UDK 612.115.3:577.114 doi: 10.15407/biotech8.06.032

PREPARATION OF CHITOSAN WITH HIGH BLOOD CLOTTING ACTIVITY AND ITS HEMOSTATIC POTENTIAL ASSESSMENT

M. D. Lootsik[1]
R. O. Bilyy[1, 2]
M. M. Lutsyk[2]
R. S. Stoika[1]

[1]Institute of Cell Biology of the National Academy of Sciences of Ukraine, Lviv

[2]Danylo Halytsky Lviv National Medical University, Ukraine

E-mail: lootsik@cellbiol.lviv.ua

Received 21.10.2015

The aim of research was the evaluation of blood clotting activity and hemostatic effect of chitosan specimens using developed set of laboratory tests and preparation of chitosan with high blood clotting activity. Chitosan specimens were powdered to the particle size less than 0.25 mm and characterized by *in vitro* tests including determination of coagulation time of heparinized blood, bulk density of material, swelling of particles in water and Tris-buffered saline, pH 7.4, as well as *in vivo* tests based on estimation of efficacy in controlling hemorrhage from wounds in mice and rats. Using of the developed tests it was revealed that available chitosan specimens (reagent grade, dietary supplements, samples prepared in laboratory) are hemostatically inactive as compared with Celox preparation. We developed a method of activation of above mentioned chitosan specimens to active hemostatic preparations. Some characteristics of chitosan that are significant for blood clotting activity are described. It was demonstrated that high molecular weight chitosan specimens are more effective as hemostatic agents comparing with low molecular weight chitosan specimens.

Key words: chitosan, Celox, hemostatic effect, laboratory tests *in vitro* and *in vivo*.

In a great number of hemostatic agents implicated in different field of medicine, the hemostatic materials based on chitosan are the most effective in stemming acute hemorrhages, especially severe ones, and are of the first order significance in the emergency and military medicine [1, 2]. Methods of chitin and chitosan isolation and purification from different sources have been described in numerous patents and papers [3–9]. Nevertheless, there is a problem in verification of the hemostatic or hemocoagulating activity of chitosan specimens purchased from different commercial sources or prepared in laboratory according to described methods. We found that chitosan as a chemical reagent or dietary supplement did not exhibit hemocoagulating or blood clotting activity. Thus, monitoring of such activity is necessary when using chitosan samples of commercial origin or samples prepared in laboratory for obtaining high quality hemostatic material whose characteristics are comparable with that of known commercial hemostatic agent Celox. Several tests for measuring hemostatic activity of chitosan preparations were proposed. They include determination of whole blood clotting time and plasma recalcification time [10], involvement of platelets (aggregation, deposition and release of ATP and other factors involved in blood clotting) [11], determination of the duration of bleeding time from pricked finger tip using chitosan soaked bandage [12], flocculation of 0.5% solution of bentonite [13], stemming of hemorrhage in a swine after groin injury with transection of the femoral vessels [14]. The last one is considered to be the most decisive, however, screening process is rather inconvenient and expensive, and the duration of bleeding time from pricked finger tip [12] is rather subjective and not suitable for the repeated performance.

In our studies, commercial chitosan specimens, as well as laboratory prepared specimens and the original Celox (Medtrade Products, UK) used as a positive control of the hemostatic agent were tested when developing different laboratory tests *in vitro* and *in vivo* that permit predicting distinct chitosan sample to be a potent hemostatic agent in clinics conditions. The *in vitro* tests included: a) clotting of blood in the presence of heparin;

b) swelling of chitosan particles in water and in saline; c) bulk density of powder (g/cm^3). While the *in vivo* tests included estimation of the efficacy of studied material in controlling of hemorrhage from wound in mice or rats.

The aim of this study was to compare properties and hemostatic activity of selected specimens of chitosan and Celox by using a developed set of tests and to elaborate the method of chitosan activation for obtaining the product possessing high blood clotting activity.

Material and Methods

The following chitosan specimens were investigated: 1) low molecular weight chitosan from crab shells (Aldrich, gift of Dr. Zaichenko O. S., Lviv National Polytechnic University); 2) chitosan Tyanshi, a dietary supplement (www.tiens.in.ua) [15]; 3) chitosan purchased from Organica (Health Products Inc., Canada) that is a dietary supplement for management of blood cholesterol level; 4) chitosan prepared in our laboratory from chitin of crab shells (obtained as described in [16]. The last preparation was subjected to hydrolysis in 40% NaOH (110 °C for 5 hours with further washing from alkali, dehydration with ethanol and drying), as described in [17]. In order to obtain reproducible results, studied material were grinded to a powder with particle size less than 0.25 mm using porcelain mortar and sieve of 60 mesh.

