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In vitro capacity of different grades of chitosan derivatives to induce platelet adhesion and aggregation

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ABSTRACT

Chitosan-derived hemostatic agents with various formulations may have distinct potential in hemostasis. This study assessed the ability of different grades and forms of chitosan derivatives as hemostatic agents to enhance platelet adhesion and aggregation *in vitro*. The chitosan derivatives utilized were 2% NO-CMC, 7% NO-CMC (with 0.45 mL collagen), 8% NO-CMC, O-C 52, 5% O-CMC-47, NO-CMC-35, and O-C 53. Samples of chitosan derivatives weighing 5 mg were incubated at 37 °C with 50 μL of phosphate buffer saline (PBS) (pH 7.4) for 60 min. The morphological features of the platelets upon adherence to the chitosan were viewed using scanning electron microscope (SEM), and the platelet count was analyzed with an Automated Hematology Analyzer. For platelet aggregation, we added an adenosine diphosphate (ADP) agonist to induce the chitosan-adhered platelets. O-C 52 bound with platelets exhibited platelet aggregates and clumps on the surface of the membrane layer with approximately 70–80% coverage. A statistically significant correlation ($p < 0.01$) for the platelet count was identified between the baseline value and the values at 10 min and 20 min. The results indicate that O-C 53 and O-C 52 were able to promote clotting have the potential to induce the release of platelets engaged in the process of hemostasis.

1. Introduction

Since the middle of World War II, half of recorded combat deaths have occurred due to exsanguinating hemorrhage. A military post-mortem study of casualties in Operation Iraqi Freedom (OIF) suggested that up to 24% of all battlefield mortality could be reduced with improved anti-hemorrhaging methods and that 85% of deaths were caused by uncontrolled hemorrhage [1]. The development of new methods or devices for hemorrhage control may contribute to a future reduction in hemorrhage morbidity and mortality [2]. Recently, chitosan-derived antihemorrhage biomaterial, which contains *N*-acetyl glucosamine (found abundantly as a major component in shells of arthropods such as crabs, shrimps, lobsters and insects) [3] was identified as having potential clinical utility.

Chitosan has become one of the most promising local hemostatic agents. It is of particular importance as it functions independently on platelets and normal clotting mechanisms. Chitosan derivatives also possesses amino and hydroxyl groups that permit them to be chemically adjusted by processes such as acylation, *N*-phthaloylation, alkylation, Schiff base formation, reductive alkylation, tosylation, *O*-carboxymethylation, *N*-carboxyalkylation, and graft copolymerization [4,5]. Although there are numerous studies on the hemostatic capacity of chitosan, to the best of our knowledge there are a limited numbers of *in vitro* studies on the capacity of chitosan derivatives acting on platelets as an absorbable surgical hemostatic agent. Driven by the significant role of adherence in platelet response during the hemostasis process, in our present study we conducted platelet adhesion and aggregation tests to characterize platelet capacity in the presence of chitosan.

The major functions of platelets in hemostasis involve their adherence at the sites of vessel injury, activation of internal signaling pathways, and formation of plugs by aggregation and clumping [6]. For platelet aggregation, we have added an ADP agonist to induce chitosan-adhered platelets. We used *N,O*-carboxymethylchitosan (NO-CMC), *O*-carboxymethylchitosan (O-CMC) and Oligo-chitosan (O-C), produced by Standard and Industrial Research Institute of Malaysia (SIRIM Berhad), with a degree of deacetylation of 75–98%. Lyostypt®, a commercial

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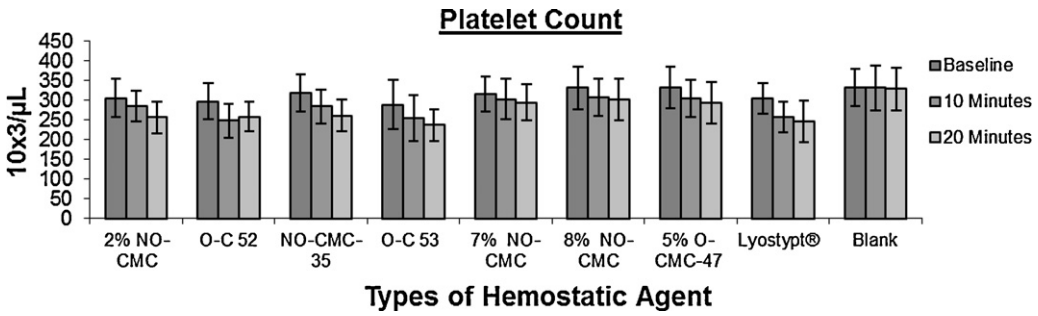

