of chitosan biomaterials is a feature of sample preparation, viscosity, molecular weight (MW), degree of deacetylation (DDA), incubation period and temperature. Platelets circulating within the blood are the essential mediators that trigger the mechanical pathway of the coagulation cascade upon encountering any damage to the blood vessels. Driven by the significant role of adherence in the platelet response during the hemostasis process, we have evaluated platelet responses to characterize platelet capacities upon the adherences of chitosan in vitro. We predict that varying formulations of chitosan-based hemostatic agents may have different potential roles in expediting hemostasis. In our research, we used \(N,O\)-Carboxymethylchitosan (NO-CMC) and Oligo-Chitosan (O-C) produced by the Standard and Industrial Research Institute of Malaysia (SIRIM Berhad) with DDA ranging between 75–95%. Lyostypt, a commercial hemostatic agent, was used as a positive control. Additionally, we demonstrated the ability of chitosan-based hemostatic agents to induce platelet and blood coagulation cascades that measure platelet adherences, platelet shape changes, platelet activation, adenosine diphosphate (ADP)-induced platelet aggregation, blood coagulation ability and coagulation profile measurements. We have also successfully reported and published that platelets respond differently to chitosan derivatives with differing MW and DDA (Periayah et al., 2013; 2014). This book chapter aims to provide the extended version of presently published content on chitosan activities towards platelet and blood coagulation mechanisms. Since we are exploring on platelet activities which involve in the coagulation process, as a new contribution to the society, chitosan-based hemostatic agents could be a new strategy biomaterial to achieve hemostasis. Knowledge on various signaling cascades in platelet thrombogenicity is very important in order to develop a novel therapeutic chitosan based treatment for hemorrhage. Since chitosan is a biodegradable biopolymer, it causes no hazard to the environment.

2 Materials and Methods

2.1 Materials

We used NO-CMC and O-C produced by the SIRIM Berhad with a DDA of 75–95%. Chitosan sponges with variable chitosan formulations (7% NO-CMC with 0.45 mL of collagen, 8% NO-CMC, O-C and one powdered type of chitosan termed O-C 53) were used. Lyostypt was used as the positive control.
2.2 Subject Selection

We recruited 14 healthy donors aged 18 to 50 who had not consumed any drugs in the previous two weeks. Informed written consent was obtained prior to the blood collection. None of the women were taking oral contraceptives when the blood samples were obtained. None of the participants had a diagnosis of a chronic disease. Prior to commencing the study, ethical clearance was obtained from the Human Ethics Committee of the Universiti Sains Malaysia (USM) (Ref Num: FWA Reg. No: 00007718; IRB Reg. No 00004494). We have assured that identifying information was not made available to anyone who was not directly involved in the study. The stricter standard was the principle of *anonymity*, which essentially indicates that the participant will remain anonymous throughout the study. All the participating donors’ personal details remained confidential.

2.3 Blood Collection

Twelve milliliters of whole blood was drawn from the antecubital vein into 3 vials of ethylenediaminetetraacetic acid (EDTA) tubes for all the studies except for the platelet aggregation, activation and coagulation profile studies. To evaluate these expressions, three-way stop-cocks were used to collect blood under minimal tourniquet pressure, and the first 1 mL of blood withdrawn was discarded. The remainder of each blood sample was aliquot into 3 tubes containing trisodium citrate anticoagulant. The subject selection was contingent on the presence of a hematocrit level between 38 and 45% and a normal platelet count between 150 $\times 10^3/\mu$L and 350 $\times 10^3/\mu$L.

2.4 Platelet Count and Morphology Studies

2.4.1 Chitosan Preparation

Chitosan samples, each weighing 5 mg, were dissolved or pre-moistened in 50 $\mu$L of (PBS) (pH 7.4) and subjected to incubation at 37 °C for 60 minutes (min) (Wagner *et al*., 1996; Zhou *et al*., 2008).

2.4.2 Platelet Count

Blood was collected from healthy donors in BD Vacutainer [K2 EDTA 3.6 mg (REF 367842)] tubes. Ten tubes were prepared, and 1 mL of blood was added per tube. The blood was introduced to the prepared chitosan samples. A 200 $\mu$L aliquot of blood was transferred from each 1 mL tube of whole blood to single vial plain tubes every 10 and 20 min. The platelet counts obtained were compared with the initial baseline counts. The results were analyzed using a Sysmex XE 5000 Automated Hematology Analyzer of Sysmex Corporation (Kobe, Japan) device. The platelet counts were analyzed using the hydro dynamic focusing method based on fluorescence flow cytometry (Sysmex, 2008; Periayah *et al*., 2013).
2.4.3 Preparation of Platelet and Erythrocyte for the Morphological Analysis of Chitosan Derivatives

Platelets from healthy donors were isolated by differential centrifugation with 150 × g for 15 min and 900 × g for 5 min at room temperature (Salas, 2000). Five hundred milliliters of isolated platelets was combined with each chitosan sample and incubated for 30 min in 12-well tissue culture plates. Each chitosan biomaterial measuring 5 mm × 5 mm was placed in a 12-well tissue culture plate. Each well was then washed with penicillin-infused phosphate-buffered saline (PBS) for 1 hour (hr), fixed in 100 µL of glutaraldehyde for 1 hr, and then washed with distilled water. Different concentrations of ethanol (30%, 70% and 100%) were added to dehydrate the chitosan biomaterials. Finally, all the samples were dried in an incubator (58 ºC) overnight. Once the biomaterials were completely dried, they were subsequently subjected to sputter-coating with gold using a gold sputter coater (Leica SCD 005, Germany) with the following conditions: vacuum millibar of 5 × 10⁻²; current of 20 milliamperes (mA); time of 150 seconds (sec). The gold sputtered chitosan biomaterials were examined under scanning electron microscope (SEM) (FEI-QUANTA FEG 450, Netherlands); in particular, their surface and cross-section morphology were examined (Okamoto et al., 2003; Periayah et al., 2013). SEM analysis is one of the robust and established technique which can detect by delivering information on biomaterial surface pattern. A drop of whole blood was placed on each slide, and peripheral blood films were prepared. The slides were stained with a Geimsa stain for 5 min and washed with PBS (pH 6.8) for 15 min. The slides were mounted, and the adhered cells were observed using light microscopy (Nikon Eclipse E200). The images were captured using Mirax Desk Zeiss (Okamoto et al., 2003; Genzen and Miller 2005).

2.5 Platelet Activation

2.5.1 Blood Sample Collection and Preparation

Blood samples were collected as described above using BD Vacutainer 0.109 M (3.2%) trisodium citrate anticoagulant tubes (REF 363083) from healthy donors. The blood samples were centrifuged at 1000 × g for 15 min. The supernatants were harvested upon centrifugation. Five hundred microliters of platelet rich plasma (PRP) was then mixed with each prepared chitosan sample for 30 min (Ritchie et al., 2000; Okamoto et al., 2003; Zhou et al., 2008; Periayah et al., 2014). The levels of P-selectin in the serum upon adhesion to the chitosan were measured using an enzyme-linked immunosorbent assay (ELISA) procedure (Cat. No. CSB-E04708 h), and the test was performed according to the manufacturer’s instructions.

