Journal of Biomaterials Science, Polymer Edition

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbsp20

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Published online: 17 Jul 2013.

To cite this article: Z. Yalinca, E. Yilmaz, B. Taneri, F. Bullici & S. Tuzmen (2013) Blood contact properties of ascorbyl chitosan, Journal of Biomaterials Science, Polymer Edition, 24:17, 1969-1987, DOI: 10.1080/09205063.2013.816929

To link to this article: http://dx.doi.org/10.1080/09205063.2013.816929

Journal of Biomaterials Science, Polymer Edition, 2013
Vol. 24, No. 17, 1969–1987, http://dx.doi.org/10.1080/09205063.2013.816929

Blood contact properties of ascorbyl chitosan

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(*Received 9 February 2013; accepted 16 June 2013*)

Ascorbyl chitosan was synthesized by heating chitosan with ascorbic acid in isopropanol. The products were characterized by FTIR and C-13 NMR spectroscopies, SEM, and elemental analysis. Blood contact properties of ascorbyl chitosans were evaluated. The ascorbyl chitosans demonstrated to have increased lipid-lowering activity in comparison to chitosan alone upon contact with human blood serum in *in vitro* conditions. Furthermore, the total cholesterol/HDL ratio was improved towards the desirable ideal values after three hours contact with ascorbyl chitosan samples. The lipid-lowering activity increased with ascorbyl substitution. The inherent nonspecific adsorption capability of chitosan due to its chelating power with several different functional groups was exhibited by ascorbyl chitosans as well. This behavior was exemplified in a simultaneous decrease in the total iron values of the volunteers together with lower lipid levels. Furthermore, ascorbyl chitosans were observed to have less hemocompatibility but increased anticoagulant activity when compared to chitosan alone. Additional *in vivo* studies are necessary to support these results and to investigate further the advantages and disadvantages of these materials to prove their safety prior to clinical applications.

Keywords: blood-biomaterial interaction; chitosan; ascorbic acid (Vitamin C); blood compatibility; hypocholesterolemic effect; anticoagulant; iron removal; chelation

1. Introduction

Chitosan is a bio-based polysaccharide which is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine units linked by β-(1–4) glycosidic bond. D-glucosamine units are predominant in chitosan chain and determine its chemical and physical characteristics. [1–6] The hemostatic activity of chitosan and chitosan derivatives have been of research interest due to their potential to be applied as a biomaterial.[5–11] Some chitosan derivatives including sulfated chitosan are anticoagulants.[12–15] Tokura et al. [5,16] produced antithrombogenic medical devices as artificial blood vessels or fibers by immobilization of sulfated chitosan oligomers on chitosan. On the other hand, Malette et al. [17] found that chitosan in acetic acid solution caused blood coagulation even in the presence of a blood thinner agent. A study by Klokkevold et al. [18] also reported that chitosan in acetic acid solution stopped bleeding in rabbits. To date, the exact molecular mechanism of chitosan's hemostatic activity has not been fully elucidated.

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Hence, the exploration of blood contact properties of chitosan and its derivatives under different conditions would contribute to the efforts for the evaluation of chitosan as a biomaterial in this regard.

Chitosan is capable of binding lipids or fats due to its chelating power via the amine and hydroxyl groups. It is edible but indigestible. There are no calories in chitosan.[19–21] Several mechanisms have been proposed to explain the hypocholesterolemic and the hypolipidemic action of chitosan.[2,19–26] These mechanisms revolve around the viscosity effect of chitosan, electrostatic interaction between positively charged amino groups on chitosan and negatively charged lipids or fat molecules, entrapment of lipid and free fatty acids, and inhibition of pancreatic lipase. Among these factors, the viscosity parameter may not be of a critical importance for lipid-lowering action of chitosan.[19,20,26] Regarding possible mechanisms of chitosan as a cholesterol-lowering agent, it should be noted that animal studies might not be predictive of outcomes in humans. Due to the presence of chitinase enzymes in the digestive systems of numerous animals, results from these animals might be different than that of humans.[27] At present, the hypocholesterolemic and hypolipidemic action of chitosan is still elusive.

Ascorbic acid, which has numerous vital physiological roles in biological systems, serves as an efficient antioxidant and functions as a cofactor in various metabolic processes. The physical and chemical instability of ascorbic acid limits its potential application. Ascorbic acid is oxidized and is converted to dehydroascorbic acid by exposure to light, air, and high temperatures.[28] A variety of ascorbic acid derivatives comprising metal salts (Na, Ca salts), ethers, esters, and the polysaccharide-based materials have been synthesized, which act as antioxidant agents. Since ascorbic acid inhibits oxidative modification of low-density lipoprotein (LDL) cholesterol, it is useful for a reduction in the risk of coronary artery diseases.[19,20,22,28,29] Antioxidants may influence coronary artery disease with different mechanisms including improvement in endothelial function, inhibition of platelet aggregation, and reduction in the threat of plaque rupture. Ascorbic acid may contribute to atherosclerosis treatment with reducing lipid peroxidation and increasing antioxidant level.[20,28] Kanauchi et al. [22] reported that the addition of ascorbic acid to chitosan causes a larger increase in fecal fat excretion without affecting protein digestibility.

This study explores the physiological role of chitosan and ascorbyl chitosans as lipid lowering and blood anticoagulate agents. The effect of chitosan and ascorbyl chitosans on total cholesterol, triglyceride, HDL cholesterol, and LDL cholesterol levels were investigated, *in vitro*. In addition to lipid-lowering activity, the effect of these agents on total iron level in human serum was also examined.

2. Experimental

Following materials, instruments, and methods were used to prepare and characterize the samples. In all blood analyses, the blood samples used were obtained from volunteers with their consent.

2.1. Materials

Chitosan (4.0×10^5 g/mol, DD 85%, Fluka, Germany), L-ascorbic acid (Supelco, USA), isopropyl alcohol (99.7%, Aldich, Germany), commercial RTU cholesterol assay kit (bioMérieux, France) for total cholesterol analysis, commercial HDL cholesterol (C-HDL Ultra) assay kit (bioMérieux, France) for HDL cholesterol analysis,

commercial LDL cholesterol (C-LDL Dir) assay kit (bioMérieux, France), and Triglycerides Enzymatic PAP 150 (TG PAP 150) assay kit (bioMérieux, France) (bioMérieux, France) for triglyceride analysis were used. Total iron was measured using a commercial Ferrimat assay kit (bioMérieux, France). The amount of total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride level, and transferrin-bound iron in serum was determined by visible spectrophotometry (BTS 310 (4.0 ver)). MT 1C coagulameter (Medical Technologic Industry) was used for the determination of prothrombin time. The complete blood cell count was measured by using the Medonic CA 530 (20 Parameter system Thor).

