# *In Vitro* Comparative Hemostatic Studies of Chitin, Chitosan, and Their Derivatives

**Wanida Janvikul,[1] Paweena Uppanan,[1] Boonlom Thavornyutikarn,[1] Jirawat Krewraing,[1] Rujiporn Prateepasen[2]**

[1]*National Metal and Materials Technology Center, Pathumthani 12120, Thailand*
[2]*Scientific and Technological Research Equipment Center, Chulalongkorn University, Bangkok 10330, Thailand*

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**ABSTRACT:** The effects of chitin, chitosan, and their derivatives on *in vitro* human blood coagulation and platelet activation were comparatively studied. The coagulation was assessed by the measure of the whole blood clotting time (WHBCT) and plasma recalcification time (PRT). The tested materials were chitin, chitosan, partially N-acetylated chitosan (PNAC), *N,O*-carboxymethylchitosan (NOCC), *N*-sulfated chitosan, *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride, and SPONGOSTAN® standard (a positive control). The results revealed that the WHBCTs of whole blood mixed with chitin, chitosan, NOCC, or SPONGOSTAN® standard were significantly decreased with respect to that of the pure whole blood (a blank control) ($P < 0.05$), while the WHBCT value of whole blood mixed with PNAC was not significantly reduced. However, the presence of PNAC significantly lowered the PRT value, similar to the addition of chitin, NOCC, or SPONGOSTAN® standard. Chitosan was found to reduce PRT, but not significantly. In the platelet adhesion and activation studies, the morphology of platelets adherent to the film surfaces of tested materials was examined using a scanning electron microscopic technique. Because of their effective coagulation activites, chitosan, PNAC, and NOCC were further evaluated to determine how platelets behaved when in contact with these film samples for given periods. It was found that NOCC activated platelets most effectively. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 445–451, 2006

**Key words:** blood coagulation; platelet activation; chitin; chitosan; derivatives; *in vitro*

## INTRODUCTION

Hemorrhage is one of the most leading causes of deaths of severely injured persons, mainly because of a primitive first aid. The use of gauze dressings, direct pressure, and tourniquets has long been adopted as a method of choice to stop bleeding in prehospital care.[1] New techniques and devices for hemorrhage control have been developed.[2–4] Fibrin dressing was shown to be effective in controlling bleeding from aortic injury in pig model equivalent to sutured repair.[4] However, this dressing comprises blood components derived from human sources; thus, it plausibly presents a high risk of disease transmission. Currently, a number of hemostatic products are commercially available, e.g., SPONGOSTAN® Standard (absorbable gelatin sponge), Surgicel (oxidized regenerated cellulose), Gelfoam (absorbable gelatin sponge), Avitene (microfibrillar collagen powder), and TachoComb (fibrin sealant dressing). Nonetheless, no single product has emerged dominant.[5]

*Correspondence to:* W. Janvikul (wanidaj@mtec.or.th).
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Chitin is one of the naturally abundant biopolymers obtained from renewable resources, while chitosan is a fully or partially deacetylated form of chitin. These versatile biopolymers are known to be nontoxic and biocompatible, enabling them to be used in a variety of application fields such as agricultural, biotechnological, and biomedical fields.[6] Chitosan bandages have been commercially developed under the name "HemCon® Bandage" for the treatment of wounded patients with severe bleeding.[7] Several studies demonstrated that chitin and chitosan had effects on *in vitro* blood coagulation and platelet activation.[8–10] The hemostasis caused by chitosan was, however, found not to be involved in the normal blood coagulation cascade, which results in fibrin formation. It was actually attributed to the physical interaction between chitosan and the cell membranes of red blood cells.[8]

