CHITOSAN ADDUCT WITH TRANEXAMIC ACID AND ITS HAEMOSTATIC EFFECT

Volodymyr Antonyuk[1,2, a]*, Nazar Manko[2, b], Lidiia Panchak[1, c], Semen Khomyak[3, d], Rostyslav Stoika[1,2,4, e]

[1]Danylo HalytskyLviv National Medical University, Pekarska Str., 69, 79010, Ukraine
[a] - ORCID: 0000-0002-3643-4957; [c] - ORCID:0000-0002-9621-3304;
[e] - ORCID:0000-0001-5719-2187
[2]Institute of Cell Biology, NAS Ukraine, Lviv, Drahomanov Str. 14/16, 79005, Ukraine
[b] - ORCOD:000-0003-1203-7479
[3]Lviv Polytechnic National University, Lviv, Yura square,3/4, 79010, Ukraine
[d] - ORCID:0000-0003-1931-1688
[4]Ivan Franko National University of Lviv, HrushevskyStr.,4, 79005, Ukraine

**corresponding author: antonyukvo@gmail.com*

Abstract

Chitosan is a cationic polymer capable of binding acidic drugs. In addition, it has haemostatic and antimicrobial activity. Chitosan derivatives withanti-fibrinolytic properties may present increased effectiveness, especially when the added substance forms an adduct with chitosan. The aim of this work was to study the haemostatic action of the chitosan–tranexamic acid complex. Two chitosan solutions (molecularweight of 250 and 625 kDa at pH 5.7 and 6.2, and after tranexamic acid had been added to chitosan solutions) werestudied. Haemostatic evaluation was performed on white outbred mice. The time to complete cessation of bleeding from the tail was determined. Chitosan 625 kDa at pH 6.2 had the best haemostatic properties. Adding tranexamic acid to the chitosan solution reduced the bleeding time. This phenomenon was more pronounced for chitosan 625 kDa. Compared with control animals, this chitosan reduced bleeding arrest time by 30% and the chitosan–tranexamic acid adduct reduced the bleeding arrest time by 75%.

Keywords:*chitosan; tranexamic acid, adduct, haemostatic effect*

Received: 26.02.2022
Accepted: 09.06.2022

1. Introduction

Chitosan is a linear polysaccharide consisting of glucosamine and *N*-acetylglucosamine units linked by 1,4-β-glycosidic bonds. The main characteristics responsible for its chemical and biological properties are its molecular weight (MW) and degree of deacetylation (DD). Depending on the source and method of preparation, the MW of chitosan may range from ≥10 to 1000 kDa, and the DD may range from 30% to 95% [1]. Chitosan is a cationic polymer capable of binding acidic drugs and has uniqueproperties,includingbiocompatibility, biodegradability, and low toxicity [2, 3].

Chitosan with a high MW can stop bleeding [4]. However, as noted by some researchers, an increase in the MW does not lead to a proportional increase in haemostatic action; the effect depends on a number of other factors [5].Protonated amino groups may be related to the haemostatic activity of chitosan. Chitin shows less effective haemostatic activity than chitosan, a finding that supports this conjecture [6].

One of the main properties of chitosan is the stimulation of regeneration. Chitosan stimulates cell proliferation and histoarchitectural tissue organisation and affects macrophage function, which helps faster wound healing.

Chitosan is the most promising material for haemostatic dressings due to its direct haemostatic properties and possible improvement of wound healing. In addition, chitosan has no disadvantages regarding the biological response. Although the haemostatic mechanism of action of chitosan remains unclear, data suggest three possible ways to influence bleeding: (1) adsorption of plasma;(2) erythrocyte coagulation; and 3) platelet adhesion, aggregation, and activation. Plasma adsorption is a key factor in chitosan application as a haemostatic agent. Chitosan can absorb from 50% to 300% liquid relative toits primary weight, meaning that it couldconcentrate erythrocytes and platelets at the injured site [7].Contact of chitosan with blood changesthe morphology of erythrocytes. They losethe typical biconcave morphology and appeared to have an unusual affinity towards one another [8]. The main cause of the haemostatic effect of chitosan is related to platelet adhesion, aggregation, and activation [9].