2.5.2 Platelet Activation Assay Procedure

One hundred microliters of the prepared standard and samples were loaded into each well, and the plate was covered using adhesive strips. The sample-loaded plate was incubated for 2 hr at 37 ºC. One hundred microliters of Biotin-Ab (1X) was loaded once
the standards and the samples were completely removed from each well. The plate was incubated for 1 hr at 37 °C. Next, Biotin-Ab (1x) was added, and each well was aspirated and washed using washing buffer (200 µL) twice for a total of three washes using a multichannel pipette every 2 min. One hundred microliters of Biotin-Av (1x) was added to each well, and the plate was incubated for 1 hr at 37°C. The aspiration or washing procedures were repeated at least 5 times as described previously. Once the final washing was completed, 90 µL of TMB substrate was added to each well, and the plate was protected from light exposure and incubated for 15 to 30 min. As a final solution to the plate, 50 µL of stop solution was added to the entire well, and the plate was gently tapped to ensure thorough mixing. Eventually, all the reactions were stopped, and the absorbance was determined at 450 nm utilizing an ELISA reader (Tecan Infinite 200 PRO NanoQuant, Switzerland). A standard curve was generated, and the concentration of each sample was determined in ng/mL. The protein expression was calculated based on the volume of supernatant obtained after clot retraction. The standard curve was generated by plotting the absorbance for each standard on the y-axis against its concentration on the x-axis and drawing a 4-parametric logistic curve-fit that was plotted through all the data points (Garbaraviciene et al., 2010). No significant cross-reactivity or interference was observed among all the measurement levels.

2.6 Platelet Aggregation

Blood was collected in BD Vacutainer 0.109 M (3.2%) trisodium citrate anticoagulant tubes (REF 363083) from healthy donors. Platelet aggregation was measured in a Chronolog lumi-aggregometer (Chrono-Log, Havertown, PA) at 37 °C, under stirring. Platelet aggregation was determined by measuring changes in the optical density (i.e., light transmittance) of the stirred chitosan-adhered whole blood after the addition of an aggregating agent to the aggregometer cuvette. Five hundred microliter samples of chitosan-adhered whole blood were diluted in normal saline and pre-warmed for 5 min in the incubation well. The baseline was determined using the platelet suspension diluted 1:1 with a platelet suspension buffer to increase the gain of the aggregometer output. The luminescence gains were decreased to the minimum value by turning the rotary switch to 0.05. The stirring speeds were set to 1200 × g. A total of 10 µL of CHRONOLUME® (ADP) was added to each sample, and the luminescence gain setting was recorded. The peak luminescence or the amplitude was recorded in ohms (Ω). Generally, each platelet aggregation ran for at least 2 min and occasionally up to 5 min. The chart speed of the recorder varies by the type of equipment, but it should be sufficiently fast to observe the change in the shape of the aggregation tracing, which is usually 2 mm or more per min. This amount of time allowed the observation of the first- and second-wave aggregation for ADP (Zhou and Schmaier, 2005; Periayah et al., 2013). The platelet aggregation test was performed within 3 hr of blood collection upon venipuncture to avoid false platelet aggregations.
2.7 Coagulation Profiles Study

2.7.1 Blood Coagulation Study

Two milliliters of blood was drawn from the antecubital vein of each of the healthy donors for a total of 12 mL, which was stored in BD Vacutainer [K2 EDTA 3.6 mg (REF 367842)] tubes. To prepare the PRP blood, it 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. Each type of chitosan was tested in duplicate. The blood samples from each donor that weighed 100 mg were dissolved in 100 µL of PBS (pH 7.4) and incubated at 37 ºC for 30 min. After 30 min, 1 mL of whole blood or PRP was added to each of the 1.5 mL Eppendorf tubes. The time at which the blood coagulated was recorded for each chitosan sample, and after 15 min, the tests were stopped (Wagner et al., 1996; Robin et al., 2012; Periayah et al., 2013; 2014). The blood clot formed on each sample was photographed with a digital camera (Fujifilm Finepix J150W, Japan).

2.7.2 Coagulation Factors Analysis

Ten milliliters of blood was collected in BD Vacutainer 0.109 M (3.2%) trisodium citrate anticoagulant tubes (REF 363083). The blood was kept warm and centrifuged at 3000 × g for 10 min at 22 ºC to obtain platelet poor plasma (PPP). The PPP was prepared in accordance with the approved guidelines of the Clinical and Laboratory Standard Institute. The PPP was briefly agitated on a vortex mixer. Five hundred milliliters of isolated PPP was combined with 10 mg of prepared chitosan that had pre-absorbed 50 µL of PBS; this mixture was incubated for 10 min. The activated partial thromboplastin time (APTT), the prothrombin time (PT), the thrombin time (TT) and fibrinogen (Fib) were measured using a STA Compact Coagulation Analyzer (Diagnostica Stago, France) according to the manufacturer’s instructions. Coagulation profiles were evaluated by pre-warming and incubating samples. The samples were calcified automatically in the machine vials and the tests were performed according to the manufacturer’s instructions. Plasma was calcified and clotting was initiated by adding the Stago reagents. The quality control measurements were set in the proper range according to standard clinical laboratory protocol prior to testing (Maurer et al., 2001; Arkin et al., 2003; Yang et al., 2007; Periayah et al., 2014).

2.8 Statistical Analysis

We used a repeated-measure analysis of variance (ANOVA) and the correlation coefficient to identify statistical trends for the platelet count test, which was compared using 3 different time intervals. Statistical significance was also compared using a one-way ANOVA, and an independent t-test. Statistical significance was defined as $P \leq 0.05$, and these values were calculated using SPSS software, version 20.0. All the quantitative experimental outcomes were elucidated in percentages and means ± standard error of the mean (S.E.M).
3. Results and Discussion

3.1 Assessment and Effects of Platelet Adhesion on the Adherences of Different Types of Chitosan

3.1.1 Platelet Count

Chitosan has become one of the most promising local hemostatic agents. It is of particular importance because it functions independently on platelets and normal clotting mechanisms. This present study was constructed to conduct a platelet adhesion test to illustrate the clear actions of chitosan on platelet counts and its morphology. Platelet counts were tested upon the adherences of chitosan biomaterials at three different time intervals including baseline, after 10 min and after 20 min. The rationale of this analysis was to study the lowest level of platelet counts because as the platelet count is reduced, the chances for the platelets to be attracted to chitosan increase. This measurement identifies materials that may be more effective as hemostatic agents (Periayah et al., 2013).