The obtained chitosan specimens were characterized by determination of the molecular weight and degree of deacetylation. Molecular weight was defined by the viscometry method, as described in [18]. Viscosity was measured at 25 °C with a viscometer of Ubbelohde type VPZh-4 (ВПЖ-4, Soyuznauchpribor, USSR) with capillar diameter = 0.56 mm. Measurements of chitosan solutions in a mixture of 0.17 M acetic acid:0.2 M NaCl (1:2) were conducted.

A degree of deacetylation of chitosan specimens was determined by the potentiometric titration based on measuring a capacity of ion exchange sorbents, as described below. Chitosan specimen was converted to base (OH^-) form by adjustment of 0.5% solution in 3% acetic acid to pH 8.5–9.0 with 10% ammonium hydroxide. Precipitated substance was collected by centrifugation (10 min at 2,000 rpm), washed with water and ethanol, and dried by evaporation on a glass surface. Otherwise, precipitate could be harvested on a glass (Schott) filter, washed with water, ethanol and dried. Dry material was powdered and stored in the dessicator over $CaCl_2$.

100 mg of chitosan base was dissolved in 10 ml of 0.1 N HCl. The dissolving of high molecular weight chitosan was proceeded slowly, for 2–4 hours under stirring. Then solution was titrated with 0.1 N NaOH using glass electrode in pH meter EB-74 (EV-74, Grodno, Belaruss). The titration curves were constructed (Fig. 1) from which the equivalent value of free HCl titrated to pH 2.5 was determined. That pH value was accepted on a basis of titration of 10 ml 0.1 N HCl. A degree of deacetylation was determined by comparison with chitosan samples of known deacetylation

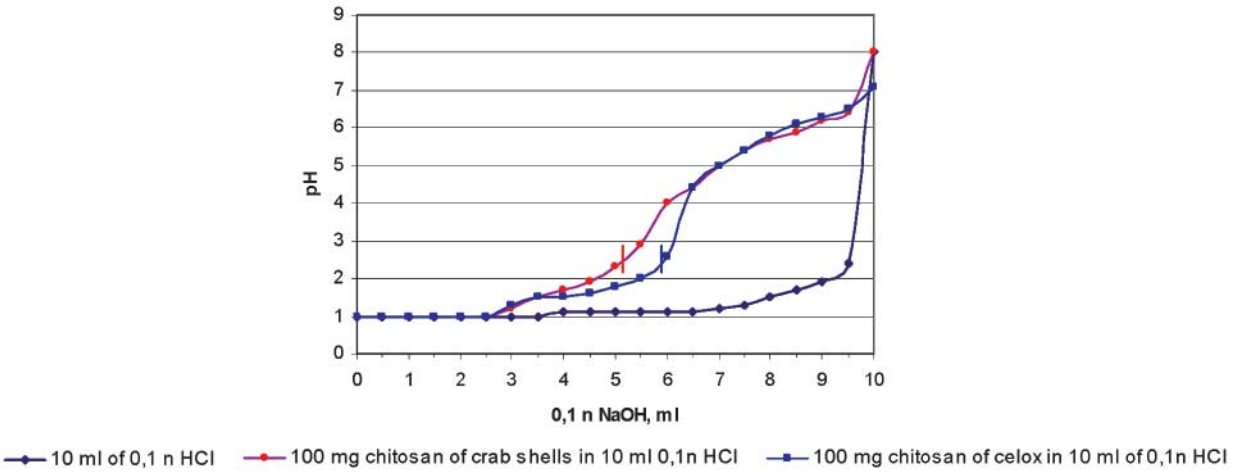

Fig. 1. **Determination of chitosan deacetylation degree as measured by the potentiometric titration:**
vertical bars on titration curves denote points which were accepted as the end of free HCl titration

level, as indicated in the accompanying certificates.

The mEq of HCl bound to chitosan base can be calculated as a difference between 1 mEq HCl (10 ml of 0.1 N HCl) and quantity of mEq titrated to pH 2.5. The obtained value was recalculated to 1 g of chitosan specimen from which the number of mEq of glucosamine in 1 g of chitosan was calculated. It was determined that 1 g of chitosan with 100% degree of deacetylation contained 6.17 mEq of glucosamine residues (considering 162 mg as 1 mEq of glucosamine residue, 1000 mg/162 = 6.17 mEq of glucosamine residues per 1 g).