The addition ofsubstances that possess antifibrinolytic action to chitosan may enhance its effectiveness, especially when the added substance forms an adduct with chitosan. This change couldfind practical application in medical care. Aminocaproic, aminomethylbenzoic, and tranexamic acids are most often used as antifibrinolytic agents. The aim of this work was to study the haemostatic effect of the chitosan–tranexamic acid complex.

2. Materials and Methods

2.1. Materials

A 10% solution of tranexamic acid – trans-4-(aminomethyl)cyclohexanecarboxylic acid, the active ingredient ofTramixand Hemotran – was purchased from a pharmacy network and met the criteria of purity of pharmaceutical drugs.

Chitosan fromTyanshi (China), with a MW of 250 ± 10 kDa, was bought from a local pharmacy and purified from the filling substances by re-precipitation.

Macromolecularchitosan was obtained from shrimp chitin by alkaline hydrolysis. Two grams of chitin (Sigma-Aldrich, USA), which was sieved through a 0.5 mm sieve, was added to 80 ml of 50% sodium hydroxide (NaOH) and boiled in a water bath for 2 h. Then, the solution was centrifuged; the precipitate was washed twice with water and then three times with 95% ethanol acidified with acetic acid (pH ≈ 6.5). The obtained washed chitosan was dried in an oven at 60°C. The MWof the obtained chitosan, determined viscometrically, was 625 ± 10 kDa, and the DD was 92.1%. The resulting chitosan is soluble in 1% acetic acid to form viscous solutions.

The following analytical grade chemicals were used in the experiments: sodium chloride, acetic acid, ethanol and diethyl ether (Ukraine, Cherkasy Plant of Chemical Reagents).

2.2. Apparatus

The viscosity of chitosan solutions was measured in a mixture of 0.1 M acetic acid and0.2 Msodium chloride (NaCl)at 25°C with a VPZh-4 Ubbeholdviscosimeter (Soyuznauchpribor, Russia) with a 0.82 mm diameter capillary.

The concentration of tranexamic acid was determined by the optical density of the reaction with ninhydrin.Ultraviolet (UV) spectra were measured on a NanoDrop 1000 spectrophotometer 3.8.1 (USA).

The Fourier-transform infrared (FT-IR) spectra of samples were recorded on a Spectrum Twospectrometer (PerkinElmer, USA) using a diamond UATR single reflection accessory. PerkinElmer Spectrum software was used to draw the spectra. The spectra (16 scans per spectrum) of the solutions were collected in the mid-infrared wavenumber range from 4000 to 400 cm^{-1}, with a spectral resolution of 4 cm^{-1}.

2.3. Methods

2.3.1. Preparation of the Chitosan–TranexamicAcid Complex

Chitosan was dissolved in 1% acetic acid overnight and then dialysed against 0.15 M acetate buffer solution(pH 5.7 and 6.2). After the chitosan solution reached the appropriate pH, an equal volume of 10% tranexamic acid solution was added to the dialysed solution. The pH of the resulting mixturewas 5.7 and 6.3, respectively. The final mixture contained 0.5% chitosan and 5%tranexamic acid.

2.3.2. Determination of theTranexamicAcid Concentration in theChitosan–Tranexam -icAcidComplex

Tranexamic acid is an amino acid and reacts with ninhydrin to form a purple colour, the intensity of which is proportional to its concentration.The presence of chitosan does not interfere with the reaction.

2.3.3. Mice

White outbred mice (6-8 weeks old) were fed with standard chow and water and kept at26°C and 60% humidity witha natural photoperiod. All *in vivo* experiments with mice were performed according to the established bioethical standards of the CIR-UADY Bioethics in Research Committee

2.3.4. Haemostatic Activity

The haemostatic activity of the chitosan–tranexamic acid adduct wasdetermined byusingwhite outbred mice weighing 18 ± 4.0 g (Bioethics protocol N 5, 05.04.2021). Each mouse was anaesthetised with an intraperitoneal injection of 100 mg/kgketamine (Pharmak, Ukraine) and fixed in a special device before the experiment. After that, itstail was cut with a sharp scalpel 10 mm from the distal end. After amputation, the wound was pressed for 60 s. Then, the wound was placed in the test solution for 3 s. Cessation of bleeding was recorded asthe time when there were no more blood dropletsat the site of the cut and a blood clot had formed.