Previous reports described comparative studies that were performed in a controlled *in vitro* environment that was associated with human blood and plasma. This study was designed to estimate the standard properties of available hemostatic agents. The interactions between the coagulation system and polymer surfaces are highly complex and require the proportional blood compatibility of biomaterials (Saito et al., 1997; Kirkpatrick et al., 1998; Sieminski and Gooch, 2000). Naturally derived polymers have the benefits of biological attributes such as cell proliferation and biocompatibility, and their application is an extension of their biological purpose.

The utilization of controlled *in vitro* techniques as screening tools aids the process of generating novel hemostatic agents. The composition of chitosan is a potential supplementary tool for the investigation of hemostasis (Cheng et al., 2009; Xiangmei et al., 2009). Platelets can adhere to the surfaces of biomaterials. Platelet number counting is an important tool for assessing hemocompatibility because the platelet number influences the formation of a hemostatic plug or thrombus (Bernacca et al., 1998; Turner et al., 2002). The outcome was analyzed using a repeated measure ANOVA test to observe the platelet counts at 3 different time intervals. A repeated measure ANOVA was employed because it is the only statistical design that could be possibly used to obtain information concerning individual patterns of change and repeatedly measure the same variable over time on the same group of subjects.

The platelet counts were observed to decrease significantly ($p < 0.05$) upon the adherences of all the tested chitosan biomaterials except for blood alone. The largest decrease was observed in O-C with the following values: baseline: $303.1 \pm 9.54 \times 10^3/\mu L$; after 10 min: $260.1 \pm 12.6 \times 10^3/\mu L$; and after 20 min: $249.1 \pm 12.7 \times 10^3/\mu L$. The next most prominent decrease was noted in O-C 53 with the following values: baseline: $308.9 \pm 12.2 \times 10^3/\mu L$; after 10 min: $275.5 \pm 10.8 \times 10^3/\mu L$; and after 20 min: $258.4 \times 10^3/\mu L$. The percentage changes in the platelet counts increased from 10.8% (after 10 min) to 16.35% (after 20 min). Subsequently, lyostypt, 7% NO-CMC and 8% NO-CMC recorded 8.7, 5.7 and 4.6% platelet count decreases during the first 10 min of the analysis, respectively. Meanwhile, 13.3, 9.6 and 10% decreases were registered after 20 min by lyostypt, 7% NO-CMC and
8% NO-CMC, respectively (Figure 1). No significant values were observed between the each of the tested biomaterials and the each time period.

![Figure 1: Mean value of platelet counts upon the adherence of chitosan. The error bars indicate the S.E.M. (n = 14).](image)

On the other hand, Pearson’s correlation was applied to measure the relationships between the 3 different time intervals. Usually, Pearson’s correlation is a measure of the strength and direction of a linear relationship between two variables. If the relationship between the variables is not linear, the correlation coefficient will not be able to elucidate the strength of the relationship between the variables (Lane, 2014). The analysis of correlation indicated that the relationship between the baseline, the 10 min interval and the 20 min interval was strong. This positive correlation indicates that for all tested biomaterials, except for blood alone, the platelet counts decreased to a significant degree. The correlation between baseline and 10 min was characterized by \( r = 0.83 \) and \( p < 0.01 \). The observed correlation between baseline and 20 min was characterized by \( r = 0.73 \) and \( p < 0.01 \). The strongest relationship was noted between 10 and 20 min, which had the value of \( r = 0.94 \) (\( p < 0.01 \)). Pearson’s correlation \( (r) \) indicates that the measurements were performed with samples and not with populations. Pearson’s \( r \) ranges from –1 to 1. An \( r \) of –1 indicates a perfect negative linear relationship between variables, an \( r \) of 0 indicates no linear relationship between variables, and an \( r \) of 1 indicates a perfect positive linear relationship between variables (Periayah et al., 2013).

Lyostypt was used as the positive control. Lyostypt is composed of collagen, which exhibits excellent biocompatibility and has a significant role in primary and secondary hemostasis. Collagen-based chitosan was utilized because collagen has the ability to initiate platelet adherences at the site of bleeding tissues (Seda et al., 2007). Collagens are crucially important for platelet adhesion and subsequent activation on the extracellular matrix of denuded endothelium (Wilner et al., 1971). In this study, 7% NO-CMC chitosan coated with 0.45 mL of ovine collagen was used. The collagen was mixed
with NO-CMC in predetermined compositions and freeze-dried to obtain a porous structure. No significant results were observed upon the adherences of the collagen coated NO-CMC in comparison with O-C. This was likely because of the different MW, DDA, functional groups and scaffold porosities of NO-CMC and O-C.

The rapid and accurate determination of platelet counts is a crucial factor in diagnostic pathology. The main reason an automated hematology analyzer was used in this study is that this analyzer is usually capable of providing accurate platelet counts with a generally good precision (Gao et al., 2013). Previously, it has been reported that chitosan is fully capable of stimulating platelet adhesion in a time-dependent manner of within a 5 to 30 min time period (Chou et al., 2003). In this present investigation on platelet quantification, the count tended to decrease upon the adherences of chitosan biomaterials within 2 different time intervals. A greater decrease of platelets resulted in more chitosan biomaterial that was capable of entrapping platelets on the surface membrane. Because platelet adhesion is an essential function in response to vascular injury and is generally viewed as the first step during the coagulation process, this quantification of platelets is useful for elucidating the significant role of chitosan biomaterials on attracting more platelets for the hemostasis process.

3.1.2 Morphological Analysis of Chitosan-Adhered Platelets

Surface membrane properties possess an enormous effect on the biocompatibility action of a biomaterial. Factors such as surface characterization, porosity evaluation, thickness and tensile strengths are the central issues in biomaterial research for correlating with biological performances. The success of using microscopic methods to characterize biomaterial surfaces is well established, and these microscopy techniques were often found in the biomaterials literature (Merrett et al., 2002). SEM and light microscopy have been used to study the morphological changes of the platelets upon the adherence of chitosan biomaterials.

The platelet morphology was observed by SEM. The greatest number of platelet attachments was observed on the tested chitosan biomaterials because nearly 90% of the platelets adhered to the chitosan surface membrane. Most platelets were irregular and pseudopodal in shape.