Bulk density of chitosan preparations was measured in small glass tubes (inner diameter 4–5 mm, volume 0.6 ml) graduated in 0.1 ml scale. Chitosan powder was introduced into tube and its mass was measured by weighting with ±0.1 mg accuracy. Then powder was packed by striking the bottom of the tube on table surface up to the constant level, and then the volume was registered. Bulk density value of specimen was presented as g/cm^3. When the amount of tested material was sufficient, the size of the tube can be choice arbitrary, e.g. standard glass conic centrifuge tube. The material under investigation was not altered and can be reutilized in further experiments.

Swelling of chitosan in water media was determined in small tubes, like ones described above. 0.5 ml of distilled water or Tris-buffered saline (TBS, pH 7.4), was introduced into a tube, and 5 mg of tested powder was poured over the liquid. The behavior of particles was observed for 1 min and checked if they are retained on the surface or drawn to the bottom. Thereafter, the content was mixed with a small hook made from glass capillary, the tube was placed vertically and a result was registered after 1 hour (volume of sediment, dissolution of particles, viscosity of mixture). Finally, the condition of mixture (complete or partial dissolution of chitosan particles and volume of sediment, if present) were registered after 12 hours.

For *blood clotting test*, 2 ml of blood were withdrawn from the peripheral vein of human volunteers after their written agree into a plastic tube containing 0.2 ml of heparin (2 mg/ml in TBS). Analysis was performed in a flat bottom glass tubes, 8×35 mm. 0.2 ml of fresh heparinized blood was introduced into a tube, 7 mg of tested chitosan powder was added, and content was mixed with a glass hook. Time of clot formation was registered by a stop watch. Blood clotting was manifested as disappearance of liquid state of blood layer, i.e. no changing of its shape during bending of the tube or putting it bottom up.

Experiments in vivo were conducted on mice and rats taking into account the approval of the BioEthics Committee at Danylo Halytsky Lviv National Medical University (Protocol N 3 dated by 2015.03.16). Animal treatment was conducted in compliance with recommendations of *The European Community Council Directive*, 24 November 1986 (86/609/EEC).

Mouse was immobilized by its placing in a special small "house", the tail of animal hanging from it freely. Bleeding was evoked by cutting off 10 mm of tail end with a sharp razor blade. Bandage was applied upon the wound, which was prepared as follows. A peace of double sheet gauze (2×2 cm size) was moistened with 10% propylene glycol solution, an excess of liquid was blotted with filter paper and 10 mg of chitosan powder was spread over the gauze surface. After drying during 15–30 min, the gauze was folded to 1×1 cm square like a filter paper funnel and stored in the closed Eppendorf tube. Just before use, additional 3–5 mg of chitosan powder was introduced into this funnel. In control, bandage was loaded with inert powder, e.g. starch. Bandage was attached to the wound by fingers for 5 min, and then it was fixed with a peace of plaster. After 10–15 min, mouse was released into a cage and observed in about 1 hour. Bandage was left further for 12–24 hours in a place of application until spontaneous detachment. The level of blood loss was evaluated semi-quantitatively by measuring hemoglobin washed out from the bandage with water.

Similar experiment on rats was conducted under general anesthesia with diethyl ether. Bandage (double sheet gauze) was of bigger size (3×3 cm) than in the case of mouse, and respectively more chitosan powder (22 + 5 mg added *ex tempore*) was used.

Results and Discussion

It was revealed that commercial preparations of chitosan (produced as chemical reagent or dietary supplement), as well as that obtained in our laboratory by using known methods, did not exhibit hemocoagulating effect towards the heparinized blood, while Celox preparation possessed such activity. When using the test of chitosan swelling in water, we revealed that inactive chitosan particles differ from Celox by a lack of swelling and solubility in

water. After application upon water column in a tube, chitosan particles rapidly fall down to the tube bottom and form small white sediment. Similar behavior was observed when acetate and succinate salts of chitosan were used. On the contrary, Celox particles were hydrated immediately with a formation of very viscous substance that did not sediment. Thereafter, Celox particles were slowly dissolved. It was suggested that a lack of swelling depended upon structure of the particles which ought to be similar to dry gel, and also depended on conditions of chitosan precipitation and desiccation at final steps of preparation. It was found that precipitation of chitosan acetate with ethanol at concentration < 50% in pH interval of 5.9–7.0 provides gel-like structure of chitosan particles that saved their swelling property. Desiccation of gel by free evaporation on flat surface of glass sheet was another revealed feature of chitosan particles. Taking into account these observations, we developed a method of chitosan activation necessary for obtaining of highly active hemocoagulating agent.

