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Effect of the Novel Biodegradable *N*, O-Carboxymethylchitosan and Oligo-Chitosan on the Platelet Thrombogenicity Cascade in von Willebrand Disease

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ABSTRACT

Introduction: Von Willebrand disease (vWD) is the second least common hemostatic disorder in Malaysia, and it has a low prevalence. This study examined the underlying platelet thrombogenicity cascades in the presence of different formulations of chitosan-derivatives in vWD patients. This paper aimed to determine the significant influence of chitosan biomaterial in stimulating the platelet thrombogenicity cascades that involve the von Willebrand factor, Factor 8, Thromboxane A_2, $P2Y_{12}$ and Glycoprotein IIb/IIIa in vWD.
Materials and methods: Variable chitosan formulations of *N*,O-Carboxymethylchitosan (NO-CMC) and Oligo-Chitosan (O-C) were tested. Fourteen vWD subjects voluntarily participated in this study after signing informed consent forms. The patient's demographic profiles, family history, type of vWD, clinical symptoms and laboratory profiles were recorded and analyzed. Enzyme-linked immunosorbent assay, flow cytometry and Western blot tests were used to determine the level of the chitosan-adhered-platelet-mechanisms.
Results: The study revealed that most patients were predominantly affected by vWD type I. The O-C group of chitosan's scaffold pores is sufficient to allow for nutrients and cells. The O-C-stimulated-mediators are capable of initiating the platelet actions and were detected to expedite the blood coagulation processes. The oligo-group of chitosans was capable of amplifying and triggering more platelet activator's pathways via the studied mediators. The present findings suggest that the ability of each type of chitosan to coagulate blood varies depending on its chemical composition.
Conclusion: The oligo group of chitosans is potentially capable of triggering platelet thrombogenicity cascades by activating platelets in vWD patients to form a platelet plug for hemostasis process.

1. Introduction

Von Willebrand disease (vWD) is the second least common hemostatic disorder in Malaysia, following only hemophilia, and it affects both sexes. It occurs at a rate of 0.002% of the 30 million in the general population. A total of five hundred fifty-four cases of vWD were reported in the Malaysian Health Registry from 1979 to 2013 [1]. vWD is a bleeding disorder that is caused by a deficiency or defect in a particular multimeric glycoprotein (Gp) addressed as the von Willebrand factor (vWF). An inadequate amount of vWF in the blood can cause low platelet adhesion in response to tissue injury due to the low plasma level of factor 8 (FVIII) from the intrinsic blood coagulation pathway [2]. vWF binds to platelets and subendothelial tissues by acting as an adhesive protein to form the platelet plug with the aid of specific regulators, such as thromboxane A_2 (TXA_2), $P2Y_{12}$ and Glycoprotein IIb/IIIa (GpIIb/IIIa). At

Abbreviations: vWD, von Willebrand disease; Gp, glycoprotein; vWF, von Willebrand factor; FVIII, factor 8; TXA_2, thromboxane A_2; GpIIb/IIIa, Glycoprotein IIbIIIa; MW, molecular weights; DDA, degrees of deacetylation; NO-CMC, *N*,O-Carboxymethylchitosan; O-C, Oligo-Chitosan; SIRIM Berhad, Standard and Industrial Research Institute of Malaysia; USM, Universiti Sains Malaysia; PDN, National Blood Centre; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immune assay; WB, Western blot; min, minutes; NC, nitrocellulose; Ab, antibodies; PBS, phosphate-buffered saline; hr, hours; RT, room temperature; FITC-, fluorescein isothiocyanate-; MFI, mean fluorescence intensity; S.E.M, standard error of means; ANOVA, analysis of variance.

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the same time, vWF also stabilizes the FVIII by adhering to and defending it from proteolysis process by mediating platelet to platelet and platelet to matrix binding [3]. vWD is an incurable disease, and managing this disease remains a difficult task for medical practitioners, particularly stopping a hemorrhage in sufficient time following any sudden injuries. Hemostatic agents are the main biomaterials and substances that are used as antihemorrhagic agents to stop bleeding because they influence the hemostasis mechanism. Among the various types of hemostatic agents exploited in medical intervention are the naturally obtained chitosan-based hemostatic dressings. This is because chitosan-formulated hemostatic agents such as HemCon, Chitoseal, CloSur, Traumastat, SyvekPatch, Celox and QuickClot have already been approved and applied medically [4]. Chitosan biomaterials showed superior thrombogenic effects by causing platelets to aggregate and form pseudopodal shapes by facilitatating the platelet activities to expedite the coagulation processes in normal subjects *in vitro* [5–8]. Chitosan is derived from the exoskeleton of marine resources, such as shrimps, crabs and lobsters. Because chitosans are capable of expediting the hemostatic mechanism, this study was constructed to examine the underlying platelet thrombogenicity cascades in the presence of chitosan-stimulation varying from different molecular weights (MWs) and degrees of deacetylation (DDA) in vWD patients. In our present work, we tested *N*,O-Carboxymethylchitosan (NO-CMC) and Oligo-Chitosan (O-C), which are produced by the Standard and Industrial Research Institute of Malaysia (SIRIM Berhad) with a DDA of 75–98%. Fourteen vWD subjects voluntarily took part in this study upon signing informed consent forms. In this study, the patient's demographic profiles, family history, type of vWD, clinical symptoms and laboratory profiles were recorded and analyzed. These data sources are the basics for verifying the platelet thrombogenicity cascades of vWD for treatment. The risk of vWD is very low among Malaysians, and no studies have been conducted on these groups of patients. The aim of this paper was to investigate the significant influences of chitosan biomaterial in stimulating platelet thrombogenicity cascades involving vWF, FVIII, TXA_2, $P2Y_{12}$ and GpIIb/IIIa in vWD. Knowledge of the various signaling cascades in platelet thrombogenicity is very important for developing a novel therapeutic chitosan-based treatment for vWD. This study mainly highlighted the mechanical action of chitosan-formulated biomaterials, which are capable of altering and elevating the platelet thrombogenicity cascades during hemostasis in vWD patients. This study explored new platelet signals that are involved in the coagulation process, and NO-CMC and O-C based hemostatic agents could be a novel strategy to achieve hemostasis.

2. Materials and Methods

2.1. Materials

Chitosan sponges with variable chitosan formulations (7% NO-CMC with 0.45 mL collagen, 8% NO-CMC, O-C, and a powdered type of chitosans, termed O-C 53) were used. Lyostypt was used as the positive control. The chitosan biomaterials were produced by SIRIM Berhad, with a DDA of 75–98%.