Fig. 1. Mean value of platelet counts upon adherence of chitosan. Error bars indicate standard error of the mean.

hemostatic agent was used as a positive control. Our results show that platelets respond differently to the presence of chitosan derivatives with differing molecular weights and degrees of deacetylation.

2. Materials and methods

2.1. Different grades and forms of chitosan

In this study, different percentage levels of chitosan sponges and two distinct powdered types of chitosan were used. The following sponge forms were used: 2% NO-CMC, 7% NO-CMC (with 0.45 mL collagen), 8% NO-CMC, O-C 52, and 5% O-CMC-47. The powdered forms of chitosan were NO-CMC-35 and O-C 53.

2.2. Subjects

Blood was withdrawn from antecubital veins and collected in vials containing 3.8% sodium citrate. We recruited 30 healthy donors aged 18–40 who had not consumed drugs in the previous two weeks. Informed written consent was obtained prior to blood collection. None of the women were taking oral contraceptives when blood samples were obtained. No healthy donor had a diagnosis of a chronic disease. Subjects were selected based on hematocrit levels between 38% and 45% and a normal platelet count between $150 \times 10^3/\mu L$ and $350 \times 10^3/\mu L$. Platelet aggregation tests were performed within 3 h of sample collection.

2.3. Chitosan preparation

Chitosan samples, each weighing 5 mg, were dissolved or premoistened in 50 μL of (PBS) (pH 7.4) [7] and subjected to incubation at 37 °C for 60 min [8].

2.4. Platelet count

Ten tubes were prepared, and 1 mL blood was added per tube. Blood was introduced to the prepared chitosan samples. A 200 μL 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 [9].

2.5. Preparation of chitosan sample for scanning electron microscope (SEM)

Based on a modification of the method used by Okamoto et al. in their study, platelets were isolated upon differential centrifugation. The isolated platelets were subjected to 30 min of incubation in 12-well tissue culture plates after the introduction of chitosan. Each well was washed with (PBS) infused with penicillin for 2 h. Chitosan was then fixed in 100 μL of glutaraldehyde for 1 h. Each well was washed with distilled water. Different percentage levels of ethanol were introduced into the wells for dehydration. Ethanol solutions of 30%, 70% and 100% were used. Finally, the samples were dried at room temperature and sputtered with gold for examination by SEM [10].

2.6. Platelet aggregation

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 stirred whole blood after the addition of an aggregating agent to the aggregometer cuvette. Samples of whole blood 500 μL each were diluted in normal saline and pre-warmed for 5 min in the incubation well. The base-line was set by 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 $1200 \times g$. CHRONO-LUME® (ADP) 10 μL was added to each sample, and the luminescence gain setting was recorded. The peak luminescence was recorded as amplitude (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 see the change in the shape of the aggregation tracing, usually 2 mm or more per minute. This amount of time allowed the observation of first- and second-wave aggregation for ADP [6].

2.7. Statistical analysis

The data are presented as means with error bars equivalent to the standard error of the mean (S.E.M.). We used repeated-measure analysis of variance (ANOVA) and the correlation coefficient to identify statistical trends. Statistical significance was defined as $p \leq 0.05$ and these values were calculated using SPSS version 18.0 software.

3. Results and discussion

3.1. Platelet count

Fig. 1 showing the most significant increase from the baseline $297.3 \pm 47.19 \times 10^3/\mu L$ was observed for O-C 52: $248.6 \pm 42.20 \times 10^3/\mu L$ after 10 min and $221 \pm 41.07 \times 10^3/\mu L$ after 20 min. The percentage of platelet counts decreased slightly from the baseline (25.66%) over the 20 min (16.38%) time interval. The next largest increase was observed for O-C 53. The percentage

Table 1
Correlation between time intervals (baseline, 10 min and 20 min).