Figure 2A–2E shows observations of these shapes under different magnifications (8000×, 6000×, 3500×, 500×, and 10000×). These varying shapes are a result of the differing surface properties of the biomaterials. The contact angle of O-C 53 (Figure 2D) under 500× 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. The platelets were appeared to have irregular shapes and did not display any granulation. Aggregated platelets had already discharged their granules, as shown in Figure 2A–2C. No single platelets were observed. Platelets clumped to form a large group, forming a fibrin clot that reinforced platelet aggregation. The results showed a positive increase compared to those of lyostypt, which was composed of highly flexible strands that allowed the platelets to form fibrin networks (Figure 2E).
Figure 2: 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]. The platelet shapes changed into irregular pseudopodals upon aggregation (solid white arrow). Fibrin meshes formed upon blood coagulation (dashed white arrow).
The SEM analysis showed that the platelets adhered to one another, clumping into irregular shapes and elongated pseudopod forms, depending on the type of chitosan material. These chitosan materials varied based on their DDA and MW. O-C appeared yellow in color, and the surface of the material was harder than those of the other types of chitosan. This distinction likely appeared because O-C was exposed to slightly higher localized temperatures during the production stage. The platelets formed abnormal shapes and exhibited varying degrees of surface roughness and surface wettability (hydrophilic and hydrophobic surfaces) (Laka and Chernyavskaya, 2006). The shapes of the platelets changed from discoid-shaped resting cells to spiculated spheres. O-C attracted more platelets to form bridges, most likely because the degree of crystallinity of O-C 53 was higher (Kuwahara et al., 2002). The broad, irregular spread of the platelets ensures firm adhesion in an irreversible mode (Periayah et al., 2013; 2014). Upon morphological analysis, platelet attachments were detected on most of the chitosan surfaces. The platelets adhered to one another and extended into pseudopodal forms to aggregate depending on their surface roughness, thickness and absorbability.

Peripheral blood smears were prepared to examine the characteristics of blood cells upon the adherences of the chitosan biomaterials. A Geimsa stain is a classic blood film stain for peripheral blood smears that is normally used to differentiate the nuclear or cytoplasmic morphology of platelets, red blood cells or erythrocytes, white blood cells and parasites. The stains or the morphology of the erythrocytes are stained in pink or red; the platelets in purple or pink; the lymphocytes in light blue; the monocytes in pale blue and the leukocytes in magenta (M15A, NCCLS, 2000; Garcia, 2001). Light microscopy employs visible light to detect tiny objects. It is easy to handle, provides superlative image quality and is cost-efficient. The images of the stained smears were captured using Mirax Desk Zeiss, which is a robust and stable base scanner capable of capturing Geimsa-stained smear slides.

Erythrocyte morphology was assessed by a peripheral blood smear (Figure 3). The clearest finding from this study was that erythrocyte aggregations were observed in all the chitosan-adhered biomaterials. Irregular aggregations of erythrocytes into grape-like clusters and the aggregation of platelet cells were observed in both NO-CMC samples (Figure 3A–3B). Fibrin meshes also formed around the erythrocyte aggregations (Figure 3A, 3E). However, in the presence of both O-Cs, the erythrocytes swelled and aggregated vigorously. Platelet aggregation was difficult to observe because the erythrocytes covered 80% of the chitosan surface (Figure 3C, 3D). Similarly, in the presence of lyostypt, the erythrocytes overlaid maximally, but in this case, the fibrin mesh was clearly visible (Figure 3E). This outcome shows that the adhesion and aggregation of erythrocytes was stimulated and induced by NO-CMC and O-C. The aggregations of erythrocytes and platelets together with the formation of a fibrin meshes in the peripheral blood films were successfully observed. The hydrophilic surface properties of chitosan derivatives promoted platelet adhesion and activation in achieving hemostasis. The purpose of this figure is to express differences between platelet and erythrocyte aggregations which distinguish the shapes and the degree of membrane coverage of the erythrocyte on chitosan biomaterials.
Recent evidence suggests that chitosan-adhered blood significantly accelerates hemostasis in vitro by decreasing the plasma recalcification time and also by accelerating fibrin formation compared to the control groups. Platelet adherence increased and was activated on the chitosan surface membrane. Chitosan formed coagulum when it adhered to whole blood samples and was capable of aggregating without lysing the erythrocytes (Malette and Quigley, 1983; Dutkiewicz et al., 1989; Rao and Sharma, 1997; Janvikul et al., 2006; Periayah et al., 2014). The data from several sources confirmed the finding that chito-oligomers stimulate hyaluronan synthesis, which stimulates cell adherence and proliferation towards morphogenesis, inflammation and wound healing. In
another study, erythrocytes formed coagulations in the chitosan-treated lingual incisions and the morphologies were noticed to be deformation shapes (Klokkevold et al., 1991; 1992). Chitosan interacted with the erythrocytes, which led to aggressive erythrocyte aggregation; at the same time, chitosan was also reported to inhibit erythrocyte lysis. The chitosan-adhered erythrocytes eventually lost their biconcave morphologies and accumulated as a clot to prevent blood flow. This study result also clearly supports the outcome hypothesizing that chitosan bio-materials are capable of promoting hemostasis by crosslinking erythrocytes together to form a lattice to ensnare the cells (Yamazaki, 2007; Periyah et al., 2014). The ionic attraction between negatively charged erythrocyte membranes and positively charged groups in chitosan is one of the possible explanations for the coagulation activities of chitosan. Consequently, positively charged chitosan is more effective as a blood coagulant (Wu et al., 2008).

3.2 Expression Levels of Cell Adhesion Molecules (P-Selectin) in Platelet Activation

Platelet activation usually occurs followed by platelet adhesion events. As an initial step in the platelet activation mechanism, platelets will undergo shape changes, cytoskeleton organization and organelle centralization (Ghoshal and Bhattacharyya, 2014). In the regulation of the hemostasis process, activated platelets clearly play an important role in facilitating the subsequent platelet aggregation step. However, uncontrolled platelet activation could possibly cause the blockage of vessels because of hypercoagulation.

P-selectin is also known as CD62P and is expressed in the α-granules of activated platelets and the granules of endothelial cells. It is the largest protein in the selectin family and has a total MW of 140 kDa. Endothelial cells rapidly stimulate inflammatory mediators such as P-selectin upon tissue injury. P-selectin also tends to be expressed during surgical traumas. P-selectin is rich in cysteine and contains a number of complex N-terminals, which are linked to oligosaccharides and play a significant role in platelet activation by combining with the external membrane. The expression period of the P-selectin protein is short-lived with a peak of only 10 min. The additional recruitment of P-selectin can be imparted by cytokine mediators. P-selectin mediates the interactions between the endothelium, platelets, and leukocytes by recruiting the phosphorylation of histidine residues on the cytoplasmic tail of the molecule (Crovello et al., 1995). The primary ligand for P-selectin is the P-selectin glycoprotein ligand-1 (PSGL-1), which is generally found on all leukocytes. The transient interaction between P-selectin and PSGL-1 allows the leukocyte cells to move along the venular endothelium.

The P-selectin protein is also involved in the leukocyte-adhesion mechanism (Berger et al., 1998). Of the multiple surface markers that can be detected upon platelet plug formation, P-selectin is one of the protein that is translocated at the outer boundary of the activated platelets. As discussed previously, chitosan is fully capable of inducing platelet adhesion by engaging in the hemostasis process. Driven by the substantial function of chitosan-derived biomaterials as an anti-hemorrhage biopolymer, the platelet activation marker P-selectin was also investigated in this study. Each chitosan biomaterial was preincubated with prepared PRP of citrated blood from healthy donors, as
previously described in the methods section. A quantitative sandwich enzyme immunoassay technique was employed. The detection range for P-selectin is 0.9 ng/mL to 60 ng/mL. The results of this study not only defined the novel role of P-selectin upon chitosan adhesion but also elucidated the significance of this protein towards the coagulation process.