Since chitin and chitosan are not dissolved in water and neutral pH range, preparations of various water-soluble derivatives with or without ionic charges, e.g., *N,O*-carboxymethylchitosan (NOCC) (—$COO^-$), *N*-sulfated chitosan ($-SO_3^-$), *N*-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (HTACC) (—$N(CH_3)^+$), and partially N-acetylated chitosan (PNAC), have been conducted.[11–14] It is of great interest to evaluate hemostatic ability and platelet adhesion and activation of these chemically modified chitin and

chitosan with respect to those of the parent biopolymers since they have never been evaluated for a hemostatic application. Hence, in this article, the *in vitro* comparative assessment of coagulation ability and platelet adhesion and activation of chitin, chitosan, and their derivatives was performed. The derivatives, herein, were those water-soluble derivatives mentioned previously. The effects of each tested material on the blood coagulation were elucidated by the measure of the whole blood clotting time (WHBCT) and plasma recalcification time (PRT) using human whole blood and platelet-poor plasma (PPP), respectively. Platelet adhesion and activation were qualitatively assessed using scanning electron microscopy. As the characteristic of surface of a contacted substrate, e.g., surface hydrophilicity, can essentially govern platelet adhesion and activation, the water contact angles on the film surfaces were also measured.

## EXPERIMENTAL

### Materials

Chitin and chitosan were locally purchased from Bannawach Bioline, Thailand. PNAC, NOCC, *N*-sulfated chitosan, and HTACC were synthesized in our laboratory according to the methods described in the literature.[11–14] The degrees of deacetylation of chitin, chitosan, and PNAC were determined by solid state $^{13}C$ CP/MAS NMR (Bruker DPX-300 spectrometer) and found to be 0.36, 0.97, and 0.56, respectively. SPONGOSTAN® Standard (absorbable gelatin sponge) was used as a positive control in the blood coagulation study. All tested samples were ground to fine particles with a size of ~0.08 mm for the coagulation study.

Only chitosan, PNAC, and NOCC were solution cast into flat films for the platelet adhesion and activation studies and surface contact angle measurement. A chitosan film was prepared by dissolving chitosan in 1% acetic acid generating a 2% w/v chitosan solution. The solution was then cast into a petri dish and dried at 60°C. The obtained film was neutralized by soaking with a sodium hydroxide solution, rinsed several times with deionized water, and air dried. NOCC or PNAC were primarily dissolved in deionized water. The viscous solution was subsequently cast in a petri dish and dried at 60°C overnight. The acquired film was later exposed to saturated steam at 115°C for 15 min. All the films were cut into $1 \times 1$ cm$^2$ pieces prior to use.

### Blood coagulation study

#### Whole blood clotting time

WHBCT was measured using a Lee and White method.[15] In brief, a given tested material was added into three glass test tubes (1.5 cm in diameter, 10 cm in length), 0.01 g each. Fresh blood was collected from a healthy volunteer using a two-syringe technique. About 1 mL of blood initially collected in the first syringe was deliberately discarded, and 3 mL of blood subsequently collected in the second syringe was introduced immediately into the tubes at a volume of 1 mL each. The tubes were incubated at 37°C in a water bath for 5 min prior to the observation of blood coagulation. The blood coagulation was defined as no blood flow when a tube was turned upside-down. The WHBCT was justified as a time from the start of the blood collection until the end of the blood coagulation in all tubes. The experiment was repeatedly performed eight times per each tested sample using blood from eight healthy donors.

#### Plasma recalcification time

PRT was measured using a hook method.[16] In brief, 450 mL of human whole blood donated from a healthy volunteer was collected and mixed with 63 mL of anticoagulant citrate phosphate dextrose adenine solution (206 mg of citric acid (hydrous), 1.66 g of sodium citrate (hydrous), 140 mg of monobasic sodium phosphate (hydrous), 1.83 g of dextrose (anhydrous), and 17.3 mg of adenine). To separate blood corpuscles and obtain PPP, the anticoagulated blood was centrifuged at $5135 \times g$ for 12 min at 4°C. Three hundred microliter of PPP was then added into a glass test tube (1 cm in diameter, 7.5 cm in length) that primarily contained 0.003 g of a given tested material. The whole tube was incubated at 37°C for 1 min. The plasma was then recalcified by adding 30 $\mu$L of 0.5*M* $CaCl_2$ solution. Fibrin formation in the plasma solution was observed by hooking the plasma solution every 30 s. The PRT was counted from the start of the addition of the $CaCl_2$ solution until the first sign of the fibrin formation on a hook. The experiment was repeatedly conducted eight times per each tested sample.