Activation of commercial and reagent grade chitosan samples to obtain blood clotting agent. Chitosan sample was dissolved in 3% acetic acid (final concentration 0.75–1.5%) under constant mechanical stirring overnight. Dissolving proceeds slowly, and diversity in denoted concentration value is due to viscosity of solution depending on the molecular weight of chitosan sample. The insoluble material, if occur, was withdrawn by centrifugation (20 min, 2,000 g). Clear chitosan solution was treated with ammonia by bubbling NH_3 gas at constant stirring until pH 8.5–9.0 is achieved, at which chitosan precipitates like cheese flakes. The precipitate was collected by filtration through a synthetic cloth that was dense enough in order to retain sediment, and then it was washed with a small volume of distilled water. The obtained material was suspended in water and treated in Potter-Elvehjem homogenizer to a uniform slurry, thereafter it was solubilized by a stepwise addition of 10% solution of the acetic acid at intensive stirring. Dissolution began at pH 6.5 and was completed at pH 5.8, giving a viscous mass. In order to destroy clumps, it was necessary to use homogenization of the mass with whatever versatile mode, and Potter-Elvehjem homogenizer was the best. pH of the obtained mixture must be 5.9–6.0, and it might be corrected with 10% solutions of acetic acid or ammonium hydroxide. Mixture was left overnight for equilibration, thereafter, ethanol was added under constant stirring up to final concentration of alcohol 25 ± 2%. Mixture was centrifuged for 15 min at 2,000 g, and sediment denoted "chitosan fraction 1" was saved. Clear supernatant was adjusted to pH 6.7 with small volume of 10% NH_4OH which induced massive precipitation of chitosan. The sediment was collected by 10 min centrifugation at 1,500 g and denoted as "chitosan fraction 2". In case of turbid supernatant, it was treated with additional quantity of ammonium hydroxide to reach pH 7.2, and small sediment, if occurred, was collected by centrifugation. Material of fractions 1 and 2 was spread in a thin layer on glass sheets and dried by spontaneous evaporation. Dry material was collected, grinded to powder that was thereafter fractionated by sieving. Fraction with particle size < 0.25 mm corresponding to 60 mesh were collected. The yield of final product was 90%. Samples were stored in the dessicator over anhydrous $CaCl_2$. Characteristics of specific chitosan specimens before and after activation are presented in Table 1.

Average molecular weight of studied chitosan samples was determined by the viscometry. The experimental data obtained for chitosan of crab shells soluble in 1% acetic acid are presented in Table 2 and Fig. 2. An intrinsic viscosity ($[\eta] = \eta_{sp}$ at C = 0) was estimated graphically and corresponded to 560 ml/g (Fig. 2). Average molecular weight of the exemplified chitosan was calculated according to Staudinger formula: $\log [\eta] = \lg K + a \log M$, where constant values in used system were:

$K = 1.8 \times 10^{-3}$, a = 0.93

$\log 560 = \log 0{,}0018 + 0.93 \log M$

$2.748 = -2.745 + 0.93 \log M$

$\log M = 5.493 / 0.93 = 5.906$

M = 805378, approx. 800 KDa.

Average molecular weight and degree of deacetylation values of studied chitosan specimens in comparison with their coagulating activity are shown in Table 3.

Obtained data show that the molecular weight of chitosan is very important for its blood clotting activity. High molecular weight chitosan preparations exhibit faster clot formation. It is suggested that chitosan obtained in our laboratory from crab shells

Table 1. **Characteristics of different chitosan specimens**

Type of chitosan specimen	Characteristics				
	Bulk density (g/cm^3)	Swelling/ solubility in water*	pH of suspension/ solution	Swelling/ solubility in TBS*	Heparinized blood clotting time**
Aldrich	0.59	Sediment 0.04 ml	5.0	Sediment 0.03 ml	No clot formation
Aldrich activated	0.26	Dissolved, viscous solution	5.0	Dissolved, viscous solution	60–120 s
Tyanshi	0.33	Sediment 0.05 ml	5.5	Sediment 0.03 ml	No clot formation
Tyanshi activated	0.27–0.43	Dissolved, moderately viscous	5.5	Dissolved, moderately viscous	2.5–3 min
Organica	0.55	Sediment 0.05 ml	5.7	Sediment 0.03 ml	No clot formation
Organica activated	0.38	Dissolved partially, sediment 0.10 ml	5.5	Sediment 0.07ml	3.5–4 min
Home prepared	0.59	Sediment 0.10 ml	5.0	0.06 ml	No clot formation
Home prepared activated	0.17–0.40	Dissolved partially, highly viscous	5.5	Dissolved partially, highly viscous	45–90 s
Celox	0.56	Dissolved partially, highly viscous	4.7	Dissolved partially, highly viscous	60–90 s

Notes. * — 5 mg of chitosan sample were introduced into 0.5 ml of water or TBS;
** — 7 mg of chitosan sample were added to 0.2 ml of heparinized blood.