Based on the assay standard operation procedure, the minimum detectable dose of human P-selectin is lesser than or equal to 0.225 ng/mL. The sensitivity of this assay, or the lower limit of detection, was defined as the lowest protein concentration that could be distinguished from 0 ng/mL. The assay procedure that was employed also had a higher level of sensitivity and excellent specificity for detecting human P-selectin. In this test, the collected samples were stored for no longer than a week to avoid protein degradation and denaturalization, which might lead to false results.

P-selectin was continuously expressed at low levels upon adherence to different forms of chitosan. The highest mean expression level of P-selectin was induced by O-C [19.3 ± 1.61 ng/mL], followed by O-C 53 [18.5 ± 2.01 ng/mL]. Both O-C and O-C 53 showed increases of 34.7 and 31.9%, respectively, compared to blood alone. Meanwhile, the level of P-selectin increased by lyostypt with only 27.6%. Among the two tested NO-CMCs, 7% NO-CMC induced the expression level by 26.3% whereas 8% NO-CMC suppressed the expression level by 22.3% compared to blood alone. Comparisons have been performed between the tested groups with blood alone to elucidate any significant values. Both the O-C and O-C 53 chitosan groups exhibited significant values of \( p = 0.002 \) and \( p = 0.015 \), respectively. However, the outcome result for the NO-CMC chitosan group did not show a significant value compared to blood alone (Figure 4).

**Figure 4:** The mean expression of P-selectin upon the adherences of chitosan biomaterials. The error bars represent the S.E.M., and the statistically significant values are clearly provided for the tested biomaterials compared to blood alone; \( n = 14 \); \( p < 0.05 \).
The measurement of the P-selectin protein on the platelet surface has been studied in many settings to determine platelet activation status. However, the recovery and survival of expressed P-selectin upon chitosan adherence in vitro is still questionable in correlation with the in vivo events upon tissue injury. This is because very few previous studies have been conducted to test the capability of chitosan derived hemostatic agents in inducing the P-selection protein molecule in activating platelets to assist in the hemostasis process. This study produced results corroborating the findings of many of the previous works. O-C affects thrombogenesis by changing the shape of the platelets to a pseudopodal shape, enabling aggregation (Periayah et al., 2014). Therefore, this platelet activation corroborated with the platelet adhesion findings that chitosan is capable of acting on the primary step of hemostasis by improving the platelet adherences and capillary restoration.

There are many reasons underlying the mechanism of action of P-selectin that must be studied and explored in detail to correlate this protein with platelet activity. P-selectin expression reported to stabilize the initial platelet aggregation formed by Glycoprotein IIbIIIa-Fib (GPIIbIIIa-Fib) interactions, permitting the accumulation of large aggregations. P-selectin expression upon platelet activation has the potential to determine the size and stability of platelet aggregates and to play an important role in thrombosis (Seda et al., 2007). At this point, P-selectin was continuously expressed at a low level upon adherence to the various forms of chitosan, with noticeable differences in the means observed between the tested groups. Investigations of P-selectin expression in PRP might be affected by mechanical platelet activation during centrifugation (Ritchie et al., 2000).

To minimize the possibility of mechanical platelet activation, three-way stopcocks were used to discard the first 1 mL of blood in the first syringe collected. Previously, it was reported that the percentage of P-selectin induced by a chitosan-heparin composite scaffold was significantly reduced compared to that induced by a chitosan scaffold alone (He et al., 2010). Additionally, the levels of P-selectin were higher in platelets exposed to chitosan compared to platelets that were isolated from blood alone. The expression of the integrin GPIIbIIIa has been reported to be elevated in platelets that adhered to chitosan (Tomihata and Ikada, 1997). The expression level of P-selectin was also noted to be dependent on the type of anticoagulant used. To study this relationship, 3.2% anticoagulant tubes (K3 tubes) were used to avoid possible errors.

As formerly reported, the platelet activation stimulated by ADP or collagen was contingent on the type of anticoagulant that was used even though the minimal expression at baseline levels for all the tested samples has been described (Schneider et al., 1997; Holmes et al., 1999; Ritchie et al., 2000). In the current study that evaluated samples without in vitro activation, expression was significantly higher in K3 EDTA than in sodium citrate blood. It is of note that in previous studies, K2 EDTA has been shown to upregulate the expression of P-selectin. EDTA has been well documented because it causes an upregulation of the platelet surface receptor and an increase of (McEver et al., 1983), which could markedly affect the coagulation pathway. It has also been shown to cause a time dependent swelling of platelets, which leads to the denaturing and
degranulation of P-selectin surface expression (McShine et al., 1990; Bath 1993; Kuhne et al., 1995; Golanski et al., 1996; Periayah et al., 2014).

3.3 Measurement of Platelet Aggregation Induced by an ADP Agonist in the Presence of Chitosan Biomeriters

Platelets readily adhere on a ruptured vessel wall in response to vascular damage by stimulating a host of events that involve additional platelets to recruit for aggregation. Platelet aggregation is stimulated by the activated receptors that are conjugated with intracellular signaling, resulting in GPIIbIIIa activation. The major functions of platelets in hemostasis involve their adherences at the sites of vessel injury by activating the internal signaling pathways, which form platelet plugs to seal the injured area (Zhou & Schmaier, 2005). ADP has long been acknowledged as providing an extension of platelet activation towards vascular damage, and it is an important agonist to detect the ability of platelets to aggregate in vivo. Meanwhile, a platelet aggregation test can be performed utilizing whole blood and PRP by employing a few agonists that are also known as platelet activators. Scientifically, agonists are described as the chemicals or signals that adhere to the receptor by stimulating the receptor to execute a biological response.

The most common agonists used to test platelet aggregations are arachidonic acid, thrombin, epinephrine, collagen, ristocetin and ADP. These agonists can be classified into two different categories: strong and weak agonists. Thrombin, ristocetin and collagen fall under the strong agonist category because they directly induce platelet aggregation, synthesize Thromboxane A2 (TXA2) and secrete platelet granules. However, ADP and epinephrine are addressed as weak agonists because they only induce aggregation without stimulating the secretions. In this study, an ADP agonist was selected to test the level of platelet aggregation upon the adherence of the chitosan derivatives. Therefore, ADP was chosen as the agonist because platelet secretion precisely follows the aggregation that is induced by a weak agonist upon the synthesis of endogenous TXA2 to be stimulated closely along platelet-to-platelet interactions throughout platelet aggregation mechanisms.

Strong agonists can potentially act as weak agonists at low concentrations, but weak agonists may not be affected even at higher concentration levels. Platelet agonist addition into blood samples can potentially activate the platelet cells by changing their shapes from a discoid pattern to spiny spheres or pseudopodal shapes, which are related to the temporary rise in the optical density of platelet aggregometry. Driven by the significant role of adherence in the platelet response during the hemostasis process, this study was conducted to evaluate platelet aggregation in the presence of ADP agonists to characterize platelet capacity with chitosan adherences. Moreover, no research has yet been conducted to test the chitosan response on platelet aggregations by adding an ADP agonist. The standard 10 µM ADP concentration was added to a chrono-log cuvette, and the results were interpreted at 5 min time intervals.