2.2. Preparation of ascorbyl chitosans

0.5 g of chitosan and ascorbic acid (0.1, 1, 5, and 100) were added into 100 mL of isopropyl alcohol (75%, v/v). The solution was mixed at 60 °C for 6 h under nitrogen gas to remove any dissolved oxygen from the solution. After 6 h, the polymer was washed with isopropyl alcohol and was dried at 25 °C. The chitosan sample which is used as blank was prepared as 0.5 g of chitosan and 100 mL of isopropyl alcohol (75%, v/v) was stirred at 60 °C for 6 h under nitrogen gas. After 6 h, the polymer was washed with isopropyl alcohol and was dried at 25 °C (Table 1).

2.3. Elemental analysis

An elemental analysis method of samples was employed at TUBITAK-MAM, Gebze.

2.4. FTIR analysis

The FTIR spectra of samples were recorded at TUBITAK-MAM, Gebze with KBr pellets on a Perkin-Elmer Spectrum One FTIR Spectrometer.

2.5. SEM analysis

SEM pictures of the samples and blood contact samples were taken in TUBITAK-MAM, Gebze using a JEOL/JSM-633F scanning electron microscope.

2.6. C-13 NMR analysis

C-13 NMR analysis of the samples was carried out at METU-Central Lab.

Table 1. Preparation conditions for synthesized ascorbyl chitosan samples (in 75% v/v isopropyl alcohol solution using chitosan solution).

Sample ID	Concentration of ascorbic acid (mM)	Composition by weight (% ascorbic acid)
Chi	–	–
ChiVC-0.1	0.1	0.35
ChiVC-1	1.0	3.4
ChiVC-5	5.0	14.9
ChiVC-100	100	78.0

Notes: Chi, Chitosan; VC, ascorbic acid, numerical values are the molarity of ascorbic acid.

2.7. Biochemical analysis

The blood samples used in the biochemical analysis were from volunteers with their consent.

2.7.1. Total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride determination in human serum

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. Ten milligrams from each examined polymer were mixed with 200 µL of human serum for 3 h *in vitro*. Total cholesterol level was determined at 670 nm, HDL cholesterol, LDL cholesterol, and triglyceride levels were determined at 505 nm by visible spectrophotometry (BTS 310 (4.0 ver)).

2.7.2. Determination of serum iron

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. 20 mg of examined samples were mixed with 400 µL of human serum for 3 h *in vitro*. The amount of transferrin-bound iron was determined by BTS 310 (4.0 ver) spectrophotometer at 562 nm. A calibration was performed for each series of tests.

2.7.3. The total calcium determination in blood serum

Blood samples of the volunteers were collected using Vacutest tubes (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing gel and clot activator. The serum was separated by centrifugation for 10 min. Ten milligrams of examined samples were mixed with 200 µL of human serum for 3 h *in vitro*. Ten milligrams of beads were mixed with 100 µL of human serum for 3 h *in vitro*. Calcium test kit (MBT, Biomer) was used. In order to measure the total calcium, 1 mL working reagent, 10 µL of standard, and 10 µL of prepared cell suspension were mixed and incubated for 10 min at 37 °C. Total calcium levels were determined by BTS 310 (4.0 ver) spectrophotometer at 670 nm.

2.7.4. Complete blood count analysis in whole blood

Blood samples of the volunteers were collected using test tubes (Meus Sri PIOVE DI SACCO, Italy) containing K_3EDTA. Twenty milligrams of each sample were mixed with 400 µL of human blood for 3 h *in vitro*. The sampling was carried out by using Medonic CA 530.

2.7.5. Prothrombin time determination in whole blood

For prothrombin time measurements, blood samples of the volunteers were collected using 3.5 mL blood vacutest tube (VACUTEST KIMA Sri ARZERGRANDE, Italy) containing Na-citrate (3.2%, 0.109 M) and plasma was separated by centrifugation for

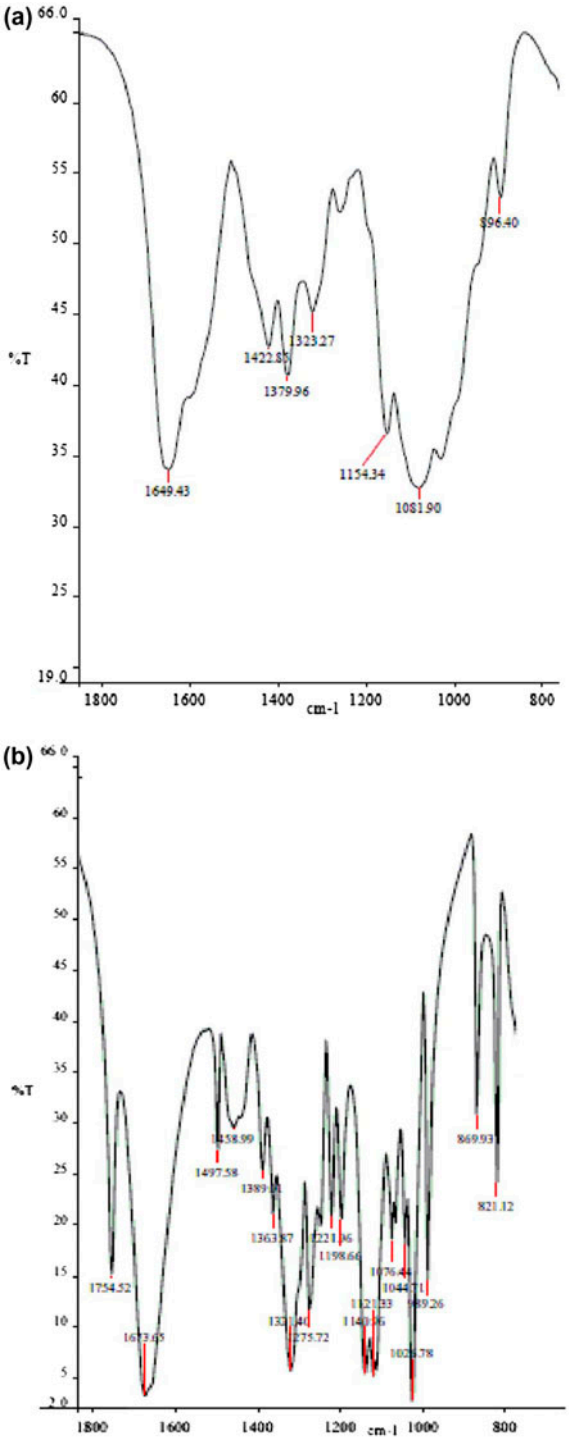


Figure 1. FTIR spectrum of (a) chitosan, (b) ascorbic acid (VC), and (c) ascorbyl chitosan (ChiVC-100).