2.2. Chitosan Preparation

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

2.3. Subject Selection

Fourteen vWD patients, aged 18 to 50, who had not consumed any drugs in the previous 2 weeks were recruited. Informed written consent was obtained prior to blood collection. None of the women were taking oral contraceptives when the blood samples were obtained. None of the participants had a diagnosis of chronic disease. Prior to starting the study, ethical clearance was obtained from the Human Ethics Committee of Universiti Sains Malaysia (USM) (Ref Num: FWA Reg. No: 00007718; IRB Reg. No 00004494) and Medical Research & Ethics Committee from Malaysian National Medical Research Registry (Ref Num: NMRR-13-873-17276). All laboratory experiments were performed in duplicate.

2.4. Sampling Time Frame and Sampling Method

The sampling time frame of this study involved Malaysian vWD patients who were diagnosed within the past 5 years (2009 to 2013). National Blood Centre (PDN) patients were identified through their previous medical reports based on their race, age, gender, state and type of vWD. Patients were categorized according to the type of vWD, which ranged from a mild to severe bleeding tendency. Their medical histories were recorded, and the obtained blood samples were subjected to laboratory investigations for research purposes.

2.5. Blood Collection

Blood was withdrawn from the antecubital veins and collected in vials containing ethylenediaminetetraacetic acid (EDTA) and citrated blood tubes. Twelve mL of whole blood was withdrawn from the antecubital vein into 3 vials of EDTA tubes for all studies, except for the expression levels of GpIIb/IIIa and TXA_2. To evaluate these, 3-way stop-cocks were used to collect blood under minimal tourniquet pressure, and the first 1 mL of withdrawn blood was discarded. The remainder of each blood sample was aliquoted into 3 tubes containing trisodium citrate anticoagulant tubes. Subject selection was contingent on having a hematocrit level between 38% and 45% and a normal platelet count between $150 \times 10^3/\mu L$ and $350 \times 10^3/\mu L$ [5].

2.6. Analyses of the Demographics, Family History, Clinical Symptoms, Type and Laboratory Profiles

This analysis was conducted with the purposes to study the demographics, family history, clinical symptoms, type and laboratory profiles of the participated vWD patients. Fourteen vWD patients were categorized based on their gender, age category, race, type of vWD and laboratory findings. The analyses were expressed in percentages. The data sources obtained via this study are the actual basic needs to verify the platelet thrombogenicity cascades and hemostatic capacity of vWD patients upon the adhesion of chitosan biomaterials.

2.7. Laboratory Profile Measurements of vWF, FVIII and TXA_2

Blood samples were collected in BD Vacutainer [K2 EDTA 3.6 mg (REF 367842)] tubes as described above. The levels of chitosan-adhered vWF, FVIII and TXA_2 were measured using enzyme-linked immune assay (ELISA) kits [vWF (Cat No. CSB-E08437h), FVIII (Cat. No. CSB-E13861h) and TXA_2 (Cat. No. CSB-E09619h) (Cusabio Biotech Co., LTD, China)] respectively. This study conducted to evaluate the expression level of chitosan-adhered vWF, FVIII and TXA_2 in vWD. Among the various types and forms of platelet mechanism, vWF and FVIII were found to be the most significant proteins to determine the level of platelet adhesion, activation and aggregation for hemostasis process. ELISA requires downstream series of procedures and usually performed to detect and quantify the presence or amount of targeted proteins.

The reagent preparation and assay procedures were performed according to the manufacturer's instructions. Hundred μL of prepared standard and samples were loaded on the each well and the plate was covered, using adhesive strips. Sample-loaded plate was incubated for 2 hours (hr) at 37 °C. Hundred μL of Biotin-Ab (1X) were loaded once the standards and the samples were completely removed from each

well. The plate was incubated for 1 hr at 37 °C. Followed by, Biotin-Ab (1X) addition, each well was aspirated and washed with washing buffer (200 μL), 2 times for a total of 3 washes using multichannel pipette for every 2 min. Hundred μL of Biotin-Av (1X) were added to each well and the plate was incubated again for 1 hr at 37 °C. Aspiration or washing procedures were repeated for at least 5 times as described previously. Once, 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. The stock solutions were used to produce a 2-fold dilution series as follows [vWF (400, 200, 100, 50, 25, 12.5, 6.25, and 0) ng/mL; FVIII (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0) ng/mL and TXA_2 (2000, 1000, 500, 250, 125, 62.5, 31.25, and 0) pg/mL]. The undiluted standard served as the high concentration of standard, and the sample diluent served as the zero standard. Once the reactions stopped, the absorbance was determined at 450 nm with 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 and pg/mL. The standard curve was generated by plotting the absorbance for each standard on the y-axis against its concentration on the x-axis,; a 4-parametric logistic curve-fit was plotted through all of the data points. No significant cross-reactivity or interference was observed among the measurement levels [6,9].

2.8. $P2Y_{12}$ Analysis

2.8.1. Sample Preparation

Blood sample collection procedures were performed as described previously. The Aurum serum protein mini kit (Bio-Rad, USA) was used to maximize the resolution of major ADP receptor $P2Y_{12}$ expression using Western blot (WB) techniques. Sample preparation consisted of the following 3 major steps: (1) column setup; (2) sample binding and purification; and (3) collection of purified samples. Purified chitosan-treated samples were then subjected to a protein quantification test.

(1) *Column setup*
Serum protein was placed in a test tube for 5 min to allow resin to settle. Cap was removed; tip was break from column and returned to test tube to start gravity flow in column. Column was washed with 1 mL of serum protein binding buffer using gravity flow. This step repeated for 2 to 3 times. Column was placed in empty 2.0 mL collection tube and centrifuged for 20 seconds at 10000 × *g* to dry resin bed. Collection tube was discarded. Yellow column tip was placed on the bottom of column and placed into a clean 2.0 mL collection tube which labeled "unbound".

(2) *Sample binding and purification*
Samples prepared in a separate tube by diluting 60 μL of prepared (Chitosan adhered-PRP) with 180 μL of serum protein binding buffer. Two hundred μL of diluted serum was added on top of resin bed in column. Column was vortexed gently and repeated after 5 and 10 min. Column was allowed to sit to an additional 5 min.