3. Results and Discussion

Tranexamic acid (Figure 1) is capable of forming adducts with chitosan.Themixture of 1% chitosan 625 kDa and 5% tranexamic acid is colourless, clear, and viscous. The mixture of 1%chitosan alone has a much lower viscosity.

To prepare the chitosan–tranexamic acid adduct,a 10% solution of tranexamic acid (0.5 ml) was added to ground chitosan (50 mg) in 0.1 M carbonate buffer (pH 8.4, 0.5 ml). The mixture was incubated for 10 min.Then, the chitosan powder was washed three times with 10 ml of 1 M NaCl.After the last wash, the supernatant was aspirated, and the chitosan

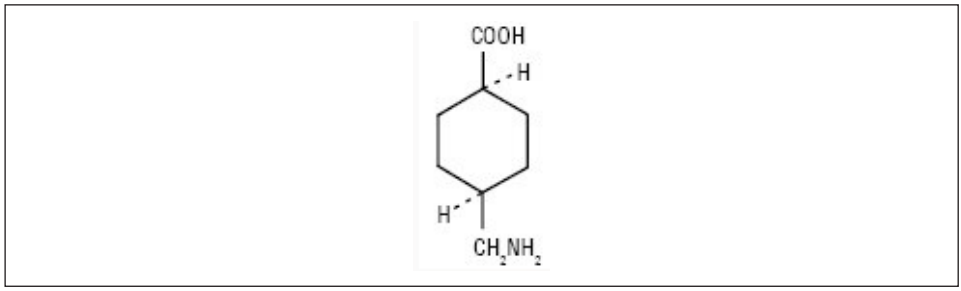

Figure 1. The structure of tranexamic acid.

powder was dissolved in 5.0 ml of 1% acetic acid.Next, the presence of tranexamic acid was determined in this solution viaa reaction with ninhydrin.UV spectra were measured on a NanoDrop 1000 spectrophotometer 3.8.1 (USA) (Figure 2).After plotting the calibration graph with known concentrations of tranexamic acid, its concentration in the adduct was determined to be ≈ 1.0%. This represents the amount of tranexamic acid that remained in the form of chitosan–tranexamic acid adducts after washing the mixture.

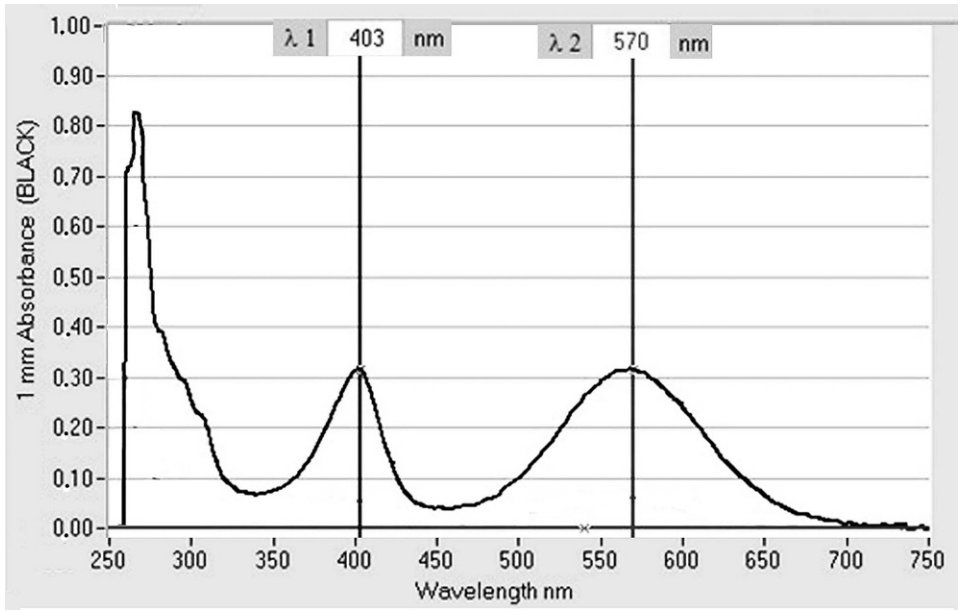

Figure 2. Ultraviolet spectrum of the reaction product of 1%tranexamic acid with ninhydrin.