### Platelet adhesion and activation studies

Anticoagulated whole blood was centrifuged at $100 \times g$ for 10 min to obtain platelet-rich plasma (PRP). The anticoagulant used was 3.2% sodium citrate solution. The PRP was immediately employed within 2 h after collected. The chitosan, PNAC, and NOCC films ($1 \times 1$ cm$^2$) were washed three times with deionized water and subsequently rinsed with 0.1*M* phosphate buffer (PB). The films were promptly introduced into the wells of 24-well tissue culture polystyrene plates and fixed to the bottom of the wells with *o*-rings. PRP (650 $\mu$L) was added onto the surfaces of each sample and incubated for 10 or 60 min at 37°C. The samples were washed again with 0.1*M* PB three times to remove any nonadhered platelets and plasma proteins. The adhered platelets were fixed with 2% glutaralde-

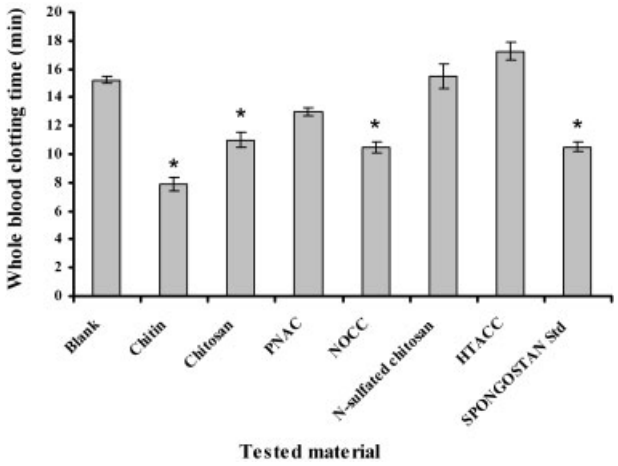


**Figure 1** Effects of all test materials on whole blood clotting time (WHBCT) [*shows significant difference ($P < 0.05$)].

hyde solution in 0.1*M* PB for 1 h. The samples were later washed three times with 0.1*M* PB, dehydrated by ethanol graded series, and dried using a critical point $CO_2$ method. Finally, the samples were coated in vacuum with gold. The platelets on each sample were examined by scanning electron microscopy (Jeol JSM-5410, Japan). The samples were tested in duplicate.

### Water contact angle measurement

The hydrophilicity of the surfaces of chitosan, PNAC, and NOCC films was comparatively evaluated by measuring the contact angles of water on the surfaces using a contact angle goniometer (Model 100–00, Mountain Lakes, NJ). Average contact angles were determined by dropping five successive drops of water on the same spot of the film surface; a contact angle between each drop of water and sample surface was swiftly measured without a receding step as water was quickly absorbed onto the sample, i.e., NOCC film. The five measured contact angles were then averaged. Averaged contact angles on three different positions per film were randomly collected. Each sample test was performed in triplicate. All obtained values were averaged again at last.

### Statistical analysis

Statistical analysis was performed using One-Way ANOVA followed by Scheffe for a multiple comparison with a 95% confidence interval. Significance was at the $P < 0.05$ level.

## RESULTS AND DISCUSSION

The histogram in Figure 1 demonstrates the ability of each tested material to induce the whole blood coagulation, compared with that of the blank control. The results revealed that the WHBCT was significantly reduced ($P < 0.05$) when whole blood was mixed with chitin, chitosan, NOCC, or SPONGOSTAN® Standard, with respect to that of the blank control. In contrast, neither *N*-sulfated chitosan nor HTACC had distinct effects on WHBCT. Surprisingly, the addition of PNAC into whole blood did not lower the WHBCT value much, but yielded significantly shortened PRT, as seen in Figure 2. Besides this material, chitin, NOCC, and SPONGOSTAN® Standard could also decrease the PRT value significantly ($P < 0.05$), while the addition of *N*-sulfated chitosan or HTACC into PPP inhibited the fibrin formation. No fibrins were generated after PPP had been mixed with each of these derivatives for 20 min. Chitosan turned to have no significant effect on PRT. When a material, like chitosan, significantly lowered WHBCT, but PRT, its hemostatic ability should be attributed to the enhancement of agglutination of red blood cells, not the acceleration of the activation of the clotting plasma proteins in the normal coagulation pathways. This was in accordance with the study in the literature.[8]

It was reported that chitosan possessed more effective canine blood coagulation ability than did chitin. However, chitin was more capable of aggregating canine platelets than did chitosan.[9] In this study, chitin appeared to have a greater human blood coagulation ability than did chitosan. This contradictory observation was possibly caused by the differences in blood type used and details of the coagulation testing procedure. In the literature, tested materials were suspended in PB saline prior to the contact with canine blood, not directly added as conducted in this study.