(3) *Collection of purified samples*
Yellow tip was removed from column and was returned to the tube. Column was centrifuged for 20 seconds at 10000 × *g* and the protein fraction was collected in "unbound" collection tube. Column was washed with 200 μL of serum protein binding buffer using the same collection tube. Column was again centrifuged for 20 seconds at 10000 × *g* and the protein fraction was collected in the same "unbound" collecting tube. Eventually, serum protein column was discarded. Combined fractions contained the albumin and Immunoglobulin G (IgG) depleted serum samples. Prepared samples subjected for further protein quantification and gel analysis.

2.8.2. Protein Quantification

Two microliters of purified chitosan-adhered samples was added to measure the protein quantity (mg/mL). Protein concentrations were determined using a NanoDrop 2000UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) at 280 nm absorbance.

2.8.3. SDS-Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was prepared according to the Bio-Rad protein user manual using the Bio-Rad Mini-PROTEAN II dual slab cell system according to the manufacturer's instructions. The required levels of the protein marker (Precision Plus Protein™ Kaleidoscope™ Standards, Bio-rad, US) (5 μL), sample (20 μL) and control (20 μL), which was the blood alone, were loaded into each well. Electrophoresis was performed for 35 min at 175 V until the tracking dye was within 1 mm of the bottom of the gel. The gel was then visualized with Coomassie blue staining or used straight for WB transfer.

2.8.4. Transfer of Proteins onto a Nitrocellulose Membrane

Before protein transfer, the gel, two filter pad sponges and a Hybond™ ECL nitrocellulose (NC) membrane were soaked in pre-chilled transfer buffer for 30 min. Complete wetting of the membrane is important to ensure proper binding. The protein transfer was then performed by layering one filter pad sponge at the bottom of a Trans-Blot SD Semi-Dry transfer cell apparatus (Bio-Rad, USA), which was followed by the NC membrane, gel and, finally, another filter sponge on top. The transfer was performed at 15 V for 90 min. The membrane was then stained with Ponceau S solution to ensure that the protein bands successfully transferred.

2.8.5. Antibodies to Determine $P2Y_{12}$

All of the antibodies (Ab) for WB testing were purchased from Abcam (England, UK). Primary Ab: anti-$P2Y_{12}$ Ab (rabbit polyclonal to $P2Y_{12}$) (ab82725), 1 : 1000; anti-β actin (rabbit polyclonal to beta-actin-loading control) (ab8227), 1 : 2000; and secondary Ab: chicken polyclonal secondary Ab to rabbit IgG-H&L (HRP) (ab6829), 1 : 2000.

2.9. Western Blot Analysis

The expression level of $P2Y_{12}$ upon the presence of chitosan biomaterial was detected by employing WB testing. WB is one of the elaborated technique detects specific protein presence in the blood and tissue samples. Followed by protein transfer as discussed above, the NC was then incubated and blocked with 5% nonfat milk diluted in phosphate-buffered saline (PBS)-Tween 20 for 2 hr at room temperature (RT) under mild agitation. The membrane was then washed four times with 1X PBS for 10 min and then incubated with the primary Ab ($P2Y_{12}$ or β-actin Ab) in blocking buffer overnight at 4 °C. On the following day, the incubated membrane was washed four times with PBS-Tween 20 (0.1%) for 10 min. The washed membrane was then incubated with the secondary Ab conjugated to HRP in blocking buffer for 2 hr at RT. Then, the membrane was again washed four times with PBS-Tween 20 for 10 min. The enhanced chemiluminescence detection kit (Amersham ECL Select WB detection reagent) was purchased from GE Healthcare life sciences (UK). The peroxidase signals were detected according to the manufacturer's instructions. Briefly, ECL detection reagent was prepared by mixing 200 μL of each detection solution (reagents A and B) and added directly to the side of the membrane carrying the protein, and the incubation was performed for 30 seconds at RT. Excess detection reagent was removed, and the membrane blot was placed and sealed in a transparent plastic wrap. The membrane

was developed with the enhanced chemiluminescence detection system using FluorChem FC2 (USA), and analyses were conducted using AlphaView Software. The band density for each test was compared to the control and the bands were analyzed using ImageJ 1.46 software [provided by NIH (http://imagej.nih.gov/ij/)]. The values were normalized to the β-actin band density [7,10,11]. Three independent experiments were performed to evaluate the $P2Y_{12}$ expression.

2.10. GpIIb/IIIa Analysis

2.10.1. Antibody Preparation

The following antibodies for flow cytometry were purchased from Becton Dickinson [(BD), NJ, USA]: anti-CD 61 (integrin beta 3 chain), PerCP (clone RUU-PL 7 F12 hybridization of mouse P3X63.Ag8.653 myeloma cells with spleen cells from BALB/c mice immunized with purified platelet membrane Gp; CD61 PerCP (340506) and a fluorescein isothiocyanate- (FITC-) conjugated monoclonal Ab to CD41a (clone HIP8, derived from hybridization of mouse P3X63 myeloma cells with spleen cells from BALB/c mice immunized with purified platelet membrane Gp) [CD41a FITC (340929)].

2.10.2. Blood Collection

Twelve milliliters of whole blood was drawn by venipuncture from both healthy donors and vWD patients into 4.5 mL vacutainer tubes containing 3.2% buffered trisodium citrate anticoagulant (REF 363083) using a butterfly needle. To evaluate the GpIIb/IIIa expression, the previously outlined blood collection procedure was used.

2.10.3. Sample Preparation

Freshly drawn blood was subjected to centrifugation at 100 × *g* at RT (20–25 °C) for 15 min. Chitosan samples, each weighing 10 mg, were dissolved in 50 μL of PBS (pH 7.4) and subjected to incubation at 37 °C for 60 min. One hundred microliters of PRP was then mixed with each prepared chitosan sample for 30 min. Ten microliters of PRP was aliquoted in 2 different FACS tubes; the first tube contained unstained cells, and the second tube was incubated with CD61 PerCP (340506) and CD41a FITC (340929) (BD, USA). Stained and unstained cells were incubated in the dark at RT for 10 min. The reaction was stopped with 500 μL of cold FACS buffer. Samples were analyzed immediately or kept at 4 °C and analyzed within 24 hr. The expression of GpIIb/IIIa was analyzed using flow cytometry.