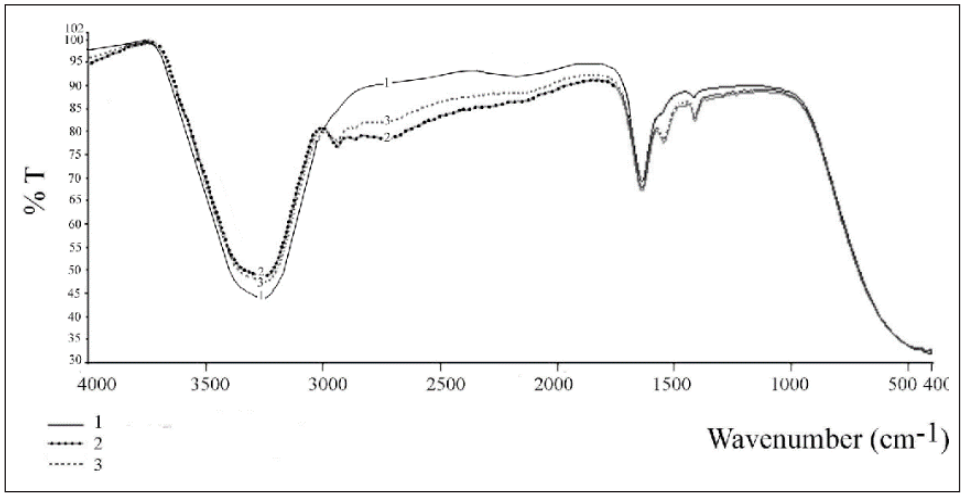

Figure 3. Fourier-transform infrared spectra of 0.5% chitosan (1),0.5% chitosan +5% tranexamic acid (adduct) (2), and5% tranexamic acid (3).

FT-IR spectra of chitosan, tranexamic acid and the chitosan–tranexamic acid adduct are presented in Figure3. The details of the spectra are presented in Table 1.

Table 1. Absorption bands of the investigated solutions.

No	Solution in water	Band position (cm^{-1}) and it assignment		
1	0.5% chitosan	2950–2820 asymmetric and symmetric *v* CH_2	1560 δ NH, *v*C-N, and *v* C-C	
2	Adduct	2938, 2824 asymmetric and symmetric *v* CH_2	1544 *v* C-N	1410 symmetric *v* C-O
3	5% tranexamic acid	2938, 2824 asymmetric and symmetric *v* CH_2	1541 *v* $H_2C\text{-}NH_3^+$	1410 symmetric *v* C-O

Because the three evaluated samples are highly dilute, their FT-IR spectra clearly show two high-intensity absorption bands at 3600-3200 and 1636 cm^{-1}, which correspond to the absorption of water, the solvent. The absorption bands of the hydroxyl (ν_{NH}, ν_{OH}) and carboxyl groups of (amide I $\nu_{C=O}$, ν_{NH}) chitosan and tranexamic acid also appear in these ranges, but they are not observed in the spectra due to overlap.In addition, in the spectrum of the aqueous solution of tranexamic acid there are absorption bands at 1635, 1541, and 1410 cm^{-1}. The absorption band at 1541 cm^{-1}corresponds to the valence oscillations of the $H_2C\text{-}NH_3^+$, bond, while the other two bands correspond to the asymmetric and symmetric oscillations of the carboxyl group COO^-. In an aqueous solution,tranexamic acid exists as an internal salt with hydrogen bonds to water molecules. The absorption bands at around 2938 and 2877 cm^{-1} can be attributed to asymmetric and symmetric C-H stretching, respectively.The spectrum of the chitosan solution show two broad absorption bands at1640-1540 and 1430-1400 cm^{-1}, which can be attributed to the oscillations of the amide and alkyl groups (stretching of amide groups and bending of alkyl groups, respectively).

The spectrum of the adduct contains all the absorption bands present fortranexamic acid. The absorption band of the C-N group is shifted towards a longer wavelength,appearing at 1544 cm^{-1}. This can be explained by the formation of hydrogen bonds of the acid with chitosan molecules, which makes the C-N bond more polar.

The results of the study of the haemostatic effect of a mixture of 1% chitosan and 5% tranexamic acid are presented in Table 2.

Table 2. Influence of the molecular weight of chitosan, the pH of the solution, and tranexamic acid on the duration of bleeding.