	Baseline	10 min	20 min
Baseline	1	0.900**	0.834**
10 min	0.900**	1	0.938**
20 min	0.834**	0.938**	1

change in the platelet count increased from 11.86% to 17.82%. Table 1 pointing the analysis of correlation indicated that the relationships between the baseline, 10 min and 20 min intervals were strong. The strongest relationship was noted between the 10 and 20 min time intervals, which had $r = 0.94$ and $p < 0.01$.

3.2. Platelet morphology

As shown in Fig. 2B, O-C 52 bound to platelets and clumped on the surface of the membrane layer, approaching 70–80% coverage. Fig. 2C and D shows clumping of platelets at 3500× and 10,500× magnification, respectively. The interaction between platelets is shown by their irregular and pseudopodal shapes. The membrane-covered protrusions from the platelet cytoplasm extended more than 1 μm. Fig. 2E, F and H shows the clumping and aggregation of platelets. Fig. 2F, where 7% NO-CMC was used, is very pale and illustrates the irregular shapes of platelets in the presence of collagen material. Meanwhile, in Fig. 2J, swelling of the fibrin networks can be visualized because Lyostypt® is made up of fine thin layers with highly flexible strands, allowing the platelets to form bridges. Fig. 2A, G and I shows selected control figures for chitosan in sponge, powder and positive control forms, which show the pores for adherence to blood cells.

3.3. Platelet aggregation

O-C 53 is the chitosan for which the lowest mean amplitude was reached, with $1.90 \pm 2.767\ \Omega$ and a difference of $11.50\ \Omega$ compared to the control level. O-C 52 reached the third lowest level with $4.60 \pm 4.926\ \Omega$. Powdered chitosan O-C 53 and NO-CMC-35 exhibited significant results ($p < 0.05$) (Table 2).

As shown in Fig. 3, chitosan adhered to whole blood was first stabilized in the cuvette. The baseline of light transmission prior to the introduction of the ADP agonist is indicated by '1'. The labeled '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 arises (indicated by '5'), when the platelet granule contents, which potentiate the primary aggregation reactions, are released.

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 due to compression [11]. A good hemostatic agent should be inexpensive, easy to utilize, long-lasting and safe. Only minor training in its use should be required. It must be adequate to accomplish the goals of reducing severe bleeding, which can potentially lead to exsanguination, and achieving hemostasis [12]. Previous reports described comparative studies performed in a controlled *in vitro* environment associated with human blood and plasma. These studies were designed to estimate the standard properties of available hemostatic agents. The interactions between the coagulation system and polymer surfaces are highly complex and require proportional blood compatibility of biomaterials [13–15].

Naturally derived polymers have the benefits of biological attributes like cell proliferation and biocompatibility, and their application is an extension of their biological purpose [16]. The utilization of controlled *in vitro* techniques as screening tools aids the process of generating novel hemostatic agents [7]. Chitosan composition is a potential supplementary tool for the investigation of hemostasis [17]. Platelets can adhere to the surfaces of biomaterials. Platelet number counting is an important tool for assessing hemocompatibility as platelet number influences the formation of a hemostatic plug or thrombus [18,19]. Although O-C 53 which is the powdered type of chitosan induced a more rapid reaction among the chitosans studied, O-C 52 in sponge form registered an equivalent result. Actually the parameter 52 and 53 is just a sequence number to differentiate the O-C from sponge and powdered type of chitosan. In this platelet adhesion study, we evaluated the adherence and aggregation of chitosan by studying the morphological features, counts at three different time intervals and platelet aggregation-induced by ADP.

In this study, we were interested in the lowest level of platelet count because, as platelet count is reduced, the chances for the platelets to be attracted to chitosan increase. This measurement indicates materials that may be more effective as hemostatic agents. This positive correlation means that for all hemostatic agents, except for the blank, the platelet counts decreased to a significant degree. The correlation between the baseline and 10 min was characterized by $r = 0.90$ and $p < 0.01$. The observed correlation between the baseline and 20 min was $r = 0.83$ and $p < 0.01$. In our present study, we used Lyostypt® as our positive control. Lyostypt® is made of collagen, which exhibits excellent biocompatibility and has a significant role in primary and secondary hemostasis.