Table 2. **Results of viscosity measurement of chitosan isolated from crab shells**

Solution	Concentration ($\times 10^{-3}$ g/ml)	Time (s)	η_r	η_{sp}	η_{sp}/C (ml/g)
So	0.00	87.2	1.000	0.000	0
S1	1.00	154.4	1.769	0.769	769
S2	0.78	135.9	1.557	0.557	502
S3	0.60	122.3	1.401	0.401	494
S4	0.47	114.0	1.306	0.306	490
S5	0.33	105.2	1.205	0.205	470

Notes. $\eta_r = T_{solution} / T_{solvent}$; $\eta_{sp} = \eta_r - 1$.

Table 3. **Effect of molecular weight and degree of deacetylation upon hemocoagulating efficacy of activated chitosan specimens**

Type of chitosan	Molecular weight (KDa)	Degree of deacetylation	Clotting time of heparinized blood
Organica	150	98%	3.5–4.0 min
Tyanshi	600	98%	2.0–3.0 min
Chitosan from crab shells	800	95%	45–90 s
Celox	500	95%	45–90 s

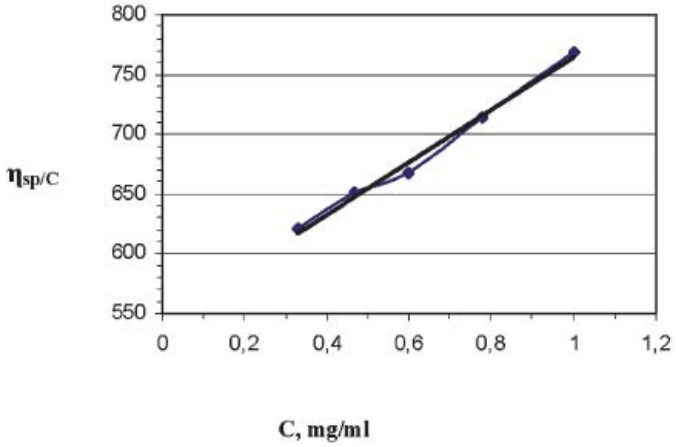

Fig. 2. **Graphical determination of intrinsic viscosity of chitosan solution on basis of relation between η_{sp}/C and concentration of chitosan solution**

exhibits high hemocoagulating activity due to the presence of high molecular weight (more then million Daltons) fractions that do not dissolve completely in 1% acetic acid and need more concentrated acetic acid for complete dissolution. Similar property was observed for Celox, while chitosans of other origin (Tyanshi and Organica) were readily dissolved in 1% acetic acid. Species specificity and mode of preparation also have an influence upon biological activity of product, as can be deduced from comparison of Tyanshi and Celox specimens.

For preparing chitosan with effective antihemorrhagic action, it is necessary to have a set of tests for monitoring process of chitosan purification and controlling quality of final product. Among different tests mentioned in the "Introduction", only the coagulation of bentonite particles [13] may be considered as a convenient screening procedure. In this investigation, we have developed several tests and indices that permit evaluating blood clotting and hemostatic activity of used chitosan preparations. They are easy enough and do not require special reagents and instruments (see below).

Clotting of the heparinized blood is the most important *in vitro* test witnessing a sufficient hemostatic activity of chitosan sample. This indicator of Celox is well known [2], but it was not applied for evaluating blood clotting activity of chitosan samples. We consider that this blood coagulation test at validation of chitosan preparations must not exceed 90 s. If the coagulation time of tested chitosan sample is 5–12 min, that means low hemostatic activity and such chitosan is not acceptable for practical application. The use of Ca-chelating anticoagulants (EDTA, sodium citrate, oxalate) for blood stabilization is not recommended, since chitosan samples themselves may contain Ca cations and, thus, can induce blood coagulation beside chitosan's action.

Interesting data were obtained when coagulation of the defibrinated blood by active chitosan and Celox was compared. It is known that defibrination causes elimination of fibrin and most of pool of blood platelets. Thus, the absoption of blood plasma, drastic increase of viscosity due to swelling of chitosan particles and binding of blood cells on the positively charged chitosan leading to appearance of the pseudoclot might be of primary significance in the mechanism of chitosan-induced blood clotting. Activation of blood platelets [12] may be involved in the coagulation process at later stages and play a role in stabilization of the clot.