Sample	Time afterwhich bleeding stopped completely(min)	% reduction in the time of cessation of bleeding when using the drug
Control (without drug)	53.20	0
5% tranexamic acid (Tramix)	49.16	7.60
5% tranexamic acid (Hemotran)	47.05	11.56
1% chitosan (molecularweight 250 kDa) in acetate buffer, pH 6.2	41.11	22.73
1% chitosan (molecularweight625 kDa) in acetate buffer, pH 6.2	37.26	29.96
1% chitosan (molecularweight250 kDa) in acetate buffer, pH 6.2 with 5% tranexamic acid	38.30	27.63
1% chitosan (molecularweight625 kDa) in acetate buffer, pH 6.2 with 5% tranexamic acid	18.75	74.76
1% chitosan (molecularweight250 kDa) in acetate buffer, pH 5.7)	41.92	21.19
1% chitosan (mol. weight625 kDa) on acetate buffer, pH 5.7	33.73	36.60
1% chitosan (molecularweight250 kDa) in acetate buffer, pH 5.7 with 5% tranexamic acid	38.38	27.86
1% chitosan (molecularweight625 kDa) in acetate buffer, pH 5.7 with 5% tranexamic acid	29.30	44.92

Note. The time is the average of three replicates.

Under the conditions of the experiment, tranexamic acid alone had a slight haemostatic effect, weaker than that of chitosan, while mixtures of chitosan andtranexamic acid significantly enhanced thehaemostatic effect (Table 1). Chitosan 250 kDa reduced the time to stop bleeding by approximately 23%, while chitosan 625 kDa reduced the time to stop bleeding by 30%. The addition of tranexamic acid to chitosan enhanced the haemostatic effect, especially in the case of chitosan 625 kDa. This effect was much more pronounced at pH6.2 than 5.7.

There is a synergistic effect probably because chitosan and tranexamic acid have different mechanisms of action regarding haemostasis.Tranexamic acid can block the interaction between plasminogen andfibrin. As a result, fibrin is not destroyed. In

addition, tranexamic acid enhances collagen synthesis, which helps preserve the fibrin matrix and increases the strength of the thrombus [10].Tranexamic acid possesses several times stronger antifibrinolytic activity than ε-aminocaproic acid [11].According to some authors, chitosan accelerates the aggregation of platelets and erythrocytes and enhances the release of transforming growth factor β1 [12].

Uncontrolled bleeding is the leading cause of death on battlefields [13] and one of themain causes of death in the civilian environment [14]. The anatomical distribution of injuries is influenced by the type of conflict, the weapons used on the battlefield, as well as the protective equipment worn by individual soldiers when deployed in the field [15]. To address these issues, haemostatic agents have been developed to treat massive bleeding in areas of the body where tourniquets cannot be used, such as the neck, the groin, and the axilla. These newer haemostatic agents have evolved into effective products to stop minor to massive bleeding.

Some countries use haemostatic bandages containing chitosan. These include ChitoGauze® XR PRO andCelox, which are used by the armies of the United States, Israel, and a number of European countries [16]. In Taiwan, the haemostatic dressing Clo-Sur PAD (Scion Cardio-Vascular, Inc., U.S.A.) and the Instant Clot Pad (Cosmo Medical Inc., Taiwan) are available. Both are used to stop trauma-related arterial bleeding and are routinely applied post-angioplasty and after wound debridement [17].

4. Conclusions

We found that 1% solutions of chitosan with different molecularweights(250 and 625 kDa) show a haemostatic effect. This effect was more pronounced at pH 6.2 than at pH 5.7 and in chitosan with a higher MW (625 kDa). When tranexamic acid is added to chitosan solutions, an adduct is formed, which enhances the haemostatic effect. The haemostatic effect of the adduct is much stronger than the separate action of chitosan and tranexamic acid. This synergismis probably due to different mechanisms of action of chitosan and tranexamic acid to stop bleeding.