2.10.4. Analysis of GpIIb/IIIa Expression using Flow Cytometry

The expression level of chitosan-adhered-GpIIb/IIIa were quantified using flow cytometer analysis. Flow cytometry is a robust cell counting and cell sorting technique widely being employed in the research field. Flow cytometric measurements of GpIIb/IIIa in platelets were performed with the FACSCanto II (Becton-Dickinson Bioscience, USA) flow cytometer. The process of sample analysis prior to flow cytometric analysis basically consists of the following 5 major steps: (1) chitosan-adhered blood sample preparation; (2) flow cytometer system and reagent setup; (3) cell fixation process; (4) cell permeabilization and immunolabeling; and (5) fluorescent staining and platelet counterstaining. A polygonal gate was created to propose each dot plot pattern. Four regions were proposed and labeled Q1, Q2, Q3, and Q4. The quadrants represent positivity for the following markers: Q1: CD 41a FITC-A; Q2: CD 41a FITC-A and CD61 PerCPA; Q3: unstained/instrument noise; and Q4: CD61 PerCP-A within the GpIIb/IIIa-positive population. At least 10,000 events were analyzed to calculate the mean fluorescence intensity (%) (MFI) of the total platelet population. The detectors were set to logarithmic amplification. The acquisition and data interpretation were accomplished using BD FACS Diva software [7].

2.11. Statistical Analysis

All of the quantitative experimental outcomes are given as the percentages and mean ± standard error of means (S.E.M.). Statistical significance was defined as $P \leq 0.05$, and these values were calculated using the Statistical Package for the Social Sciences (SPSS) software, version 20.0. The significant differences for each tested biomaterial were analyzed using the analysis of variance (ANOVA); paired sample t-test and were expressed as a p value less than 0.05.

3. Results

3.1. Demographics, Family History, Clinical Symptoms, Type, and Laboratory Profile Analyses of vWD

Fourteen patients, between 21 and 50 years of age, were identified from the PDN registry because they voluntarily donated 12 mL of blood after signing informed written consent forms. The median age of the subjects is 28 years. The cohort consisted of 5 males (35.7%) and 9 females (64.3%), resulting in a male: female ratio of 1: 1.8. The age distribution was categorized into 3 different levels, which were 21–30, 31–40 and 41–50 years. Of the 14 patients, 64.3% of the vWD patients were younger than 21–30 years old. Although the Malaysian population has multiple races, the majority of the Malaysian population was of the native ethnicity, referred to as Malays. Eight patients (57.1%) from the Malay race and 6 patients (42.9%) from the Chinese race took part in this study. The majority of vWD cases (11) had blood group of O (78.6%). A family history of a bleeding disorder is the primary risk factor for vWD. The diagnosis of vWD is very challenging, and it fully depends on the family's bleeding history and laboratory diagnoses. Only 9 patients (64.3%) from the study were aware of their family records; the remaining patients were not aware that vWD occurred within their family history. The clinical symptoms of the vWD have been categorized into the following 5 clinical presentations: easy bruising, gum bleeding, epistaxis, menorrhagia and severe hemorrhage. The subjects who participated in this study complained of the aforementioned symptoms for more than one episode. The majority of the patients (71.4%) have experienced bleeding gums and 50% of patients expressed discomfort with menorrhagia and easy bruising difficulty.

Six of 14 patients (42.6%), complained about severe hemorrhages with injury or surgical interventions. The treatment for vWD relies on the severity of the bleeding tendency, which can be classified into the following 4 types: types I, II, III and pseudo-type. Approximately 78.6% of the subjects who took part in this study were diagnosed with type I vWD. Patients with type 1 vWD can lead normal and healthy lives. Although type III is the most serious form of vWD, which is very rare, and only 2 (14.3%) of the subjects presented with this type of vWD. vWF and FVIII are the major laboratory profiles evaluated to detect the deficiency and abnormality levels of vWD. The highest activities of vWF and FVIII were recorded from the ranges of 31-60% (35.8%) and 61-100% (57.2%), respectively, of the 14 subjects (Fig. 1). The clinical severity of vWD ranges from mild to severe and can be closely correlated with the laboratory measurements.

The level of vWF was analyzed using the t-test, expressed as the mean ± S.E.M. vWF was continuously expressed at a lower level upon the adherence of chitosan. The highest mean expression level of vWF was induced by O-C, which was 14.5 ± 1.79 ng/mL. Blood alone recorded the second highest expression, which was 11.1 ± 2.81 ng/mL, followed by O-C. The vWF expression level of the O-C increased 30.6% from the blood alone and decreased compared to blood alone. The biomaterials, 8% NO-CMC, O-C 53, lyostypt and 7% NO-CMC, were reduced by 21.4%, 25.6%, 43.4% and 45.7%, respectively. The vWF expression level of all tested biomaterials was significantly different compared with the blood alone, and the analyses were conducted using the paired sample t-test ($p < 0.05$) (Fig. 2A). O-C was recorded as the highest chitosan-expressed-FVIII, which was 1.06 ± 0.20 ng/mL. OC 53 and blood alone

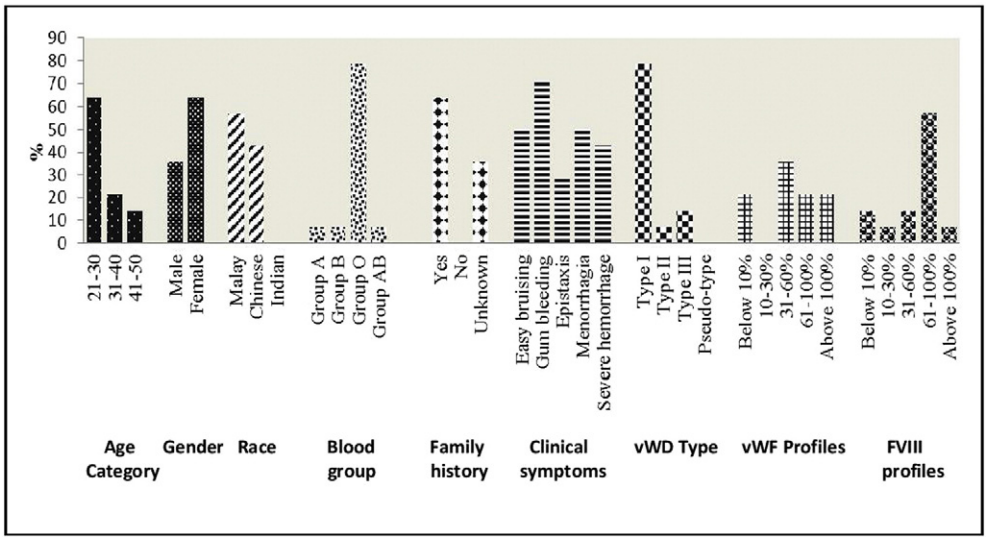

Fig. 1. Percentages of age, gender, race, blood group, family history, clinical symptoms, type of diagnosis, laboratory profiles in vWD subjects; (n = 14).