Swelling of chitosan powder in water is the next important indicator of chitosan's hemostatic activity. The particles of chitosan's active samples are retained on the surface of water column after introduction into the tube, and after mixing, they swell rapidly and form viscous jelly-like medium that becomes optically homogenous after later dissolution of material. In case of using inactive chitosan samples, the particles rapidly fall down on the bottom of the tube and, thus, do not cause medium viscosity. The swelling of chitosan powder in water correlates closely with its hemocoagulating/ hemostatic activity.

pH of chitosan solution/suspension in water is also important, and it is advisable that it is close to neutral, since an excessive acidity will irritate tissues underlying a wound. Celox provides pH 4.5–4.8, our preparations show pH 5.0–5.4. Although in buffered saline (pH 7.3) swelling of chitosan particles is less than in water, Celox and hemostatic chitosan samples are well swollen and dissolve gradually in the TBS producing highly viscous solution (phosphates must be omitted as they induce precipitation of chitosan). While inactive chitosan samples swell weakly that is in one half of their swelling capability in water.

Bulk density is another useful index of chitosan samples, since it reflects a porosity of material. Lower value of density corresponds to higher porosity and subsequently higher surface of particles available for contact and their higher coagulation efficacy. Besides, equal quantity of lighter powder of chitosan permits covering bigger area of injury than its dense powder. Bulk density does not correlate with blood clotting activity. Some commercial chitosan samples having low bulk density (e. g. 0.33 g/cm^3) show no blood clotting activity, while Celox with bulk density of 0.56 g/cm^3 is a highly active hemostatic agent.

Chitosan obtained by us differs from Celox specifically by its lower bulk density that corresponds to a higher porosity.That is a useful property since it permits covering bigger areas of tissue lesion with less quantity of material (chitosan). Lower bulk density of chitosan samples was achieved by using special conditions of precipitation and dessication. It was found that treatment of chitosan sediment with high concentration of ethanol or aceton leads to an increase in bulk density and, subsequently, to a decrease of porosity of gel particles, probably due to marked dehydration and tighter package of chitosan's polysaccharide chains.

Thus, chitosan preparations obtained in this study could be used as a starting material for production different chitosan-based materials of the biomedical implication, first of all, as hemostatic bandage and wound dressing. Now-a-day, it is proved that Celox used as bandage is more convenient and pfererable in field conditions than Celox used as powder [2]. Chitosan powder can be used for impregnation of gauze since its particles are highly sensitive to moisture and firmly attach to wet cotton fibers and threads.

In general chitosan is highly perspective material especially in biomedical aspects [19–28]. Ukraine has a potential and it is desirable the own industrial production of chitin and chitosan for making them accessible to a broader community of reserchers and inventors.

Method of activation of chitosan specimens to a products with high blood clotting activity was developed. It is based on a proposed set of laboratory tests *in vitro* and *in vivo* for characterization of hemocoagulating/hemostatic effect of the sample. It was shown that high molecular weight chitosans exhibit higher hemocoagulating activity than those with low molecular weight. The first ones are preferable for preparation of hemostatic agents. Biological activity of chitosan is also dependent upon its species specificity and method of preparation.