We used collagen-based chitosan in our study as we were aware of the ability of collagen to initiate platelet aggregation at the site of bleeding tissue [20]. Collagens are crucial for platelet adhesion and subsequent activation on the extracellular matrix of denuded endothelium [21]. In our study, we used 7% NO-CMC chitosan coated with 0.45 mL ovine collagen. Collagen was mixed with O-C in predetermined compositions and freezes dried to obtain a porous structure. No significant result was observed in comparison with O-C due to its molecular weight <45 660 Da. We found that chitosan enhanced blood coagulation [10] due to its considerable properties as a functional, renewable, nontoxic, bioabsorbable [22] and biodegradable biopolymer [23].

SEM analysis showed that platelets adhered to one another, clumping into irregular shapes and elongated pseudopod forms, depending on the chitosan material presents. These chitosan materials varied based on their degree of deacetylation and molecular weight. O-C 52 appeared yellow in color, and the surface of the material was harder than those of other types of chitosan. This distinction probably appeared because O-C 52 was exposed to slightly higher localized temperatures during the production stage. Platelets formed abnormal shapes and exhibited varying degrees of surface roughness and surface wettability (hydrophilic and hydrophobic surfaces) [24]. The shapes of the platelets changed from discoid-shaped resting cells to spiculated spheres. The shape change was followed by platelet aggregation and granule secretion which led to the release of more ADP and several other substances [25].

As mentioned above, we used ADP in the platelet aggregation test as an agonist to induce platelet aggregation. ADP is probably 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 platelets, leading to the primary wave of aggregation. The secondary wave reflects the release of ADP from platelet storage granules. Low-dose ADP induces only primary aggregation, and the effect is reversible. The molecular mechanism of the effect of ADP on platelets remains unclear. To verify the hypothesis that ADP reduces platelet counts, we have added ADP as an agonist to