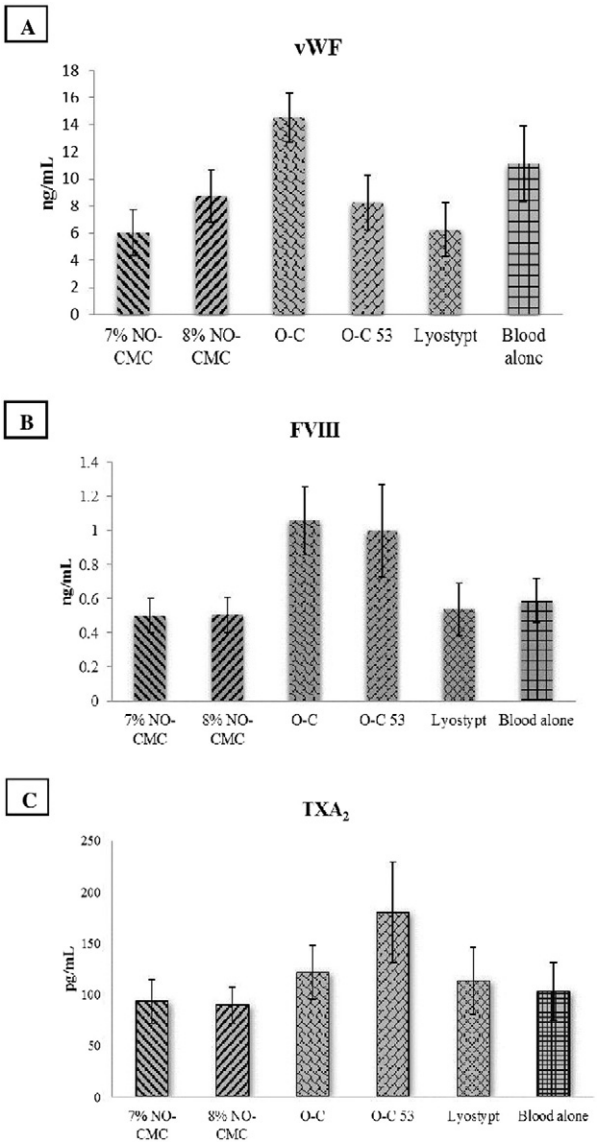

Fig. 2. Mean expression of vWF, FVIII and TXA_2 of vWD patients upon the adherence of chitosan biomaterial. Error bars represent the S.E.M; (n = 14).

were 0.99 ± 0.27 ng/mL and 0.60 ± 0.13 ng/mL, respectively. The remaining tested biomaterials continued to be expressed at the lower point compared to blood alone and the O-C group of chitosans. The lyostypt, 8% NO-CMC and 7% NO-CMC levels for FVIII were 0.53 ± 0.15 ng/mL, 0.50 ± 0.10 ng/mL and 0.49 ± 0.10 ng/mL, respectively. Although the expression level of FVIII varied for each group of chitosan, there were no significant differences within the tested chitosan and blood alone (Fig. 2B). The median expression of TXA_2 was 113.79 pg/mL. Lyostypt registered the highest mean expression for TXA_2 and was followed by O-C 53, whose values compared with blood alone were 18.2% and 10.4%, respectively. Contrary to the expected result, the NO-CMCs, i.e., 7% NO-CMC and 8% NO-CMC, had reduced expression levels compared with blood alone, with mean differences of 9.2% and 12.7%, respectively. There were no significant differences in the TXA_2 expression between each of the tested biomaterials in vWD (Fig. 2C).

The analysis ($n = 3$) for the $P2Y_{12}$ expression in vWD patients was evaluated using WB experimentation. The test outcome revealed that both the O-C type of chitosans (O-C 53 and O-C) and lyostypt were capable of upregulating the mean value of the $P2Y_{12}$ expression by 1.29 ± 0.20-fold, 1.14 ± 0.13-fold and 1.05 ± 0.06-fold, respectively, compared with blood alone. Both NO-CMCs (7% NO-CMC and 8% NO-CMC) expressed $P2Y_{12}$ by displaying very light bands, which resulted in down-regulated expressions, with 0.94 ± 0.03 and 0.87 ± 0.18-fold changes, respectively. The dotted line in Fig. 3 depicts the expression level in blood alone, which corrected to 1 when calculating the band densities in ImageJ, and the values were normalized to the β-actin band density. The reference range of the quantification of this $P2Y_{12}$ protein expression is based on levels below and above 1. The expression level below 1 was equivalent to the downregulation of $P2Y_{12}$, and the expression level above 1 referred to the upregulation of $P2Y_{12}$. Fig. 3A shows the expression levels of $P2Y_{12}$ in the presence of chitosan biomaterials. Fig. 3B shows the representative mean ± S.E.M from 3 independent experiments ($n = 3$). The dotted line depicts the expression level in blood alone (1-fold) (Fig. 3).

The GpIIb/IIIa expressions in vWD patients ranged from 0.12% to 80.6%. The median range detected in the vWD measurement was 9.45%. The expression level of GpIIb/IIIa in vWD patients for blood alone was a very low level (3.45 ± 0.85%). The O-C group of chitosan recorded the highest mean expression levels, O-C 53 (48.2 ± 5.27%) and O-C (33.4 ± 5.21%), respectively. Both oligo types of chitosans had an increased level of GpIIb/IIIa, by 92.8% and 89.7%, compared with blood alone. Subsequently, 8% NO-CMC, lyostypt and 7% NO-CMC had only slight increases in the mean expression of GpIIb/IIIa, which were