REFERENCES

1. *Achneck H. E., Bantayehu Sileshi, Jamiolkowski R. N., Albala D. M., Shapiro M. L., Lawson J. H.* A comprehensive review of topical hemostatic agents: efficacy and recommendations for use. *Ann. Surg.* 2010, 251 (2), 217–228.
2. Hemostatic material «Celox». A comphensive manual from SURV24. http://www.surv24.ru/blogs/2014/11/21/. (In Russian).
3. *Kuzin B. A., Babievsky C. K., Prokhorenkova G. K., Kuzin A. B.* Method of obtaining of chitosan. *R. F. Patent Ru 2 067 588*. October 10, 1996. (In Russian).
4. *Kasjanov G. I., Kvasenkov O. I., Nikolayev A. I., Kasjanova E. E.* Method of obtaining of chitosan. *R. F. Patent Ru 2 116 314*. July 27, 1998. (In Russian).
5. *Ivanov A. V., Gartman O. R., Tsvetkova A. V., Poltoratskaya E. B.* Method of obtaining of chitosan. *R. F. Patent Ru 2 117 673*. August 12, 1998. (In Russian).
6. *Bannikov V. V., L'vovich F. I., Frayman D. B.* Method of obtaining of chitosan. *R. F. Patent Ru 2 073 017*. February 10, 1997. (In Russian).
7. *Solodovnik T. V., Unrod V. I.* Method of obtaining of chitosan. *Ukraine Patent UA 33 765*. Fubruary 15, 2001. (In Ukrainian).
8. *Gartman O. R., Vorobyova V. M.* Technology and properties of chitosan from Hammarus crayfish. *Fundamental investigations.* 2013, N 5–6, P. 1188–1192. (In Russian).
9. *Nemtsev S. V., Zuyeva O. U., Hismatullin M. R., Albulin A. I., Varlamov V. P.* Obtaining of chitin and chitosan from honey bees. *Applied biochem microbiol*. 2004, 40 (1), 46–50. (In Russian).
10. *Janvikul W., Uppanan P., Thavornyutikarn B., Krewraing J., Prateepasen R. In vitro* comparative hemostatic studies of chitin, chitosan and their derivatives. *J. Appl. Polym. Sci.* 2006, 102 (1), 445–451. doi: 10.1002/app.24192.
11. *Okamoto Y., Yano R., Miyatake K., Tomohiro I., Shigemasa Y., Minami S.* Effects of chitin and chitosan on blood coagulation. *Carbohydr. Polym.* 2003, 53 (3), 337–342. doi: 10.1016/S0144- 8617(03)00076-6.
12. *Sanandam M., Salunkhe A., Shejale K., Patil D.* Chitosan bandage for faster blood clotting and wound healing. (Determination of the duration of bleeding from pricked finger tip using chitosan soaked bandage). *Int. J. Adv. Biotechnol. Res.* 2013, 4 (1), 47–50.
13. *Hardy C., Johnson E. L., Luksch P.* Hemostatic material. *U. S. Patent US 7 981 872*. July 19, 2011.
14. *Littlejohn L. E., Devlin J. J., Kircher S. S., Lueken R., Melia M. R., Johnson A. S.* Control of hemorrhage in a swine model of penetrating trauma, i.e. groin injury with transection of the femoral vessels. *Acad. Emerg. Med.* 2011, 18 (4), 340–350. doi: 10.111/j.1553-2712.2011.01036.x.
15. http://tiens.in.ua/products/kapsuly-s-hitozanom-tyanshi.
16. *Lazurievsky G. V., Terentieva I. V., Shamshurin A. A.* Practical exercises in chemistry of natural compounds. *Moskva: Vysshaya shkola*. 1966, P. 88–91. (In Russian).
17. *Skriabin K. G., Vikhoreva G. A., Varlamov V. P.* Chitin and Chitosan. Production, properties and usage. *Moskva: Nauka*. 2002, 368 p. (In Russian).
18. *Costa C. N., Teixeira V. G., Delpech M. C., Souza J. V., Costa M. A.* Viscometric studies of chitosan solutions in acetic acid/sodium acetate and acetic acid/sodium chloride. *Carbohydr. polym*. 2015, V. 133, P. 245–250. doi: 10.1016/j.carbpol. 2015.06.094.
19. *Felse P. A., Panda T.* Studies on application of chitin and its derivatives. *Biopr. Engin*. 1999, V. 20, P. 505–512.
20. *Majeti N., V Ravi Kumar.* A review of chitin and chitosan applications. *React. Funct. Polym*. 2000, 45 (1), 1–27.
21. *Hiroshi Sashiwa, Sei-ichi Aiba*. Chemically modified chitin and chitosan as biomaterials. Review article. *Progr. Polym. Sci*. 2004, 29 (9), 887–908.
22. *Rinaudo M*. Chitin and chitosan: properties and applications. *Progr. Polym. Sci*. 2006, 31 (7), 603–632. doi: 10.1016/j. progpolymsci.2006.06.001.
23. *Tikhonov V., Stepnova E., Lopez-Llorka L., Gerasimenko D., Avdienko I., Varlamov V.* Bactericidal and antifungal activities of low molecular weight chitosan derivatives. *Carbohydr. Polym*. 2006, 64 (1), 66–72.
24. *Dasha M., Chiellinia F., Ottenbriteb R. M., Chiellinia E*. Chitosan — a versatile semi-synthetic polymer in biomedical applications. *Progr. Polym. Sci*. 2011, 36 (8), 981–1014.
25. *Jayakumar R. Prabaharan, Sudheesh Kumar P. T., Nair S. V., Tamura H*. Biomaterials based on chitin and chitosan in wound dressing applications. *Biotechnol. Adv*. 2011, 29 (3), 322–337.
26. *Pogorielov M. V., Kornienko V. V., Tkachenko Yu. A., Oleshko O. M.* Materials to treat the skin defects: chitosan derivatives and perspectives for their application (literature review). *J. Clin. Experiment. Med. Res.* 2013, 1 (3), 275–284. (In Ukrainian).
27. *Xiaosong Li, Min Min, Nan Du, Ying Gu, Tomas Hode, Mark Naylor, Dianjuo Chen, Nordquist R. E., Wei R. Chen.* Chitin, chitosan, and glycated chitosan regulate immune responces: the novel adjuvants for cancer vaccine. *Clin. Developm. Immunol.* 2013, Article ID 387023, 8 p. http://dx.doi.org10.1155/2013/387023.
28. *Smith A., Perelman M., Hinchcliffe M*. Chitosan: a promising safe and immune enhancing adjuvant for intranasal vaccines. *Hum. Vaccin. Immunother.* 2014, 10 (3), 797–807.