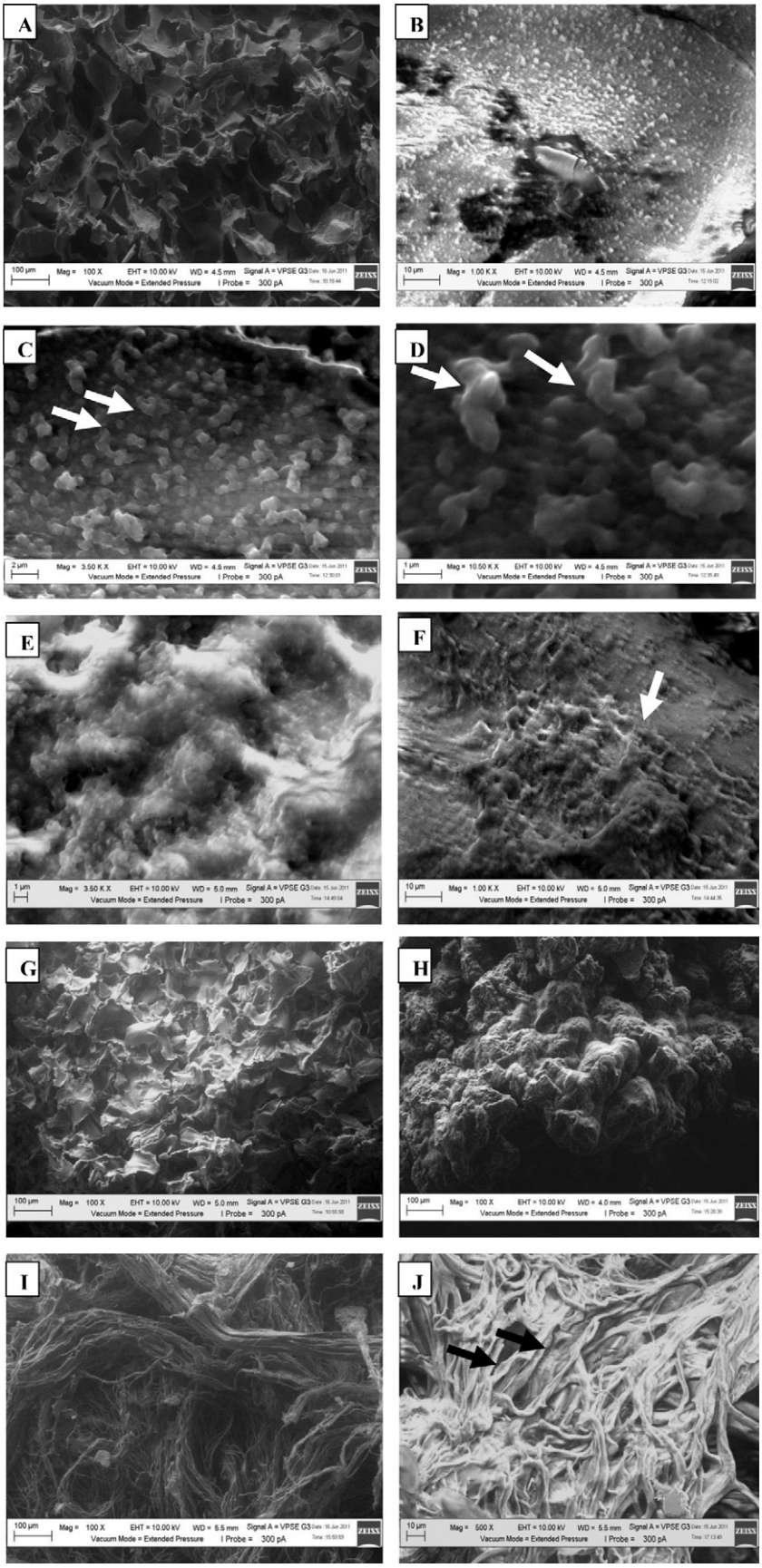

Fig. 2. Effect of different types of chitosan on platelet morphology, as shown by SEM analysis (A) O-C 52 (Control) (100×), (B) O-C 52 (1000×), (C) O-C 52 (3500×), (D) O-C 52 (10,500×), (E) 8% NO-CMC (3500×), (F) 7% NO-CMC (0.45 mL of collagen) (1000×), (G) O-C 53 (control) (100×), (H) O-C 53 (100×), (I) Lyostypt® (Control) (100×) and (J) Lyostypt® (500×).

Table 2
Mean values of chitosan-adhered platelet aggregation induced by ADP.

Types of hemostatic agents and control	Amplitude (Ω)	Types of hemostatic agents and control	Amplitude (Ω)
2% NO-CMC	11.90 ± 4.202	5% O-CMC-47	3.40 ± 2.066
Control	18.00 ± 4.830	*Control*	9.70 ± 5.813
7% NO-CMC	9.60 ± 5.461	NO-CMC-35	3.50 ± 5.759
Control	15.60 ± 5.038	*Control*	12.30 ± 6.343
O-C 52	4.60 ± 4.926	O-C 53	1.90 ± 2.767
Control	13.30 ± 3.020	*Control*	13.40 ± 8.168
8% NO-CMC	12.40 ± 5.502	Lyostypt®	7.40 ± 4.575
Control	17.80 ± 8.804	*Control*	14.24 ± 6.523

Error bars indicate the standard error of the mean.

induce the platelet aggregation of blood samples adhered to chitosan derivatives.

O-C 53, NO-CMC-35 and O-C 52 were the chitosan groups that showed significant results, as indicated by the lowest amplitude mean level ($p < 0.05$). 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, we compared each sample with a negative control to ensure that the ADP agonist did not provide false positive results. To obtain optimal results, all studies were performed within 2 h of blood collection. Storage time was avoided because the physiological integrity of platelets decreases with prolonged storage [26]. Examples of changes in light transmission during ADP-induced platelet aggregation in the presence of O-C 53 and Lyostypt® are shown in Fig. 3. This result shows that O-C 53 and Lyostypt® perform their functions as hemostatic agents but that these *in vitro* platelet aggregations do not precisely reflect *in vivo* platelet function.