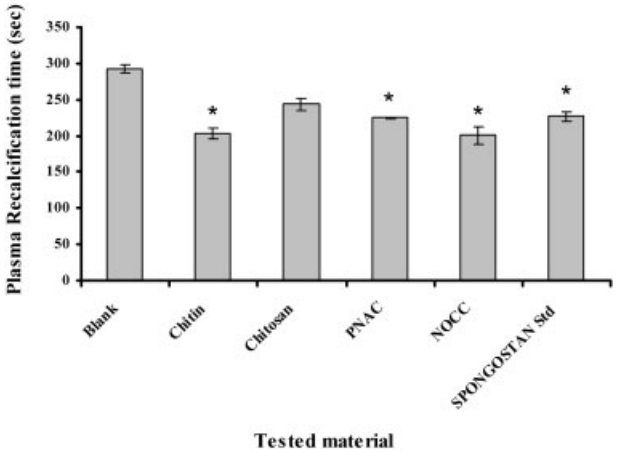


**Figure 2** Effects of all test materials on plasma recalcification time (PRT) [*shows significant difference ($P < 0.05$)] [PPP mixed with *N*-sulfated chitosan or HTACC did not generate fibrins or clots within 20 min (data not shown)].

Moreover, the quantity of the materials in contact with the canine blood was much smaller. The hemostatic performance of chitin seemingly declined when it was subjected to the chemical modifications. The results indicated that chitin possessed the greatest hemostatic ability. Deacetylation of chitin, yielding chitosan, diminished its coagulation ability. Further chemical modifications of chitosan to yield water-soluble derivatives progressively worsened the clotting ability of the resulting offspring, except for NOCC. This derivative could satisfactorily accelerate the blood coagulation. The effect of NOCC on the blood coagulation could be attributed to its physical and chemical properties. In the normal contact activation of the clotting cascade, factor XII (inactive), also termed Hageman factor, is activated on contact with negatively charged surfaces of subendothelial collagen.[17,18] The negatively charged surface of NOCC probably readily induced the contact activation of clotting proteins and initiated the clotting cascade, resulting in the quick formation of fibrins. Although carrying negative charges, *N*-sulfated chitosan prohibited the formation of fibrins. It was reported that sulfated chitosan showed a strong anticoagulant activity, because of its strong surface charge of its functional group similar to the mechanism observed in heparin therapy.[19] The sulfate groups could bind with the positive charges of antithrombin, inactivating thrombin. As a consequence, fibrin formation did not occur. HTACC was also found to prolong WHBCT and inhibit fibrin formation. The positive charges of ammonium chloride groups bound tightly with negative charges on the red blood cell surface, resulting in the separation of the blood cells from plasma as observed during the experiment. Not only negative charges from red blood cells but ones from clotting proteins could possibly tie with the positive charges, inhibiting the normal activation of the clotting cascade.

In normal hemostasis, platelets play a primarily crucial role. They respond to vessel damage by adhering to exposed subendothelial tissues of vessel wall at the site of injury, such as collagen. Later, the platelets at the damage site are physiologically stimulated, resulting in a change in their shape. They extend their pseudopods and become aggregated eventually to form the primary hemostatic plug. Also, they stimulate local activation of plasma clotting factors, generating the formation of a fibrin clot that reinforces the platelet aggregates.[17,18] It has long been known that because of the poor solubility property of chitin, direct uses of this material become limited. Hence, its deacetylated product, chitosan, has gained much more applicative attentions, e.g., development of chitosan bandages for the treatment of wounded patients with severe bleeding. Among the tested derivatives, PNAC and NOCC were further studied to evaluate their abilities to activate human platelets, because of their observed effective hemostatic abilities. Chitosan was selected as a control in this study as Spongostan® Standard is a porous sponge. The platelet activation on each sample was qualitatively assessed based on the morphology of the adhered platelets. Figures 3 and 4 illustrate the platelets after contacting with the surfaces of the samples for 10 and 60 min, respectively. At short-term (10 min) contact of platelets with the samples, the number of platelets adhered on the chitosan film was relatively lowest among the tested materials. Although platelets adherent to the surface of NOCC and PNAC were not much different in number, the platelets on the NOCC appeared more aggregated. At long-term (60 min) contact, the number of platelets attached on the chitosan film increased drastically, roughly about that on the PNAC film. By manual counting from micrographs, the number of human platelets per area ($mm^2$) on the chitosan film was found to be comparable with that reported in the literature.[20] The greatest number of attached platelets was observed on the NOCC film. Most of the platelets observed on the NOCC film possessed numerous pseudopods binding to each other in an aggregating form. This indicated that NOCC attracted and activated the platelets most effectively, probably because of its carboxyl functional group similar to that of collagen fibers in the vascular endothelium. Low density polyethylene modified to have carboxyl groups was also found to promote platelet adhesion and activation more readily than unmodified low density polyethylene.[21]