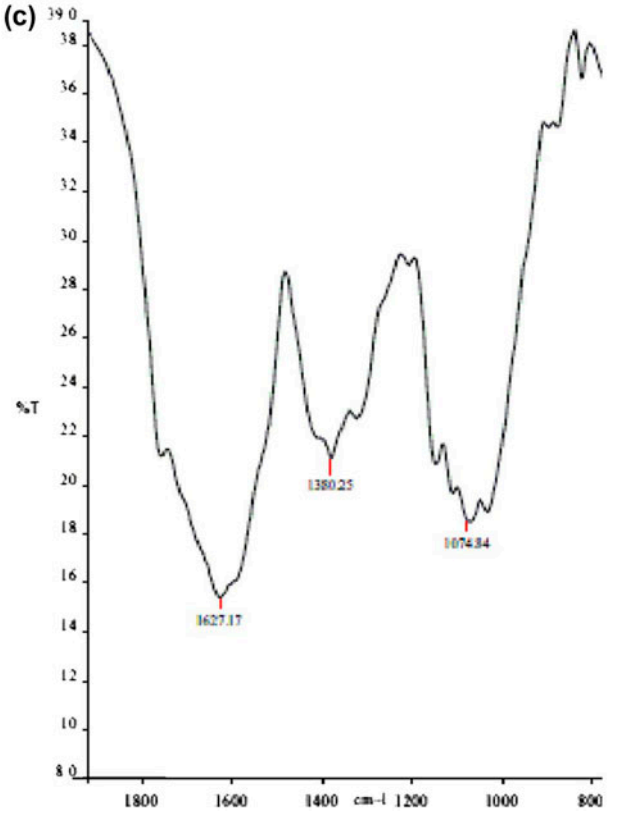


Figure 1. (*Continued*).

10 min. The test was performed by adding thromboplastin reagent (trini clot pt excel, rabbit brain). Ten milligrams from each examined polymer were mixed with 200 µL of whole blood for 3 h *in vitro*. Prothrombin time analyses of the separated plasma were done by MT 1C coagulameter.

3. Results and discussion

3.1. FTIR analysis

In the FTIR spectrum of chitosan shown in Figure 1(a), a characteristic amide band at 1649 cm^{-1}, the C–H bending vibrations 1400–1500 cm^{-1} region, the $–CH_3$ bending at 1380 cm^{-1}, and the pyranose C–O–C and C–OH stretching vibrations of chitosan in the region 1100–900 cm^{-1} are observable. The ascorbic acid spectrum in Figure 1(b) exhibits all characteristic absorption bands of –C–C, –C=C–, C–H, C–O, and O–H linkages in addition to the lactone absorption bands at 1754 and 1673 cm^{-1}. The ascorbyl chitosan shown in Figure 1(c) exhibits the absorption bands at 1720 and 1627 cm^{-1}, which have been taken as evidence of ester and amide bond formation between chitosan and ascorbic acid. The C–H bending vibration at 1400–1300 cm^{-1} region including the $–CH_3$ bending at 1380 $cm^{-1}$and the pyranose ring absorptions are available in the 1100–900 cm^{-1} region, similiar to parent chitosan.