Although chitosan was found to form coagulum, the adherence and aggregation of platelets did not precisely reflect blood coagulation. The effects of chitosan on the coagulation profile are due not only to physical consequences, but are also associated with their chemical structure, particularly the amine residue [10]. This result indicates that the amine residue is important in the aggregation of platelets to form a clot. Once the platelets crosslinked and formed pseudopods, the pore sizes of the composite scaffold of the chitosan became smaller, a three-dimensional network structure was formed and the microstructure scaffold density increased slightly [16].

Chitosan was shown to induce significant induce platelet adhesion and aggregation at 5 and 30 min in a concentration-, time- and dose-dependent manner in addition to activating intrinsic blood coagulation [24,27]. The experimental results explain the interaction of platelets to chitosan in damaged tissues [24]. Relative to NO-CMC-35, O-C 53 attracted more platelets to form bridges, most likely because the degree of crystallinity of O-C 53 is higher [28]. The broad, irregular spread of platelets ensures firm adhesion in an irreversible mode. This effect is desirable for the development of a superior thrombogenic state by inducing platelet aggregation and accumulation at the site of bleeding [29]. The degrees of modification to the cell shape and spread area are associated with the surface energetics of the polymer materials [30]. Chitosan adherence may

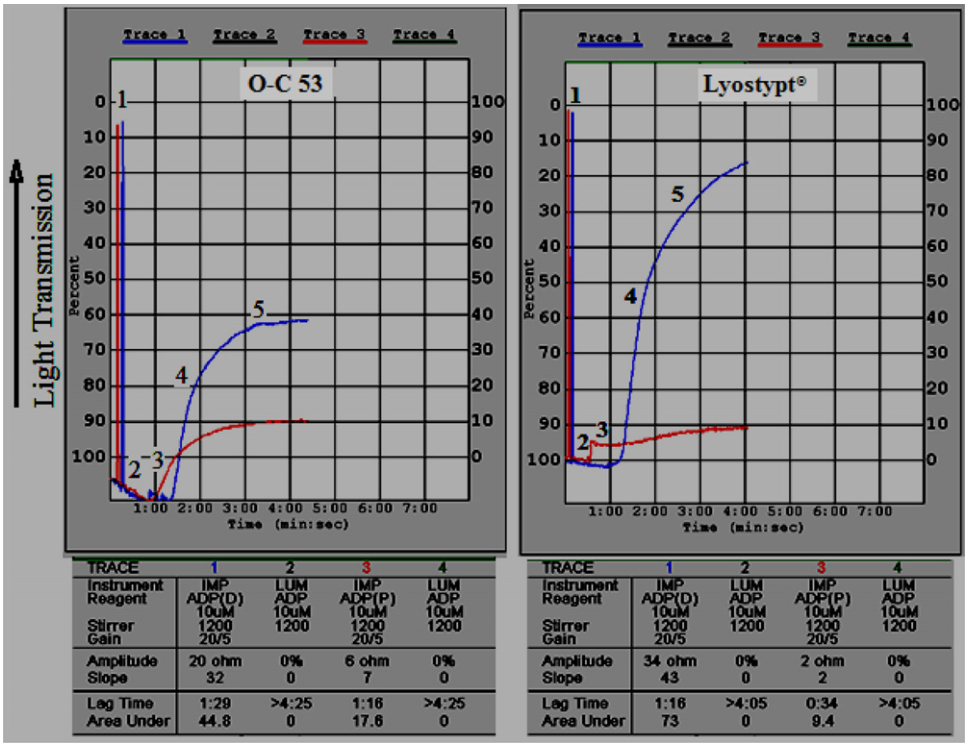

Fig. 3. Examples of changes in light transmission during platelet aggregation induced by ADP upon the presences of O-C 53 and Lyostypt®. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

be able to induce intracellular signaling reactions for the activation of Glycoprotein IIb/IIIa and discharge of thromboxane A2/ADP, which enhances platelet spreading and stability of adhesion [31].

4. Conclusion

Chitosan derivatives exert a combined effect on thrombogenesis by causing platelets to aggregate and form pseudopodal shapes. Various formulations of chitosan exhibited different capabilities *in vitro* based on the chemical composition of the material. We conclude that O-C 52 and O-C 53 were superior to other types of chitosan in achieving hemostasis. Further studies are needed to elucidate the precise mechanism of action of chitosan derivatives on platelets.

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