Several studies revealed that the surface properties of substrates, such as surface hydrophilicity and surface chemical composition, greatly influenced platelet adhesion and activation.[21–24] To understand the effect of surface properties of the samples on the behavior of platelets, the contact angles between water and surface of each sample were measured. As shown in Figure 5, the average water contact angle (31.36 $\pm$ 0.23°) observed on the NOCC film was smallest. The hydrophilicity is inversely proportional to the water contact angle. Thus, NOCC appeared most hydrophilic among three tested materials. The greatest hydrophilicity of NOCC was due to the presence of carboxylate groups. Although chitosan possessed a higher quantity of hydrophilic —$NH_2$ groups, compared with PNAC, its average contact angle (79.72 $\pm$ 0.33°) was slightly smaller that of PNAC (87.46 $\pm$ 1.07°). A difference in a physical property, i.e., crystallinity, between these two materials must be taken into account. N-acetylation of pure chitosan involves the production of a less crystalline sample, namely, PNAC, than solid-state deacetylation of chitin, producing chitosan. The crystallinity of chitosan certainly impeded water spreading onto its sam-

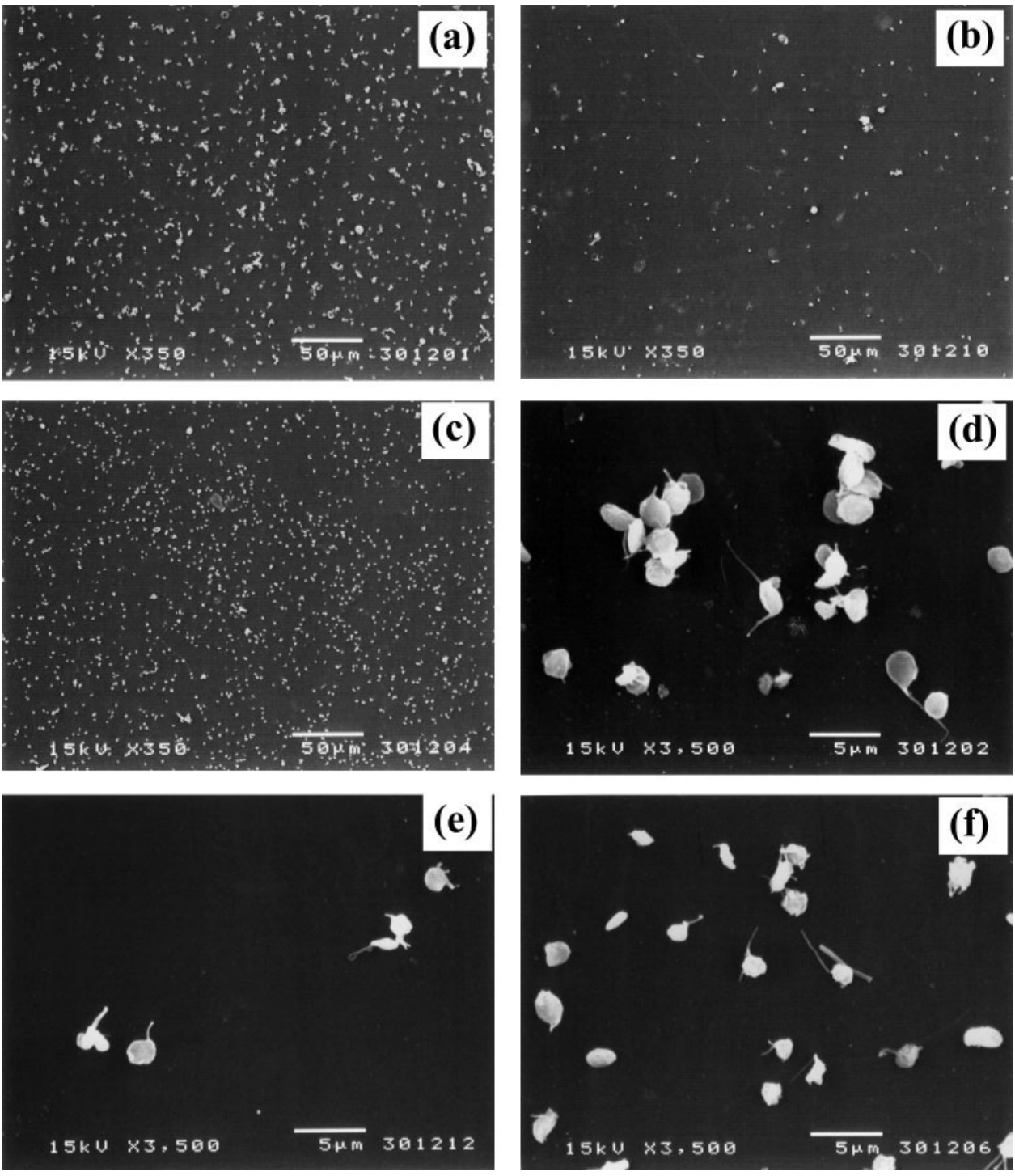


**Figure 3** SEM micrographs of platelets after in contact with samples for 10 min: (a,d) NOCC; (b,e) chitosan; and (c,f) PNAC, at original magnification ×350 and ×3500.

ple surface. As mentioned earlier, once blood vessels are damaged, platelets contact with collagen and become stimulated. Plasma proteins adsorbed on collagen, such as fibrinogen and fibronectin, act as bridges between platelets and substrate surface for supporting adhesion.[17,25] The moderate hydrophilic surface did appear to promote amounts of fibrinogen adsorption, resulting in the increased platelet adhesion and thrombus formation.[23,24] It was likely that the hydrophilic surface of the NOCC film was favorable for the platelet attachment and activation, leading to hemostasis. Consequently, less hydrophilic materials, i.e., chitosan and PNAC, fairly promoted platelet adhesion and activation.