Platelet aggregation was measured using a Chronolog lumi aggregometer. This aggregometer measured and quantified platelet functions using electrical impedances. Generally, this is the diagnostic tool used to address the aggregation dysfunction that
occurs during the hemostasis process for certain types of hemostatic abnormalities such as vWD, Bernard Soulier Syndrome and Glanzmann Thrombasthenia. The measurement of platelet aggregation also illustrates the process of dense granule release by providing better insight into the mechanisms of the platelet response. The amplitude levels of the aggregated platelets were expressed in ohms (Ω). O-C 53 was the chitosan for which the lowest mean amplitude was reached at \( 1.57 \pm 0.64 \) Ω and a difference of 11.93 Ω compared to the control level. O-C reached the second lowest level at \( 4.14 \pm 17.71 \) Ω. Meanwhile, 7 and 8% NO-CMC recorded the highest amplitude release at \( 9.79 \pm 1.30 \) Ω and \( 11.71 \pm 1.18 \) Ω, respectively. Large differences in the mean amplitudes were noted between both the O-C and NO-CMC chitosan groups. All the tested groups of biomaterials exhibited significant differences compared to blood alone, which was only tested with the addition of ADP \((p < 0.05; \text{Figure 5})\).

As shown in Figure 6, chitosan adhered to whole blood was stabilized first in the cuvette. The baseline of light transmission prior to the introduction of the ADP agonist is indicated by ‘1’. The label ‘2’ indicates the addition of the agonist. The platelet shapes changed initially (shown by ‘3’), resulting in a reduction of light transmittance, followed by an initial wave of aggregation (indicated by ‘4’). If the stimulation of ADP is strong, a secondary wave of ADP-induced platelet aggregation occurs (indicated by ‘5’) in which the platelet granule contents, which potentiate the primary aggregation reactions, are released. Only the results exhibited in channel number 3 are noted. This is because of the computed on-screen instructions. The slope, lag time and area under the curve were not taken into account to depict the level of platelet aggregation because they only illustrate the graph, which expresses the amplitude mode.
Although O-C 53, the powdered type of chitosan, induced a more rapid reaction among the types of chitosan studied, O-C in sponge form also registered an equivalent result. The shape change was followed by platelet aggregation and granule secretion, which led to the release of more ADP and several other substances (Mill et al., 1968). ADP was used in this study because it is the best studied and most commonly used agonist. It is released from dense granules during platelet activation, and its initial binding results in the release of intracellular calcium and a change in the shapes of the platelets, leading to the primary wave of aggregation. The secondary wave reflects the release of ADP from the platelet storage granules. Low-dose ADP induces only primary aggregation, and the effect is reversible. The molecular mechanism of ADP on platelets remains unclear.

The chitosan groups O-C and O-C 53 both showed significant results, as indicated by the lowest amplitude mean levels ($p < 0.05$). This result indicates that ADP could not induce platelet aggregation because most of the platelets were attracted to chitosan, reducing the number of platelets remaining in the tested blood sample. To elaborate more on this platelet aggregation test, if the ADP stimulus was not sufficiently strong, the platelets failed to aggregate. Therefore, a comparison was performed for every sample with a negative control, which was blood alone, to ensure that the ADP agonist did not provide false positive results. To obtain optimal results, all the studies were performed within 2 hr of the blood collection. Storage time was avoided because the physiological integrity of platelets decreases with prolonged storage (Kohli et al., 1998). Examples of changes in light transmission during ADP-induced platelet aggregation in the presence of O-C 53 and lyostypt are shown in Figure 6. This result showed that the O-C chitosan group performed their functions as hemostatic agents; however, these in vitro platelet aggregations do not precisely reflect in vivo platelet functions. Chitosan-adhered whole blood samples were placed in the cuvette, followed by the addition of distilled water at an equivalent ratio of 1:1. Strictly, infusion saline should not be mixed with the whole blood sample because it contains an improper osmolality, benzyl alcohol and other preservatives that could affect platelet functions. Cell counter diluents are also inappropriate to load with the whole blood for analysis because they contain EDTA, which could inhibit platelet aggregations. Although chitosan was found to form coagulum, the adherences and aggregation of the platelets did not precisely reflect blood coagulation. The effects of chitosan on the coagulation profile occur not only because of physical consequences but because of their chemical structure, particularly the amine residue (Okamoto et al., 2003). This result indicates that the amine residue is important in the aggregation of platelets to form a clot (Periayah et al., 2013).

In the previous morphological analysis, the membrane coverage of platelet aggregation on the surface of the chitosan scaffold was pictured clearly. At that level, the O-C chitosan group registered the biomaterials that stimulate more platelet adherences towards aggregation. ADP addition in the chitosan-adhered blood samples, which was evaluated using a turbidimetric aggregometry assessment, is one of the conformational tests used to denote and strengthen the hypothesis on platelet shape changes to form aggregation to seal an injury in vivo.
Figure 6: Examples of changes in light transmission during platelet aggregation induced by ADP in the presence of O-C 53 and lyostypt. The highlighted cells indicate the recorded amplitude (Ω) of platelet aggregation.

### 3.4 Effects of chitosan on coagulation ability and coagulation profiles

Hemorrhage remains the leading cause of combat death and is a major cause of death from potentially survivable injuries. Great strides have been made in controlling extremity hemorrhage with tourniquets, but not all injuries are amenable for tourniquet application. Topical hemostatic agents and dressings provide great success in controlling extremities. In many hospital settings, maintaining a good hemostatic balance in bleeding patients remains a major challenge. Successful approaches in hemostasis research may contribute to a significant reduction in hemorrhage-related fatalities. Hemostatic agents have been widely applied in surgical settings. The FDA recently issued a warning regarding a few types of hemostatic agents that had been reported to cause
adverse effects such as swelling, paralysis and nerve injury because of compression (Periayah et al., 2013).

The development of new methods or devices for hemorrhage control may contribute to a future reduction in hemorrhage morbidity and mortality. A successful hemostasis completely depends on the successful balance between coagulation and complementary and fibrinolytic pathways, with complex interactions between plasma proteins, blood cells, blood vessel endothelium and blood flow and viscosity (Deuel et al., 1982; Berger et al., 2004; Alfars and Khashjoori, 2008; Periayah et al., 2013; 2014). Therefore, alternative or naturally derived wound dressings have been intensely researched to identify and resolve challenges in treating hemorrhage. Chitosan was introduced as an antihemorrhagic biomaterial because it is cationic and insoluble at a higher pH, but when reversibly sulfated, it becomes anionic, which has water-soluble properties that initiate blood coagulations (Suh and Matthew, 2000). Hence, developing novel bioadhesives and hemostatic agents has been a continued priority for reducing hemorrhage complications.