5. References

[1] Cheung RCF, Bun Ng T,Wong JH, Chan WY; (2015) Chitosan: an update on potential biomedical and pharmaceutical applications. Marine Drugs 13, 5156-5186. **DOI**:10.3390/md13085156

[2] Wang W, Meng Q, Li Q, Liu J, Zhou M, Jin Z, Zhao K; (2020)Chitosan derivatives and their application in biomedicine. Int J MolSci 21, 487.**DOI**:10.3390/ijms21020487

[3] Antonyuk V, Manko N, NektegaevI, Stoika R; (2021) Pharmacokinetics of ethacridine conjugated with chitosan in rats.Methods Objects Chem Anal 16(1), 41-47 **DOI**:10.17721/moca.2021.41-47

[4] Lootsik MD,Bilyy RO,Lutsyk MM, StoikaRS; (2015)Preparationof chitosan with high blood clotting activity and its hemostatic potential assessment.BiotechnolActa 8(6), 32-40. **DOI**:10.15407/biotech8.06.032

[5] Kadyseva OV, BykovVN., Strelova OY, Grebenyuk AN; (2021) Study of the effect of the physicochemical properties of chitosan on its haemostatic activity. Progress ChemAppl Chitin Deriv26, 112-120. **DOI**:10.15259/PCACD.26.010

[6] Singh R, Shitiz K, Singh A; (2017). Chitin and chitosan: biopolymers for wound management. IntWound J 14(6), 1276-1289. **DOI**:10.1111/iwj.12797

[7] Pogorielov MV, Sikora VZ; (2015). Chitosan as a hemostatic agent: current state. Eur J Med B 2(1), 24-33.**DOI**:10.13187/ejm.s.b.2015.2.24

[8] Klokkevold PR, Fukayama H, Sung EC, Bertolami CN; (1999). The effect of chitosan (poly-N-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. J Oral Maxillofacial Surg57(1), 49-52. **DOI**:10.1016/S0278-2391(99)90632-8

[9] Wang XH, Li DP, Wang WJ, Feng QL, Cui FZ, Xu YX, Song XH, van der Werf M; (2003) Crosslinkedcollagen/chitosan matrices for artificial livers. Biomaterials 24, 3213-3220. **DOI**:10.1016/S0142-9612(03)00170-4

[10] McCormack PL; (2012) Tranexamicacid. A review of its use in the treatment of hyperfibrinolysis. Drugs 72, 585-617. **DOI**:10.2165/11209070-000000000-00000

[11] Klauss M, Knorr J, Breuer T, Gertler R, MacGuil M, Lange R, Tassani P, Wiesner G; (2011) Seizures after open heart surgery: comparison of e-aminocaproic acid and tranexamic acid. J CardiothoracVascAnesth25(1), 20-25.**DOI**:10.1053/j.jvca.2010.10.007

[12] Suresh MR, Valdez-Delgado KK, VanFosson CA, Trevino JD, Mann-Salinas EA, Shackelford SA, Staudt AM; (2020). Anatomic injury patterns in combat casualties treated by forward surgical teams. J Trauma Acute Care Surg89(2S), S231-S236. **DOI**:0.1097/TA.0000000000002720

[13] Liu JL, Li JY, Jiang P, Jia W, Tian X, Cheng ZY, Zhang YX; (2020). Literature review of peripheral vascular trauma: is the era of intervention coming? Chin J Traumatol 23(1), 5-9. **DOI**:10.1016/j.cjtee.2019.11.003.

[14] Hauschild VD, Schuh-Renner A, Lee T, RichardsonMD, Hauret K, Jones BH; (2019) Using causal energy categories to report the distribution of injuries in an active population: an approach used by the US Army. J Sci MedSport, 22(9), 997-1003. **DOI**:10.1016/j.jsams.2019.04.001

[15] Okamoto Y, Yano R, Miyatake K, Tomohiro I, Shigemasa Y, Minami S; (2003) Effects of chitin and chitosan on blood coagulation. CarbohydrPolym 53, 337-342. **DOI**:10.1016/S0144-8617(03)00076-6

[16] Boulton AJ, Lewis CT, Naumann DN, Midwinte MJ; (2018) Prehospital haemostatic dressings for trauma: a systematic review. Emerg Med J 35(7), 449-457. **DOI**:10.1136/emermed-2018-207523

[17] Kang P-L, Chang SJ, Manousakas I, Lee CW, Yao C-H, Lin F-H, Kuo SM; (2011) Development and assessment of hemostasis chitosan dressings. CarbohydrPolym 85(3), 565-570. **DOI**:10.1016/j.carbpol.2011.03.015