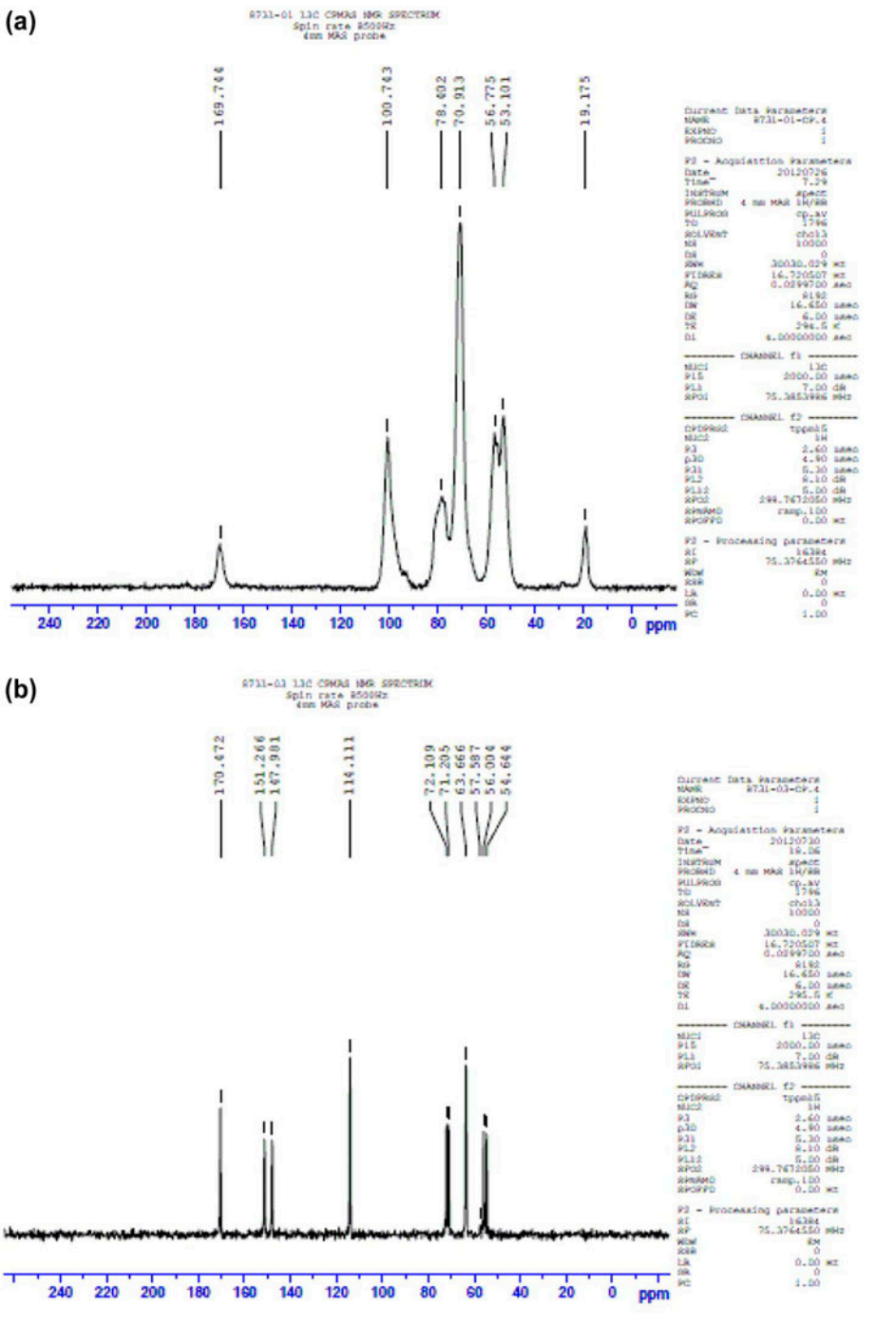


Figure 2. C-13 NMR spectrum of (a) chitosan, (b) ascorbic acid (VC), and (c) ascorbyl chitosan (ChiVC-100).

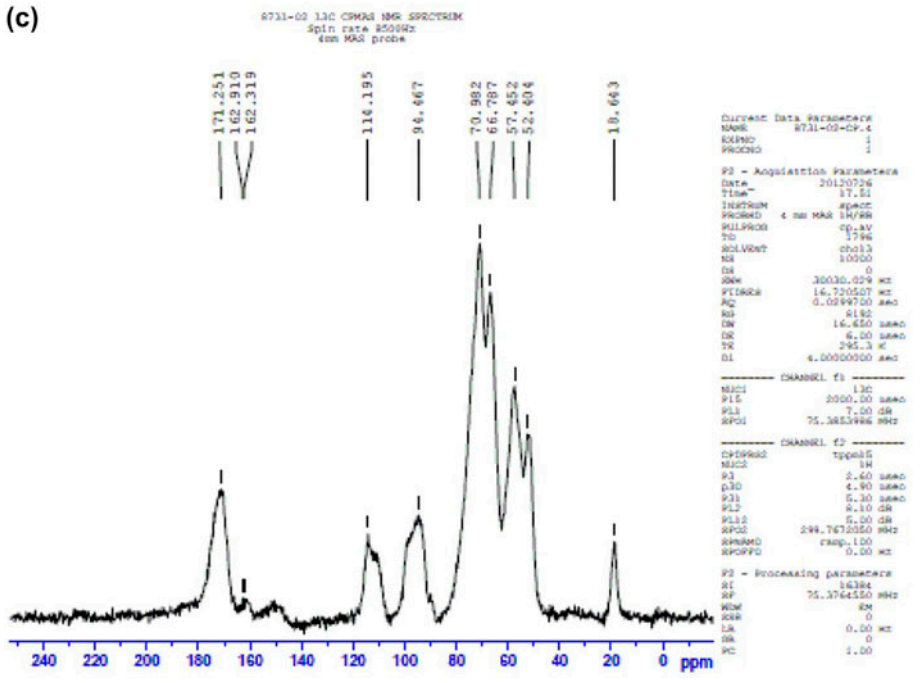


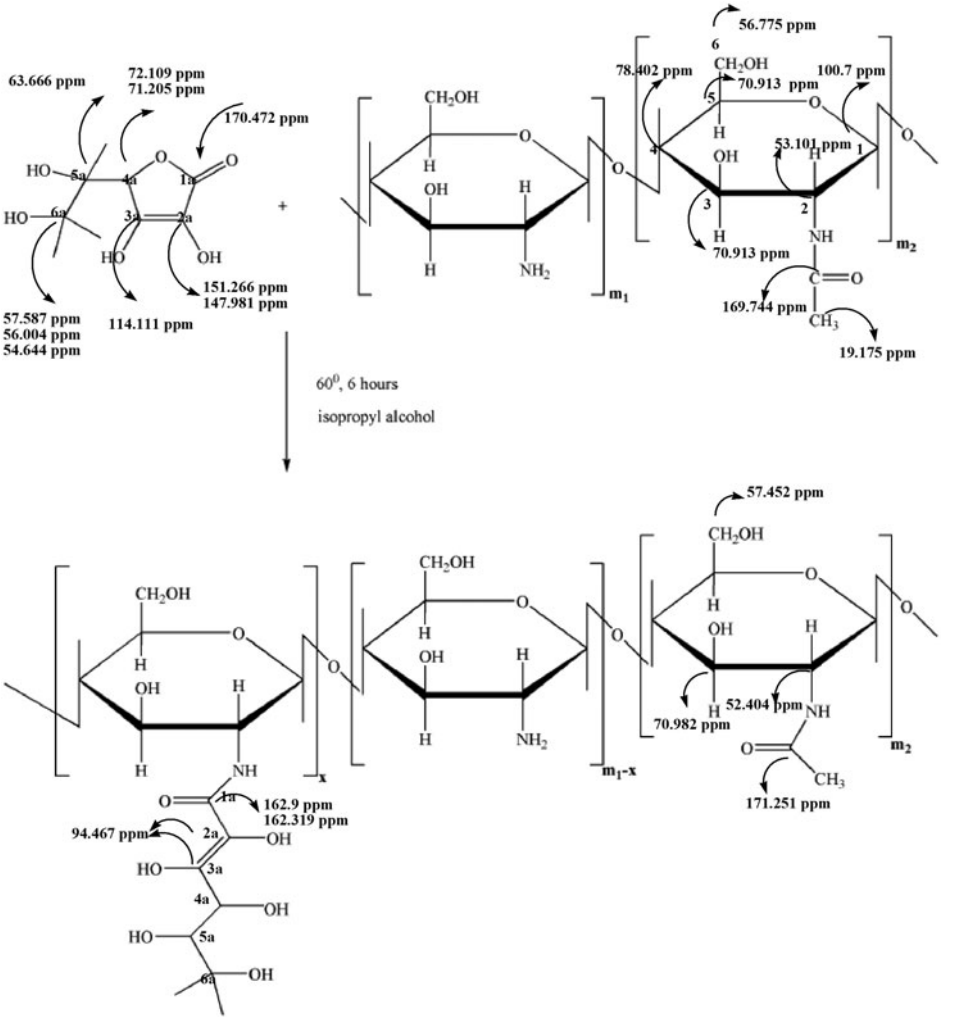
Figure 2. (*Continued*).