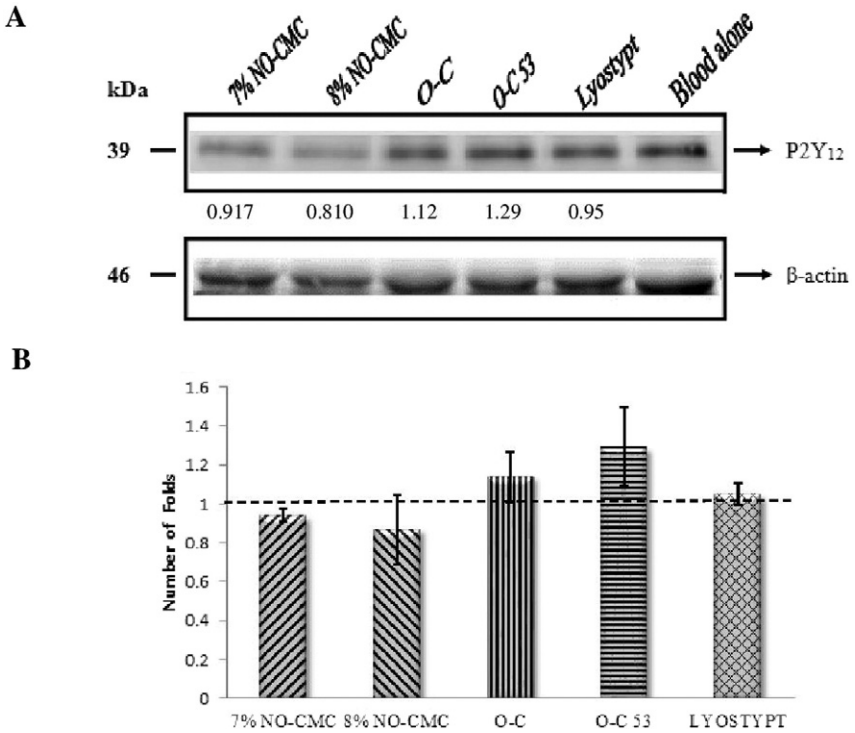

Fig. 3. Expression of $P2Y_{12}$ upon the adherence of chitosan biomaterials in vWD patients using the WB test. [A] The expression levels of $P2Y_{12}$ in the presence of chitosan biomaterials are shown as the fold change compared with blood alone (1-fold). [B] The blots are representative of the mean ± S.E.M from three independent experiments ($n = 3$). The dotted line depicts the expression level in the blood alone (1-fold).

13.6 ± 4.01 %, 6.67 ± 1.31 % and 5.6 ± 1.98 %, respectively. Significant differences were noted for all of the tested biomaterials compared with blood alone, except for 7% NO-CMC ($p < 0.05$); ($n = 14$) (Fig. 4).

The representation of the dot plot results for the tested biomaterials are presented in Fig. 5 (CD 41a FITC-A × CD 61 PerCP-A) and indicate that the population is positive for GpIIb/IIIa. The Q2 region was positive for GpIIb/IIIa expression (values are presented as the percentages and highlighted in red). Blood alone detected GpIIb/IIIa at a lower percentages. The oligo group of chitosan continued to show a positive and higher expression of GpIIb/IIIa among vWD subjects (Fig. 5). Based on the flow cytometry analysis, both antibodies CD 41a and CD 61 were used to determine the GpIIb/IIIa expression, which is depicted in red fluorescence emission in the above dot plots (Fig. 5A-F). The small portion of black-colored dots visualized in Fig. 5D represents the background fluorescence of the antigen-negative cells or other particles that are mostly referred to as noise, unstained cells and debris.

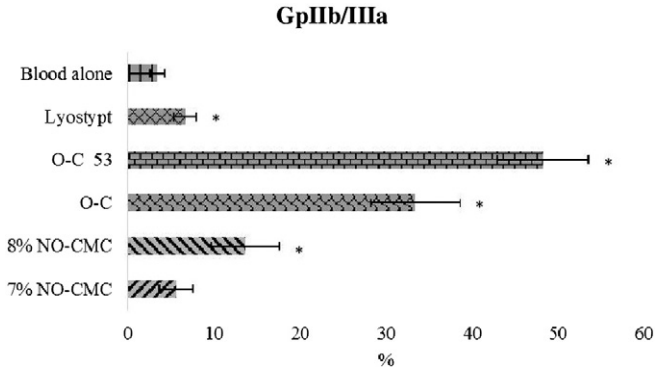

Fig. 4. *In vitro* expression levels of GpIIb/IIIa upon the adherence of chitosan biomaterial in vWD patients. Depicted is the mean ± S.E.M of 14 fluorescence measurements, as obtained by flow cytometry. The independent t-test was used to determine statistical significance ($p < 0.05$)* for the tested biomaterials compared with blood alone, and the values are clearly shown in the above figure.

4. Discussion

vWD develops primarily from the qualitative and quantitative deficiency of vWF, which is a multimeric protein required for the initial platelet adherence. vWF is the primary clotting protein that binds with FVIII to form a platelet plug. Fourteen vWD subjects who were from two different races, were aged 21–50 years old, and met both the inclusion and exclusion criteria were recruited. The prevalence of vWD in the study subjects was highest in Malays and 14.2% lower in Chinese subjects. The prevalence of the vWD subjects was significantly correlated with the gender, age category, type of diagnosis and laboratory findings. Most patients were predominantly affected by vWD type I.

Patients with vWD are generally at a higher risk of hemorrhage, but when it occurs internally, the bleeding can be deadly [12]. In such a scenario, vWD patients require hemostatic dressings to stimulate or trigger their coagulation cascades. A successful biomaterial should possess several important characteristics, such as being biocompatible, antimicrobial, non-toxic, non-carcinogenic, and inexpensive, to promote better drug delivery. Chitosan-derived biomaterials are unique marine polysaccharides that have a variety of physiochemical and biological properties, which allow for their application in various biomedical fields [13]. They have been widely studied as biodegradable hemostatic agents. Among the various types and forms of platelet mechanisms, vWF and FVIII were found to be the most significant proteins for determining the level of platelet activities for hemostasis process. vWF is the primary clotting protein that binds with FVIII to form a platelet plug [14]. O-Cs and NO-CMCs were studied to examine the efficacy of vWF and FVIII blood proteins in vWD patients blood in expediting the coagulation process *in vitro*. The laboratory analysis showed that O-C was capable of inducing the expression of vWF and FVIII to expedite the blood coagulation process in vWD patients. In this study, O-C-triggered-vWF binds to platelets and subendothelial tissues by acting as an adhesive protein, forming the platelet plug. In addition, O-C has a negatively charged surface that induces the clotting activation proteins, such as vWF and FVIII, to form fibrin meshes. As a result, this group of

biomaterials appears to be effective by creating physical compression to block the excessive blood loss in fibrin networks. This statement is also clearly supported by our previous findings which consists of the microscopy evaluation of the formation of fibrin network and platelet plug [15]. The blood absorbability of chitosan biomaterial is solely facilitated by the porous membrane, MW and DDA [8,17]. Based on the scaffold characterization testing which conducted on the previous analysis, the NO-CMC chitosans differ from one another because of their different porosity sizes. However, the pore size of O-C chitosan was smaller than that of 7% NO-CMC. A smaller pore size results in higher platelet attachment, which also elevates the chemical mediator reactions [16]. This hypothesis highly support our other outcomes which conducted on the platelet morphology, cytokine and growth factor release [5,6].