ОТРИМАННЯ ХІТОЗАНУ З ВИСОКОЮ ГЕМОКОАГУЛЯЦІЙНОЮ АКТИВНІСТЮ ТА ОЦІНКА ЙОГО ГЕМОСТАТИЧНОЇ АКТИВНОСТІ

М. Д. Луцик[1]
Р. О. Білий[1, 2]
М. М. Луцик[2]
Р. С. Стойка[1]

[1]Інститут біології клітини НАН України, Львів
[2]Львівський національний медичний університет ім. Данила Галицького, Україна

E-mail: lootsik@cellbiol.lviv.ua

Метою роботи було вивчення гемостатичної дії різних препаратів хітозану за допомогою розроблених лабораторних тестів для визначення його гемокоагуляційної активності. Із застосуванням тестів *in vitro* зразки хітозану (порошки з розміром частинок менше 0,25 мм) характеризували за такими показниками: час коагуляції гепаринізованої крові, насипна щільність порошку, ступінь набухання частинок порошку у воді та в трис-забуференому фізрозчині, pH 7,4. У разі застосування тестів *in vivo* визначали ефективність зупинки кровотечі з рани у лабораторних мишей або щурів. За допомогою запропонованих тестів виявлено, що зразки хітозану, доступні як комерційні реагенти, харчові добавки або отримані в лабораторії, не виявляють гемокоагуляційної активності порівняно з препаратом Целокс. Розроблено метод активації хітозану й отримання препаратів з гемокоагуляційною активністю на рівні препарату Целокс. Розглядаються властивості хітозану, суттєві для виявлення його гемокоагуляційної дії. Показано, що препарати високомолекулярного хітозану мають вищу гемокоагуляційну активність порівняно з низькомолекулярним і є більш придатними для одержання гемостатичних засобів.

Ключові слова: хітозан, Целокс, гемостатична активність, лабораторні тести *in vitro* та *in vivo*.

ПОЛУЧЕНИЕ ХИТОЗАНА С ВЫСОКОЙ ГЕМОКОАГУЛИРУЮЩЕЙ АКТИВНОСТЬЮ И ОЦЕНКА ЕГО ГЕМОСТАТИЧЕСКОЙ АКТИВНОСТИ

М. Д. Луцик[1]
Р. А. Билый[1, 2]
М. М. Луцик[2]
Р. С. Стойка[1]

[1]Институт биологии клетки НАН Украины, Львов
[2]Львовский национальный медицинский университет им. Данила Галицкого, Украина

E-mail: lootsik@cellbiol.lviv.ua

Целью работы было изучение гемостатического действия различных препаратов хитозана с помощью разработанных лабораторных тестов для определения его кровосвертывающей активности. С использованием тестов *in vitro* образцы хитозана (порошки с размером частиц менее 0,25 мм) характеризовали по следующим показателям: время свертывания гепаринизированной крови, насыпная плотность порошка, степень набухания частиц порошка в воде и в трис-забуференном физрастворе, pH 7,4. С применением тестов *in vivo* определяли эффективность остановки кровотечения из раны у лабораторных мышей или крыс. С помощью предложенных тестов установлено, что образцы хитозана, доступные как коммерческие реагенты, пищевые добавки или полученные в лабораторных условиях, не проявляют гемостатической активности при сравнении с препаратом Целокс. Разработан метод активирования образцов хитозана и получения препаратов с гемостатической активностью на уровне препарата Целокс. Рассматриваются свойства хитозана, существенные для проявления его кровосвертывающего действия. Показано, что препараты высокомолекулярного хитозана проявляют более высокую гемокоагулирующую активность по сравнению с низкомолекулярным и более предпочтительны для получения гемостатических средств.

Ключевые слова: хитозан, Целокс, гемостатическая активность, лабораторные тесты *in vitro* и *in vivo*.