3.2. C-13 NMR analysis

C-13 NMR spectra of chitosan, ascorbic acid, and ascorbyl chitosan are shown in Figure 2(a), (b), and (c). In the spectrum of chitosan, the signals at 169.744 ppm and 19.175 belong to the carbonyl carbon and the methyl group of the acetamide groups, respectively. The signals at 100.743, 78.402, 70.913, 56.775, and 53.101 ppm are assigned to the ring carbons: C-1, C-4, C-3,5, C-6, and C-2, respectively. In the spectrum of ascorbic acid, the signal at 170.472 ppm belongs to the carbonyl carbon (C-1). The doublet signals at 151.266 and 147.981 ppm are assigned to C-2. The signal at 114.111 ppm belongs to C-3. The signals at 72.109 and 71.205 ppm are assigned to belong to C-4. The signal at 63.666 ppm belongs to C-5. The triplet signals at 57.587, 56.004, and 54.644 ppm belong to C-6. In the spectrum of ascorbyl chitosan, the carbonyl carbon of the acetamide group of chitosan is observed at 171.251 ppm. New signals appear at 162.910 and 162.319 ppm, which can be attributed to the amide and ester carbonyls of the ascorbyl group. Bis-hydroxy functionalities exhibit themselves at 94.467 ppm. The primary and secondary alcohol carbons of the ascorbyl group and chitosan produce overlapping signals in the 50–70 ppm range. The methyl group of chitosan is observed at 18.643 ppm. The proposed structure of the product based on FTIR and C-13 NMR data is shown in Scheme 1.

3.3. Elemental analysis

Elemental analysis results of ascorbyl chitosan samples are shown in Table 2. Chitosan was reported to have 39.350% C, 7.170% N, and 7.150% H. The ascorbyl chitosan shows decrease in % C, % N, and % H values with increasing ascorbyl group content,



Scheme 1. The proposed structure of the ascorbyl chitosan based on FTIR and C-13 NMR data.

Table 2. Elemental analysis of chitosan and ascorbyl chitosan samples.

Sample ID	C%	N%	H%	O%
Chi	39.350	7.170	7.150	46.330
ChiVC-0.1	39.320	7.140	7.040	46.500
ChiVC-1	39.490	6.970	7.110	46.430
ChiVC-100	38.570	4.050	5.987	51.393

indicating that chitosan repeat units have been modified with ascorbyl group causing an increase in the average molar mass of the repeat unit. The samples were characterized with respect to ascorbyl content by weight and degree of substitution (i.e. degree of ascorbylation), based on elemental analysis results. The theoretical and experimental values are given in Table 3. It should be noted that the accuracy of the experimental

Table 3. Compositions of ascorbyl chitosan samples.

Sample ID	Theoretical ascorbyl content (% weight)	Theoretical degree of substitution (mole fraction)	Experimental ascorbyl content (% weight)	Experimental degree of substitution (mole fraction)
ChiVC-0.1	0.35	0.0033	0.42	0.00465
ChiVC-1	3.5	0.033	2.37	0.0269
ChiVC-100	78	3	43.5	0.853

results are only reliable with the confidence limits of the method used. Hence, a slightly higher experimental value than the theoretical value has been reported for the first sample. The results show that not all of the ascorbic acid present initially has been substituted on the chitosan backbone.

3.4. SEM analysis

The surface morphologies of Chi, blood contact Chi, ChiVC-1, blood contact ChiVC-1, ChiVC-100, and blood contact ChiVC-100 were examined using SEM micrographs

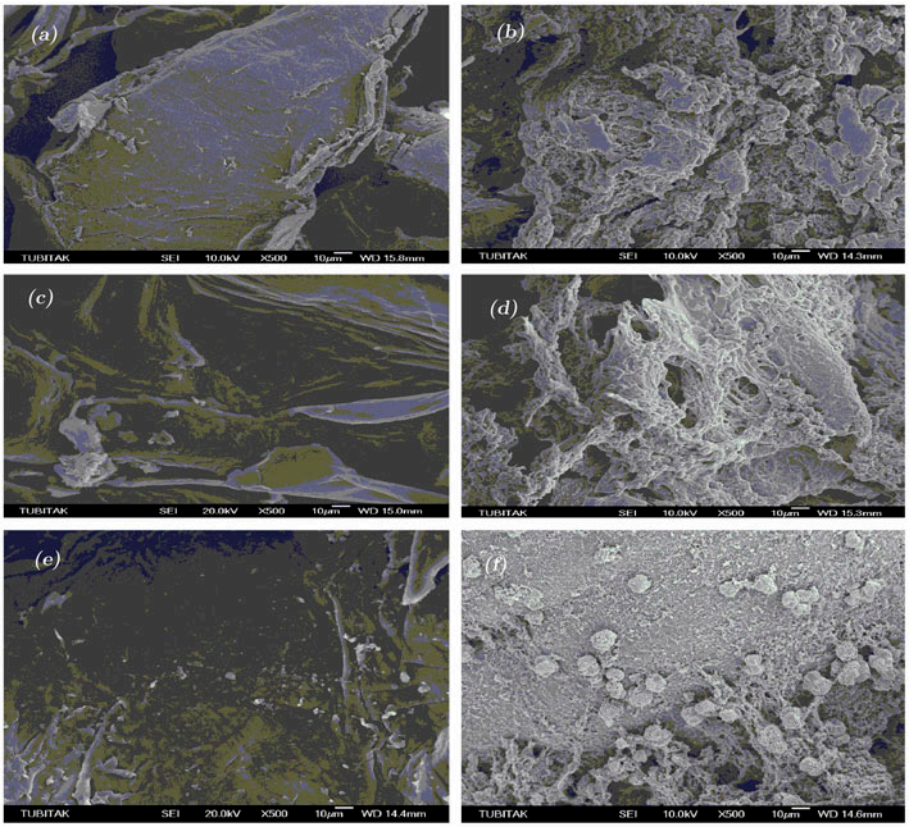


Figure 3. SEM micrograph of (a) Chi, (b) blood contact Chi, (c) ChiVC-1, (d) blood contact ChiVC-1, (e) ChiVC-100, and (f) blood contact ChiVC-100.