Although many studies have been conducted to clarify the hemostatic effectiveness of the application of chitosan, the hemocompatibility of chitosan derivatives still remains unexplained. In the present study, the feasibility of the chitosan biomaterials towards blood coagulation abilities and coagulation profiles were screened by employing two different methods. Whole blood and PRP were utilized to test the blood coagulation abilities. A method that mimics the Westergren method and a dynamic rheological experiment were applied to test both types of prepared blood. These classic techniques are the most common and modified methods that are applied to test the erythrocyte and PRP sedimentation rate by measuring the timed fall of the level of blood within 15 min of the time interval observed macroscopically.

This test is often referred to as a nonspecific measurement in monitoring blood diseases and assisting in a particular diagnosis. The Westergren method is not a well-understood phenomenon and can only be described in 3 different stages: aggregation, precipitation and packing. Any of the factors affecting these 3 crucial phases could possibly influence the number and shape of the blood cells and the plasma viscosity (Vennapusa et al., 2011). The study of the flow of matter-like substances in solid, semi-solid and liquid conditions in which they could react favorably together with a plastic flow instead of elastic deformation upon force application is referred to as rheology (Schowalter, 1978). The rheology application between the coagulation system and the polymer surfaces of the chitosan derivatives are of a highly complex nature and largely depend on the blood compatibility of the biomaterial properties such as plasmatic enzymes, cellular elements and flowing conditions (Saito et al., 1997; Kirkpatrick et al., 1998; Sieminski and Gooch, 2000; Periayah et al., 2014).

The blood clotting process is a host defense mechanism that helps to protect the integrity of the vascular system after tissue injury in parallel with the inflammatory and repair responses. Coagulation profile evaluations, which include APTT, PT, TT and Fib, were measured on an STA Compact Coagulation Analyzer (Diagnostica Stago, France) according to the manufacturer’s instructions. Normally, these profiles are examined in a patient with a sufficient amount of coagulation activity to ensure the blood clotting
process occurs within a necessary period. Therefore, the significance of this test was to evaluate the above mentioned profile actions upon chitosan biomaterials and exhibit its effectiveness in expediting the clotting time.

3.4.1 Blood Coagulation Study

Figure 7 shows the blood coagulation ability of NO-CMC, O-C, lyostypt and blood alone. As discussed, a test that imitates the Westergren method and a dynamic rheological experiment were employed. The blood samples were allowed to settle, and blood coagulation was tested by inverting the tubes and observing erythrocyte and platelet aggregations. Upon macroscopic observation, both NO-CMC samples were able to aggregate fully within 7 min (Figure 7A, 7B). The faster aggregations were noticed because of the surface properties that influence the blood with the NO-CMC adherences. The NO-CMC surface membrane appears to be very hard and possesses a relatively greater porosity compared to the O-C chitosan group. The O-C samples fully aggregated the 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, the powdered-type O-C 53 was able to coagulate just 50% (Figure 7D). The 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 were detected to promote blood coagulation but at unequal levels.

![Figure 7: Blood coagulation test. (1) Whole blood 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].](image)
In the previous test that was conducted on platelet plug formation with chitosan adherences, O-C was noted to promote better platelet stimulation by engaging in the hemostasis process. The NO-CMC biomaterials were able to swell and degrade compared to the O-C group which had a positive aggregation; this was practically visible through the naked eye. Theoretically, platelet mechanical activities are believed to be concluded upon the quantification of any particular device assistances.

Chitosan is a biopolymer that is known to act on negatively charged, low-MW plasma proteins to promote aggregation (Etienne et al., 2005). All the screened chitosan biomaterials in this study accelerated erythrocyte and PRP aggregation. The NO-CMCs activated the platelet and erythrocyte activities faster than O-C. O-C 53 was only partially able to aggregate erythrocytes and platelets by extending the coagulation time. The optimal biomaterial weight of 100 mg was employed, and the 7% and 8% NO-CMCs were both able to aggregate 1 mL of erythrocytes or PRP within 7 min, which was consistent with a recent study by Ina Maria, 2013 in which blood in an inverted test tube became gel-like because of an increased viscosity and the presence of blood clots within 10 min of incubation with chitosan-coated films. Chitosan likely triggers hemostasis through its ionic affinity (Maria, 2013). Chitosan sponges strongly adhere to the surface membrane of muscles upon the rabbit dorsal vein wound. Throughout the implantation, the chitosan sponges were noted to promote flexibility and were resistant to wound breakage (Alfars et al., 2008). This shows that chitosan did not directly injure the surface membrane of the muscle upon application and that it is possible to generate the wounded tissues. In another study, chitosan tended to expedite the bleeding time of blood drops within 2 – 2.5 min using a bandage that was dipped in chitosan solutions (Sanandam et al., 2013).

Arand et al. reported that erythrocytes attach to one another and capture cells to build artificial clots upon chitosan adherence (Arand & Sawaya, 1986). Rao and Sharma also reported that platelets exposed to chitosan displayed a distinct adhesion to the chitosan surface within 30 to 60 sec (Rao & Sharma, 1997). This aggregation response results from the fact that chitosans are composed 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 (Fischer et al., 2004). Therefore, we discovered that it is possible for O-C, O-C 53 and lyostypt to coagulate to the desired level if the weight of the biomaterials is adjusted depending on their type and thickness.

### 3.4.2 Coagulation Profiles

The normal ranges of PT, APTT and TT in human serum are 10–21 sec, 30–45.8 sec and 11–21 sec, respectively. In this experiment, only blood alone without any biomaterial adherence expedited the coagulation times that were measured in sec. All the chitosan-adhered samples prolonged the clotting time but did not show any abnormal coagulation profile ratios. O-C 53, followed by 7% NO-CMC, was the chitosan that extended the clotting time. Followed by blood alone, lyostypt was shown to be significant by exhibiting its coagulation ability for PT within the mean period of 14.5 ± 0.67 sec; $p = 0.02$. Sub-
sequently, O-C and 7% NO-CMC depicted a clotting period of 14.9 ± 0.45 sec; \( p < 0.02 \), and 15.0 ± 0.52 sec; \( p = 0.01 \), respectively. Although the powdered type of O-C 53 was capable of measuring the PT within the normal range, compared with all of the examined biomaterials, O-C 53 extended the PT up to 16.2 ± 0.53 sec; \( p = 0.01 \).

Blood alone was the best at expediting the APTT test at 39.9 ± 1.19 sec. Among the tested biomaterials, lyostypt, O-C and 7% NO-CMC were discovered to prolong the APTT profile with increases of 5.6, 6.2 and 6.3%, respectively. Meanwhile, 8% NO-CMC and O-C 53 were noted to extend the APTT outcome upon the chitosan adherences with times of 45.1 ± 1.24 sec and 45.4 ± 1.38 sec, respectively. Likewise, 8% NO-CMC and O-C 53 elucidated significant values comparable to blood alone at \( p < 0.05 \).