The expression level of TXA_2 was studied to discover the potential for chitosan biomaterial to activate more new platelets and to monitor the beneficial effects of chitosan in assisting with platelet clumping. Compared with the NO-CMC group of chitosans, O-C was discovered to activate TXA_2 expression at higher level compared to blood alone. Previously, Chou et al. suggested that the initial platelet adhesion towards chitosan can generate the intracellular signaling of gpiibiiia and TXA_2 discharge, which strengthens the platelet plug formation [17]. This investigation is the first to establish the association of chitosan-

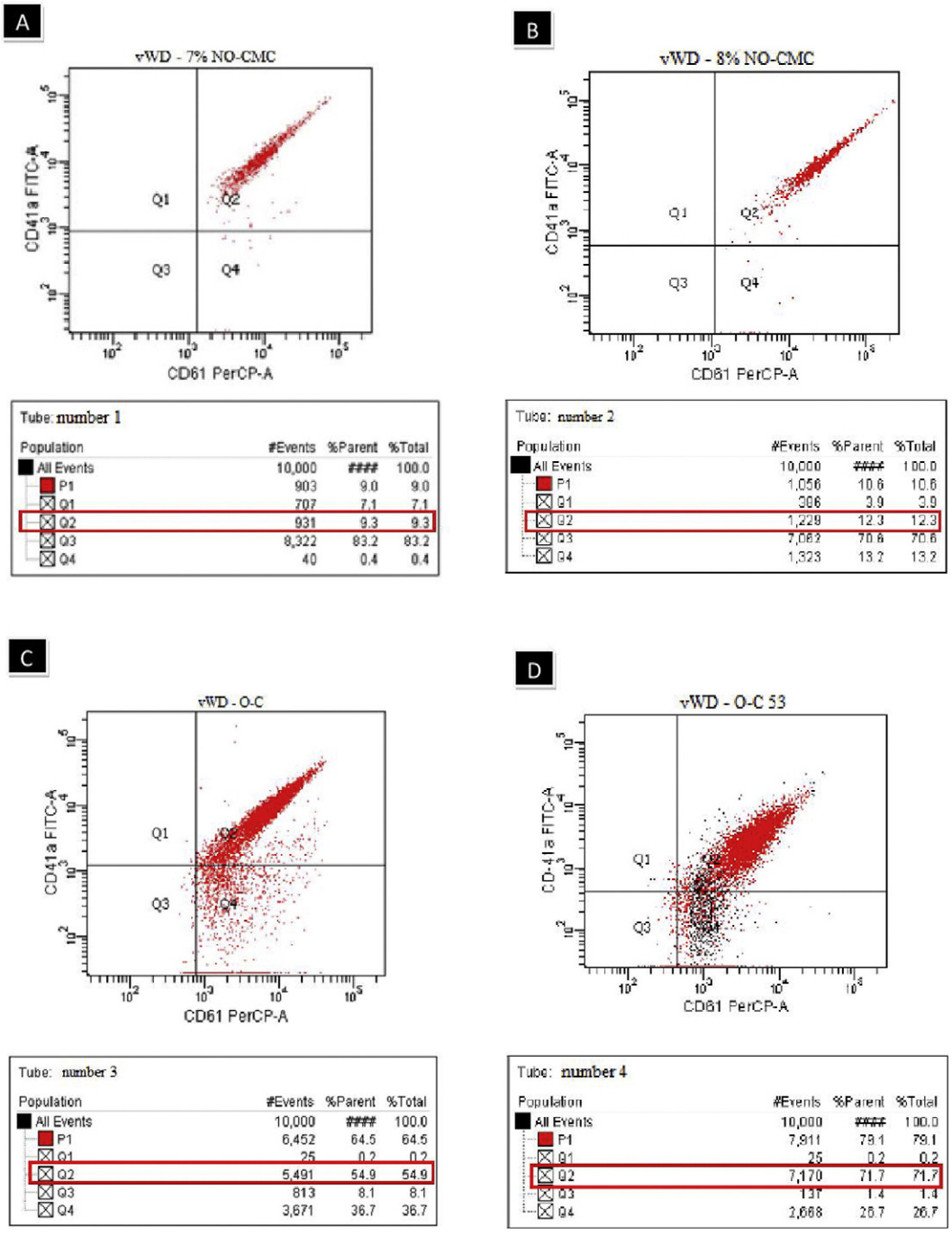

Fig. 5. GpIIb/IIIa expression levels demonstrated upon the stimulation of chitosan biomaterials in vWD patients according to flow cytometry. The expression levels of GpIIb/IIIa are represented by the dot plot results (CD 41a FITC-A × CD 61 PerCP-A), and the GpIIb/IIIa-positive population is expressed in the Q2 region. The events and percentages of each tested biomaterial are indicated by red boxes. The same gating and parameter settings (FITC × PerCP) were used for all tests ($n = 14$); [(A) 7% NO-CMC; (B) 8% NO-CMC; (C) O-C; (D) O-C 53; (E) lyostypt; and (F) blood alone].

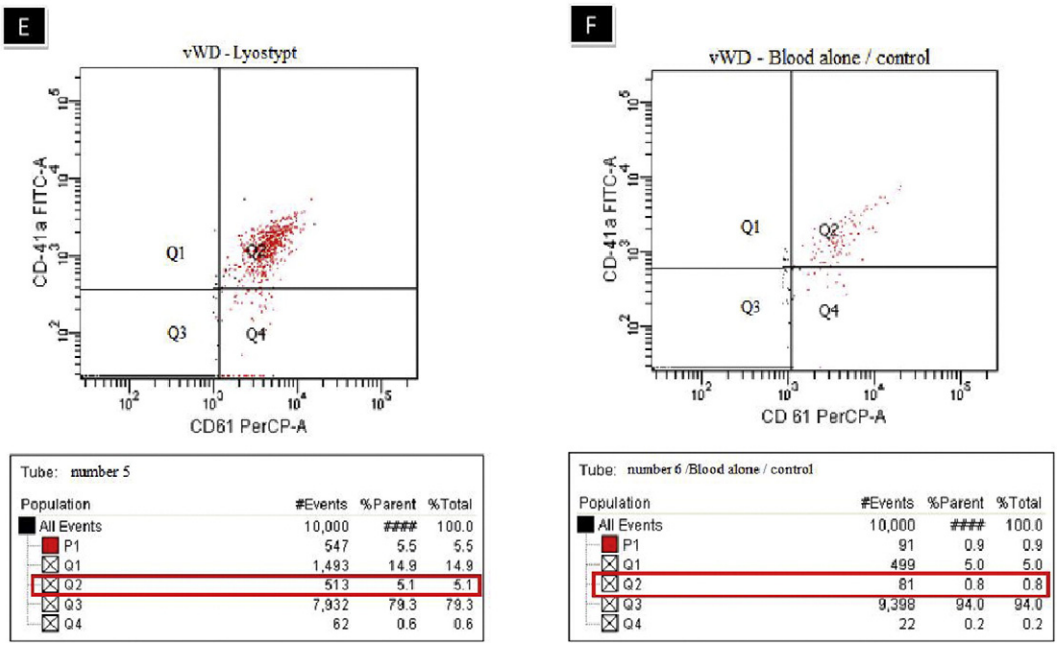

Fig. 5 (*continued*).