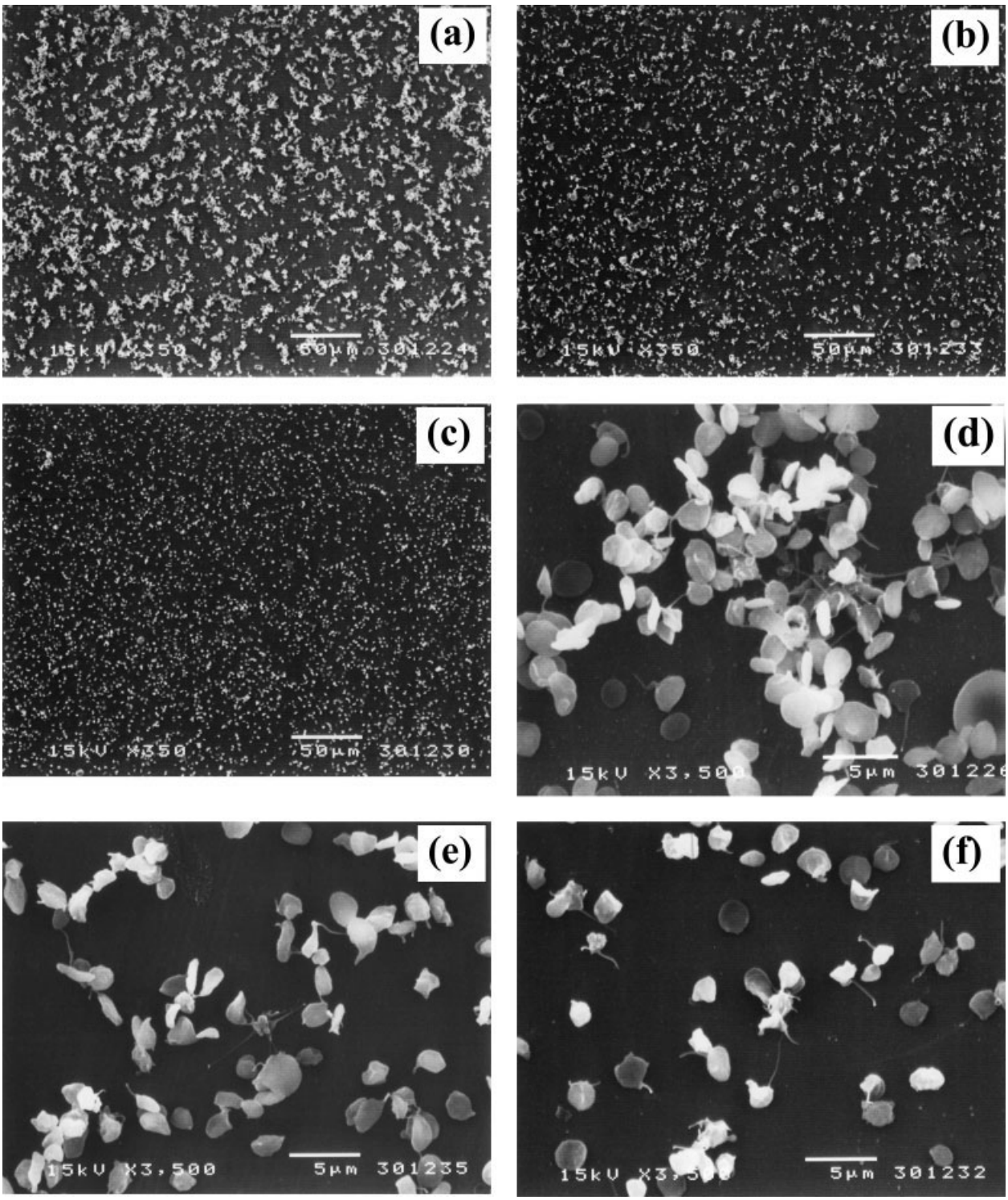


**Figure 4** SEM micrographs of platelets after in contact with samples for 60 min: (a,d) NOCC; (b,e) chitosan; and (c,f) PNAC, at original magnification ×350 and ×3500.

## CONCLUSIONS

It was found that chitin, PNAC, and NOCC could significantly accelerate the *in vitro* human coagulation process by reducing the PRT values; fibrin formation proceeded more quickly than usual. The mechanism of hemostasis caused by chitosan did not follow the normal coagulation cascade as the PRT was not significantly affected by the addition of chitosan. The other tested derivatives had no favorable influence on the WHBCT value and even hindered the formation of fibrins. The hemostatic ability of PNAC and NOCC was more vividly observed in the platelet adhesion

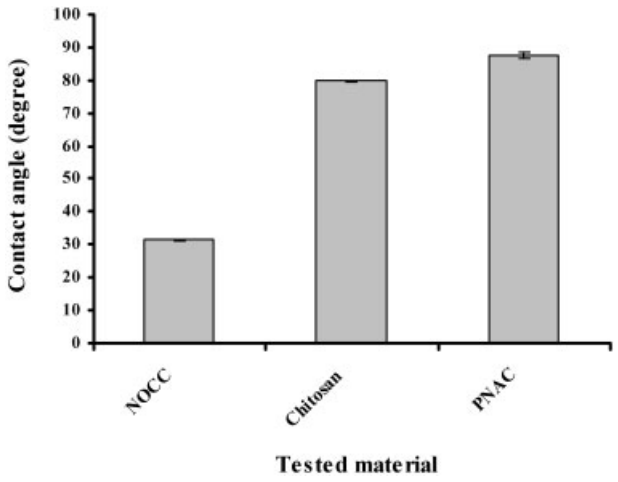


**Figure 5** Average contact angles between water and surface of tested films.

and activation studies. The platelets on the NOCC film were highest in number and most effectively activated, compared with those on the PNAC and chitosan films.

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