The time between the addition of the thrombin and the blood clotting was registered as the thrombin clotting time. Again, blood alone registered with a mean TT of 15.5 ± 0.49 sec, followed by O-C, lyostypt and 8% NO-CMC with recorded times of 16.7 ± 0.54 sec, 17.0 ± 0.43 sec and 18.4 ± 0.40 sec, respectively. In this test, O-C 53 and 7% NO-CMC continued to prolong the TT by 24.4% and 25.7%, respectively. All the tested biomaterials were compared to blood alone and exhibited significant values; at this level, 7% NO-CMC, 8% NO-CMC and lyostypt were demonstrated to be significant at \( p < 0.05 \). All the tested biomaterials were discovered to protract the coagulation profiles. A one-way ANOVA was employed to investigate the significant values between the coagulation profile groups. Significant differences were found between the tested groups for PT (\( p = 0.024 \)), APTT (\( p = 0.021 \)) and TT (\( p < 0.05 \)). The asterisk (*) symbol indicates the significant values of each biomaterial compared to blood alone (Figure 8).

![Figure 8](image_url)

**Figure 8:** Coagulation profiles of PT, APTT and TT showing the means, with error bars presented as the S.E.M. The asterisk (*) symbol indicates the significant values noted compared to blood alone; \( n = 14 \); \( p < 0.05 \).
Fib measurement was expressed in grams per deciliter (g/dL). The normal range of Fib is 14–21 g/dL. Lyostypt, O-C and O-C 53 had values of 16.6 ± 0.85 g/dL, 16.5 ± 0.84 g/dL and 16.4 ± 0.89 g/dL, respectively. Both NO-CMCs (7% NO-CMC and 8% NO-CMC) reported decreased levels of Fib by 7.1 and 9.4%, respectively, compared with blood alone. No significant differences were noted between the tested biomaterials and blood alone (Figure 9). Fib determination with thrombin clotting time originates from the method identified by Clauss. The Clauss method is a functional assay based on the time required for fibrin clot formation.

![Graph showing Fib levels of each tested biomaterial](image)

**Figure 9:** The mean expression of Fib of each tested biomaterial. The error bars indicate the S.E.M. ($n = 14$).

To complement the blood coagulation test outcomes, the coagulation profiles of PT, APTT, TT and Fib were measured. These are the parameters that are commonly measured in blood coagulation profiles to diagnose certain disorders. These tests are sensitive to both 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 the intrinsic, extrinsic and fibrin formation pathways in plasma to the presence of chitosan. All the tested biomaterials promoted coagulation compared to blood alone (Figure 8 & 9). However, the findings of this study do not support the erroneous prolongation of PT and APTT levels because this test measured whole blood counts before the blood was subjected to further testing. The normal values of TT suggested that no anticoagulants were present in the blood samples. The coagulation profiles were not significantly different among the tested biomaterials, with the exception of PT. At times, PT and APTT are likely to be prolonged if the blood samples contain high levels of hematocrit (55%) or are collected in under-filled collection tubes, which is why hematocrit levels must be considered in this analysis. Therefore, the hemostasis induced by chitosan did not involve the normal blood coagulation cascade that leads to fibrin formation. In recent research, sulfated
chitosan was shown to supply sufficient hemostatic effects to accelerate coagulation (Subhapradha et al., 2013).

The present results are also consistent with those of other studies such as that by Romani et al., who showed that sugar-modified chitosan measured by coagulation profiles did not affect coagulation pathways (Romani et al., 2013), supporting the idea of chitosan-modified biomaterials functioning independently without intervening with existing coagulation mechanisms. Moreover, chitosan also sustains cell proliferation and endothelial adhesions (Khan et al., 2000; Romani et al., 2013), which was attributed to the physical interaction between chitosan and the cell membranes of erythrocytes. Hemostatic capacity conveyed by chitosan biomaterials enhanced only the aggregation of erythrocytes but did not accelerate the activation of the clotting time in normal coagulation pathways (Rao and Sharma, 1997). Future Fib measurement studies on chitosan could lead to a prevention of abnormal fibrinolysis in coagulopathy patients. However, the data in this study do not rule out the possible role of higher Fib levels contributing to a hypercoagulable state. The effect of chitosan biomaterials on coagulation profiles depends on their physical and chemical structure and properties, particularly the amino residues (Okamoto et al., 2003). This fact is very encouraging to support our results because the biocompatible chitosans tested were found to contain N- and O- positions in their structure. In this coagulation profile study, the coagulation ability and coagulation profiles of PT, APTT, TT, and Fib upon two different groups of chitosan biomaterials were successfully demonstrated. Although the chitosan biomaterials were able to enhance the coagulation ability macroscopically, they tended to extend the examined coagulation profiles, which are the crucial screening tests for abnormal blood clotting. All the tested chitosan biomaterials recorded to coagulate within the normal clotting time range by not extending the measurement period.

4 Conclusion

Various formulations of chitosan exhibit different capabilities in vitro based on the chemical composition of the material. We concluded that O-C and O-C 53 were superior to other types of chitosan in achieving hemostasis. The most striking result that emerged from our present data was that O-C was superior to NO-CMC in activating platelets to form the primary hemostatic plug prior to coagulation. Novel O-C and NO-CMC were produced with varying MW, DDA and physical and chemical properties in response to different temperatures and pH levels. The platelet count noticed to decrease upon the adherences of chitosan biomaterials in a time-dependent manner and upon the addition of ADP agonist. The more the number of platelets decrease, the more the chitosan biomaterial capable to entrap platelets on the surface membrane. This suggests O-C group of chitosan have better quality for platelet adherence. O-C bound platelets formed abnormal-specified shapes and clumped on the membrane surface layer by occupying almost 80% of the chitosan surface membrane. Irregular aggregations of erythrocytes into grape-like clusters were successfully observed for all the studied biomaterials. At the same time, O-C induced the expression level of P-selectin protein mol-
Ecule indicate the prompt stimulations of platelets to activate. Both O-C and NO-CMC group of chitosan capable to coagulate within the normal clotting time range. The impact of the tested biomaterials on the coagulation depended on the physical and chemical properties of the chitosan group, particularly on the amine residues. O-Cs exert a combined effect on thrombogenesis by causing platelets to adhere, activate, aggregate and form insoluble fibrin network to strengthen platelet plug formation in normal subjects. However, NO-CMC and O-C were detected to promote blood coagulation not in equivalent level. Based on the outcome of this research, the studied novel O-C and O-C 53 stimulated hemostasis process and worked better and equal to the commercially available lyostypt. Further investigation and experimentation must 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 becoming the most effective naturally obtained biodegradable hemostatic adhesive yet. The relevance of these enhancements in platelet function was underscored by our current findings. Further studies are required to elucidate the precise mechanism of action of chitosan derivatives on platelets.

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Conflicts of Interests

The authors have declared that no competing interests exist.

References