Table 4. Total cholesterol, triglyceride, HDL choletserol, LDL cholesterol, ferrimat (total iron), and total cholesterol/HDL ratio in the blood samples of healthy volunteers after 3 h of contact *in vitro*.

Sample ID	Total Cholesterol (mg/dL)	Triglyceride (mg/dL)	HDL cholesterol (mg/dL)	LDL cholesterol (mg/dL)	Ferrimat (μg/dL)	Total cholesterol/HDL ratio
Volunteer 1 (female)						
Initial	203	158	90	81.7	120	2.25
Chi	199	154.3	88	80.4	118	2.47
ChiVC-0.1	196	147	86.7	78.1	114.5	2.50
ChiVC-1	188	134	85.4	75.9	108	2.48
ChiVC-5	163	89	83	62.4	98.8	1.96
Volunteer 2 (female)						
Initial	286	176	70	179.1	69	4.18
Chi	244	159	67.6	145.7	67	3.61
ChiVC-0.1	232	103	64.6	140.9	64	3.59
ChiVC-1	177	85	63	113	60	2.80
ChiVC-5	114.8	47	60.9	53.6	53.2	1.88
Volunteer 3 (male)						
Initial	276	151	66	181.8	58	4.2
Chi	269	147	64	172	57.3	4.2
ChiVC-0.1	266	140	62.3	170	56.2	4.3
ChiVC-1	260	136	60.9	168.8	54	4.3
ChiVC-5	209	90	57	134	49.9	3.66
Volunteer 4 (male)						
Initial	265	159	64	177	47	4.1
Chi	262	157	60.9	175.5	46.7	4.3
ChiVC-0.1	260.4	155.2	60.6	173	46.4	4.3
ChiVC-1	256	147.1	59.2	170.4	44	4.3
ChiVC-5	152	101.9	56.3	140.9	42.3	2.69

with a magnification of 500× as shown in Figure 3. All samples have varying degrees of surface roughness. The smoothness of the surface increases upon ascorbylation due to the decrease in the crystallinity.

The surface morphologies of the samples after blood contact were examined using SEM micrographs with a magnification of 500× as shown in Figure 3. Blood contact samples showed a completely different surface after treatment with blood. The adsorption of blood components on the surfaces was observed in all SEM micrographs of blood contact samples, showing that the samples have poor hemocompatibility.

3.5. Biochemical analysis

3.5.1. Physiological role of chitosan and its derivatives in human blood

Chitosan and its derivatives have the potential to be used as biomaterials. Hence, the interaction of chitosan and ascorbyl chitosan with blood cellular components, such as blood cells and blood proteins, the transferrin-bound iron chelation capacity, calcium ion removal capacity, the lipid-lowering activity, and the influence on blood coagulation were investigated.

3.5.2. Total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride determination in human serum

According to the results given in Table 4, upon contact with chitosan, the total cholesterol level in the blood sample of *volunteer 1* decreases from 203 to 199 mg/dL, which is equivalent to 1.9% decrease. In the same volunteer's sample, ascorbyl chitosans; ChiVC-0.1 and ChiVC-1, decrease the total cholesterol level from 203 to 196 mg/dL, and to 188 mg/dL, respectively. These values correspond to 3.45 and 7.39% decrease in the total cholesterol level. ChiVC-5 reduced the total cholesterol level by 19.7%. Ascorbic acid showed the highest reduction in the total cholesterol level causing 55.67% decrease in the total cholesterol value. For *volunteer 1*, triglyceride level decreased by about 2.34, 6.97, 15.2, and 43.7% upon contact with Chi, ChiVC-0.1, ChiVC-1, and ChiVC-5, respectively. It was found out that ascorbylation resulted in higher reduction in LDL cholesterol compared to chitosan. A similar behavior was observed in the other examined blood samples (*volunteer 2*, *volunteer 3*, and *volunteer 4*) as shown in Table 4.

Our results demonstrate that ascorbylation improves lipid-lowering activity of chitosan (Table 4). Chitosan alone causes the lowest reduction in total cholesterol levels. The increasing extent of ascorbylation enhances the lowering activity of the samples on total cholesterol, triglycerides, and HDL cholesterol levels. The total cholesterol/HDL ratio was reduced with increasing ascorbylation. Smaller ratio of total cholesterol/HDL indicates a less risk for cardiovascular diseases.

A comparison of the lipid-lowering activity of ascorbyl chitosans with that of a hypocholesterolemic agent, cholestryamine, would illustrate the value of the findings reported here. Cholestryamine is a bile acid binding resin of polystyrene cross-linked with divinylbenzene and bearing quaternary ammonium groups. There are several studies reported in the literature comparing the lipid-lowering activities of chitosan and cholestryamine in rats. In a study by Jennings et al. [30] the lipid lowering and intestinal morphological effects of cholestryamine, chitosan, and oat gum were investigated in male Sprague-Dawley rats fed on a cholesterol-enriched diet. It was found that while chitosan was as effective as cholestryamine in reducing the serum cholesterol level, it

did not induce any negative changes in the intestinal mucosa unlike cholestryamine. Both agents did not change hemoglobin or serum iron levels. Similarly, Sugano et al. [31] investigated the lipid-lowering activity effect of male Wistar rats. Hence, chitosan is a hypocholesterolemic agent comparable to cholestryamine according to animal studies. Reports are available for the cholesterol lowering effect of cholestryamine alone or in combination with other agents in patients suffering from hypercholesterolemia. Total cholesterol and LDL cholesterol of heterozygous patients with familial hypercholesterolemia were reported to decrease by 20 and 28%, respectively, after cholesteryamine treatment for 2–16 months.[32] Serum LDL cholesterol of 18 patients with xanthomatous familial hypercholesterolemia was reduced by 35% when treated with cholestryamine as reported by Gylling et al. [33]. In another study by the same authors,[34] the total and LDL cholesterol levels were observed to be lowered by 16 and 21%, respectively, in the patients under cholestryamine treatment. Serum triglycerides, VLDL, and HDL cholesterol levels were unchanged. Among our samples (in *volunteer 2*) reported in this study, the total cholesterol- and LDL cholesterol-lowering efficiencies of ChiVC-5 are comparable to those of cholestryamine with 59.9 and 70.1% lowering values, respectively. The advantage of ascorbyl chitosan over cholestryamine is that it also reduces the triglycerides, while the drawback is that it reduces the HDL cholesterol to some considerable extent, by 13.0%.