mediated-TXA_2 in vWD patients. Based on the outcome of this study, the oligo group of chitosans was capable of amplifying and triggering more platelet activator pathways via the TXA_2 receptor signal. This signaling pathway assists with platelet aggregation. Occupying the TXA_2 receptor leads to a series of downstream events that ultimately induce the expression level of the mediators, mainly platelets, to promptly achieve hemostasis.

The $P2Y_{12}$ expression levels for type II and III vWD could not be analyzed because only one vWD type II patient was recruited and only 2 patients vWD type III patients, who belonged to the same family, were recruited. This analysis requires replicates of at least 3 independent experiments. This part of study aimed to determine the expression level of $P2Y_{12}$ in vWD, not to emphasize the degree of treatments based on the disease type. O-C-induced-$P2Y_{12}$ is one of the main reasons for quick, effective platelet aggregation *in vitro*. Although chitosan biomaterials are thought to work in mysterious ways, the exact mechanical functions have not been revealed in any studies to date. However, the hemostatic properties of chitosan biomaterials rely on several important aspects, including the MW, DDA, solubility, chitosan concentration, positive charge density, chemical structure modification, pH, temperature, incubation period, and hydrophilic and hydrophobic characteristics [5,16,18].

Platelet aggregation is mediated by the GpIIb/IIIa receptor, which is an abundant cell surface receptor and represents 15% of the total surface proteins [19,20]. Additionally, no studies have reviewed the role of GpIIb/IIIa mediators in assisting platelet adhesion and aggregation in the presence of chitosan-derived biomaterials. Chitosan stimulation of GpIIb/IIIa expression varies between individuals. Both 7% and 8% NO-CMCs induced low levels of GpIIb/IIIa expression. For the overall outcome, O-C induced-GpIIb/IIIa expression and function was equal to that of blood alone. In this test, blood samples were collected via the three-way stop cock collection method to minimize the platelet activation upon collection. Although more care was taken during blood collection and preparation, the expression level of GpIIb/IIIa was very low when reviewing the outcome from blood alone in vWD patients. One reason for this low expression level is that the platelet activity and morphologies can be sensitive to the chitosan biomaterials.

The platelets might become activated upon centrifugation and transportation prior to analysis. To address possible errors, platelet morphological studies need to be conducted in the future. Chitosan-derived biomaterials were observed to stimulate and increase the expression level of GpIIb/IIIa compared with blood alone. At this point, among the examined biomaterials, the O-C group of chitosan plays a role in elevating the expression level of GpIIb/IIIa, which mediates the platelet binding capacity to a variety of cells and substrates. O-C-stimulated GpIIb/IIIa could possibly bind to fibrinogen /vWF, which eventually organizes molecular bridges that interconnect aggregated platelets to prevent further hemorrhage. GpIIb/IIIa and $P2Y_{12}$ analysis revealed that the O-C group of chitosan is capable of activating platelets by providing a good surface for blood coagulation mediators and signals to facilitate thrombin generation, which is then able to stimulate platelet activation. This activation further stimulates intracellular calcium mobilization and platelet shape changes. Although the exact mechanism of chitosan has not been clearly outlined in any studies, chitosans were believed to function through vasoconstriction, the rapid mobilization of RBCs, clotting receptors and platelets to achieve hemostasis [8]. The release of coagulation mediators also considered to be dependent on the degree of the membrane injury of platelets [6,8]. Previously, we have employed Fourier Transform Infrared Spectroscopy to analyse the functional groups and scanning electron miscroscopy to examine the scaffold membrane properties of both O-C and NO-CMC group of biomaterials. The absorption band and the significant peaks for each functional group of the chitosan biomaterials were categorized into 17 distinct groups. The functional groups presented in the O-C group of chitosans mimics the standard types of chitosan. Meanwhile, NO-CMC group of chitosan was found to be absent of many functional groups following significant alterations and the deletion of chemical structures. In our study, the mean diameter pores on the entire chitosan scaffold ranged from 50–90 μm. Polygonal and elongated pores were observed randomly in the chitosan scaffolds. Consequently, in this scenario, the integration of the functional groups, structure and the scaffold pore size of chitosan biomaterials typically believed to influence the biological reactions [16].

The present findings suggest that the ability of each type of chitosan to coagulate blood varies according to its MW, DDA, chemical composition, thickness, hardness and biodegradability. However, NO-CMC and O-C did promote blood coagulation at the same level. Based on the outcome of this research, the novel O-C and O-C 53 stimulated the

hemostasis process and worked better and as well as, respectively, the commercially available lyostypt in vWD patients.

5. Conclusions

Chitosan-derivatives with various formulations can alter and stimulate the blood coagulation mechanisms in vWD. vWD is a low prevalence hereditary bleeding disorder that occurs in Malaysia, and most patients are predominantly affected by vWD type I. The NO-CMC and O-C groups of chitosan were investigated in this study. The oligo group of chitosan could trigger platelet thrombogenicity cascades by inducing the expression of vWF, FVIII, TXA_2, $P2Y_{12}$ and GpIIb/IIIa in vWD patients *in vitro*. These adhesive mediators initiate platelet action and were detected to expedite the blood coagulation processes in vWD patients. Further investigation and experimentation are needed to determine the tensile strength and exact mechanical properties of the O-Cs before considering whether they are applicable in the medical field *in vivo*. In the future, these improvements may make chitosan the most effective, naturally obtained, biodegradable hemostatic-adhesive. More advanced studies using animals and *in vitro* models are needed to establish and elucidate the properties of chitosan derivatives. Such research will provide a basis for the use of chitosan in human clinical trials.

Competing Interests

The authors have no competing interests to declare.

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