3.5.3. *Determination of serum iron*

Iron removal from blood samples of healthy volunteers was performed by following the serum iron content in the blood serum, which is a measure of the transferrin-bound iron. The behavior of the ascorbyl chitosan was compared to that of chitosan and ascorbic acid. The results are shown in Table 4. It was found that ascorbic acid alone reduced total iron, down to a level below standard which is 60 μg/dL minimium. On the other hand, chitosan removed only 2 μg/dL in blood sample of *volunteer1*, which corresponds to 1.67% decrease and did not have any significant iron removal capacity alone. It was observed that the ascorbylation increased the iron removal capacity of chitosan considerably. The ascorbyl chitosan samples; ChiVC-0.1 and ChiVC-1, decreased the serum iron level from 120 to 114.5 μg/dL, and to 108 μg/dL, respectively. These values are within normal range (60–170 μg/dL) and correspond to 4.58 and 10.0% decrease in the serum iron levels. ChiVC-5 reduced the serum iron level by 17.7%. Ascorbic acid showed the highest reduction in the serum iron level, 44.2%. A

Table 5. Prothrombin time levels (in seconds) after 3 h of blood contact chitosan and ascorbyl chitosans.

Sample ID	*Volunteer 4*		*Volunteer 9*
	Total calcium (mg/dL)	Prothrombin time (s)	Prothrombin time[a] (s)
Initial	9.4	12	34
Chi	6.3	14.4	38
VC	3.2	>200	>200
ChiVC-0.1	5.7	19.8	45
ChiVC-1	5.0	27.6	46.1
ChiVC-5	4.9	32.4	49
ChiVC-100	3.6	48.8	70.5

Note: [a]*Volunteer 9*, who is using warfarin medicine.

similar trend was observed in the other examined blood samples (*volunteer 2*, *volunteer 3*, and *volunteer 4*) as shown in Table 4. In a previous study by the authors, up to 80% decrease in serum iron was reported by iron-imprinted chitosan tripolyphosphate beads. [35] The iron-imprinted chitosan tripolyphosphate beads have the advantage of being selective towards iron-containing molecules. No cholesterol or lipid-lowering activity was observed with these samples.

3.5.4. Total calcium analysis in serum

Removal of calcium ions was observed in all tested samples. It was found that the highest removal capacity belongs to ascorbic acid, which chelated 65.96% of the total calcium ions. The ascorbyl chitosans chelate more calcium in comparison to chitosan. The higher removal of total calcium ions reduced coagulation by ascorbyl chitosan samples with increased ascorbyl groups as shown in Table 5. The ascorbyl chitosan samples; ChiVC-0.1, ChiVC-1, and ChiVC-5 decreased the total calcium level from 9.4 to 5.7 mg/dL, 5.0, and to 4.9 mg/dL, respectively.

3.5.5. Complete blood count analysis in whole blood

A blood contact material is required to have hemocompatibility. That is, it should cause minimium change in the complete blood count compositions. Red blood cell count (RBC), white blood cell count (WBC), platelets (PLT), hematocrit (HCT), the mean corpuscular volume (MCV), and hemoglobin (HGB) are shown in Table 6.

Our findings from complete blood count compositions demonstrate that chitosan alone is the most hemocompatible sample in terms of negligible interaction between blood cells and materials mixed with blood. Ascorbyl chitosans contain worse hemocompatibility compared to chitosan alone. This observation was illustrated by the decrease in RBC count (Table 6). The increase in the MCV values is indicative of the volume increase of the RBC when chitosan alone is used. Additionally, the increase in the HCT value with chitosan alone may also be considered as an increase in the RBC volume during incubation time.

Hemolysis was observed when ascorbic acid came into contact with blood after 15 min of incubation at 10 mg/200 μL as illustrated in Table 6 for RBC count when ascorbic acid alone is added. Ascorbic acid made blood darker compared to blood which was blank. Additionally, the blood which included ascorbic acid became more gelatinous in comparison with control and other blood contact examined materials. Due to these problems associated with ascorbic acid, we had concluded that blood was ascorbic acid incompatible. On the other hand, chitosan and its derivatives did not show hemolysis over 3 h of incubation at 10 mg/200 μL. This may be explained as ascorbic acid having a stronger electrostatic interaction with blood components compared to ascorbyl chitosans. Negatively charged biological molecules on blood cell membranes may electrostatically interact easily with ascorbic acid mixed with blood than with blood mixed with raw chitosan and ascorbyl chitosans due to its more cationic structure. Besides, the nonspecific binding of negatively charged biomolecules on blood cell membrane and adsorbed blood components cause the variation in blood characteristics.

A reduction was observed in PLT values dramatically with ascorbic acid alone. This may be the indication of prolonged prothrombin time and extension of the clotting time (Table 5). Ascorbic acid, chitosan, and ascorbyl chitosans values have shown to delay blood coagulation and might accelerate fibrinolysis process. Therefore, ascorbic acid,

Table 6. The percent changes* in red blood cell count (RBC), white blood cell count (WBC), platelets (PLT), hematocrit (HCT), the mean corpuscular volume (MCV), and hemoglobin (HGB) levels in the blood samples of healthy volunteer (*volunteer 4*) after 3 h of contact *in vitro*.

M	Normal ranges	Initial	VC	Chi	ChiVC-1	ChiVC-5
RBC	3.5–5.50 (10^6/mm^3)	4.97	1.2 (−%75.9)	4.88 (−%0.2)	4.3 (−%13.5)	4.29 (−13.7)
HCT	35.0–55.0 (%)	36.20	6.4 (−%82.3)	36.4 (+%0.55)	30.4 (−%16.0)	28.3 (−%21.8)
WBC	3.5–10.0 (10^3/mm^3)	13.90	2.3 (−%83.5)	13.2 (−%5.04)	10.04 (−%27.8)	9.01 (−%35.2)
HGB	11.5–16.5 (g/dL)	12.80	1.9 (−%85.2)	12.5 (−%2.3)	12.42 (−%3.125)	12.2 (−%4.7)
PLT	100–400 (10^3/mm^3)	295.00	32 (−%89.2)	291 (−%1.36)	270 (−%8.5)	265 (−%10.2)
MCV	75.0–100.0 (μm^3)	76.70	50 (−%34.8)	79.98 (+%4.3)	72.03 (−%6.09)	72.01 (−%6.11)

Note:* increase or decrease in percent changes for investigated blood cell components is shown as + and −, respectively.

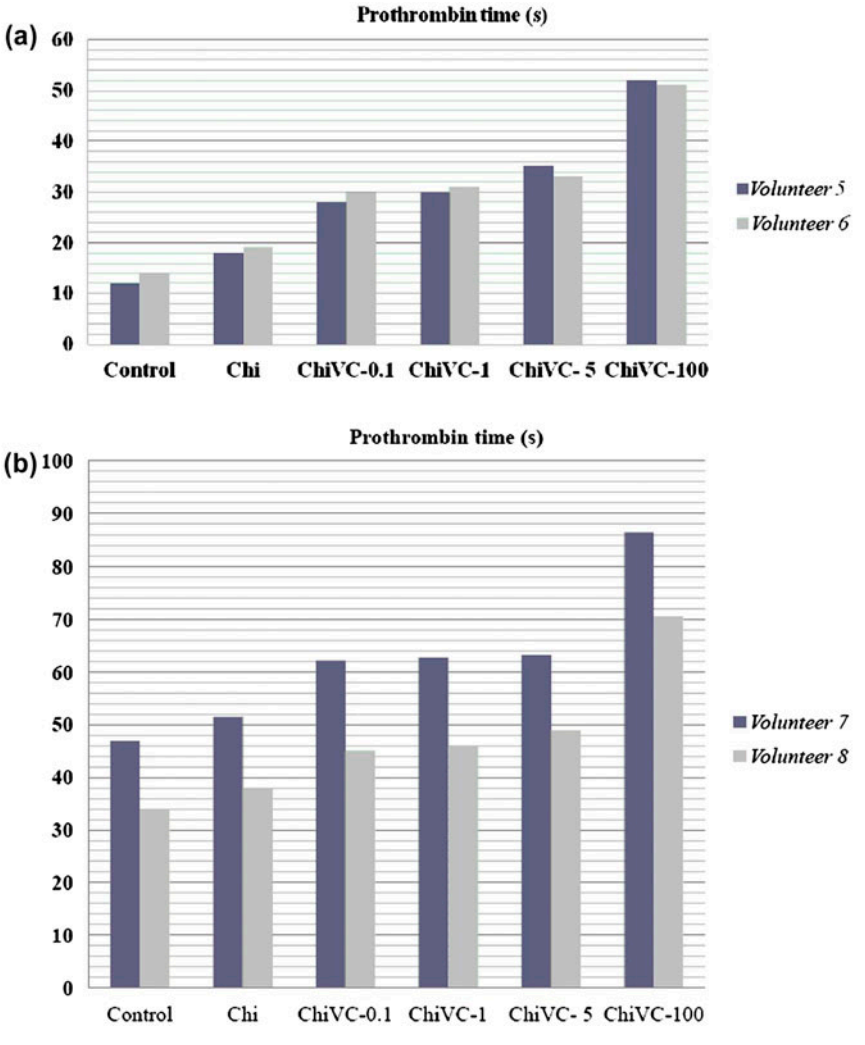


Figure 4. Bar graph of prothrombin time levels after 3 h of blood contact ascorbyl chitosan samples (a) with two healthy volunteers and (b) with two volunteers using warfarin.

chitosan, and ascorbyl chitosans may interfere with the blood coagulation due to complex biochemical process in various ways.

3.5.6. *Prothrombin time determination in whole blood*

The prothrombin time measures the integrity of the extrinsic pathway. The blood samples used were selected from two healthy volunteers and two volunteers using warfarin. The examined samples prolonged the prothrombin time of the analyzed blood from healthy and warfarin-user volunteers compared to the control as shown in Figure 4 and Table 5. Therefore, chitosan, ascorbic acid, and ascorbyl chitosans exhibited anticoagulant effect on the blood and deactivated hemostasis. Ascorbic acid had the strongest blood-thinning effect.

In our investigation (Table 5), chitosan had the minimum anticoagulant effect. We found that ascorbic acid had the highest anticoagulant activity due to huge increase in prothrombin time. We observed that increase in ascorbic acid content prolonged blood coagulation. The increased anticoagulant activity is caused by the removal of calcium ions which are necessary for the extrinsic and intrinsic blood clotting. Due to calcium ion-chelating ability of chitosan, ascorbyl chitosans, and ascorbic acid, the function of vitamin K is blocked, this leads to a delay or prevents coagulation completely.

Reduction in PLT prolonged prothrombin time and increase in clotting time were measured. The decrease in blood proteins such as hemoglobin and other proteins that are essential for blood clot formation might influence the biological process in blood. Moreover, reduction in coagulation factors, which are required for thrombosis, lead to more bleeding and increased prothrombin time.

4. Conclusions and future perspectives

Bio-based polymer chitosan was ascorbylated in this study to examine its potential to interact with blood *in vitro*. This study had potential applications when the examined samples facilitated to lower lipid levels (such as total cholesterol and LDL cholesterol), and showed a good hemocompatibility without causing adverse reactions (such as thrombosis, hemolysis, or inflammation). Examined ascorbyl chitosan had the ability to lower lipid (total cholesterol, LDL cholesterol, and triglyceride) levels in blood. However, their high anticoagulant activity may lead to hemophilia disorders. Additionally, decreasing HDL cholesterol provided drawbacks for examined ascorbic acid and its ascorbic acid derivatives with chitosan.

Further confirmatory *in vivo* studies are required to understand the action of ascorbyl chitosan at the molecular level, and to gain insight into the unknown biochemical functions of these chitosan-based products. Additionally, it is needed to investigate these materials for toxicological evaluation, which is crucial for the safety and biocompatibility before it is used in clinical applications. Moreover, from our observations it could be interpreted that chitosan and its derivatives display a nonspecific binding affinity towards biomolecules used in this study. Therefore, it should be utilized with caution when considered as a bioselective material. Further studies are necessary to improve the utility of ascorbyl chitosan in order to achieve selective binding capacity.

Acknowledgment

We would like to acknowledge all the blood donors for their blood used in our study.

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