Development of Chitosan Hemostatic Sponges with Different Solvents and Tranexamic Acid

Maksym Pogorielov,
Volodymyr Deineka,
Oleksandr Oleshko,
Irina Liubchak,
Katerina Dedkova
Medical Institute
Sumy State University
Sumy, Ukraine
m.pogorielov@gmail.com

Roman Vasyliev, Dimitri Zubov
State Institute of Genetic and
Regenerative Medicine NAMS of
Ukraine, Kiev, Ukraine
Medical company *ilaya*®
rvasyliev.ilaya@gmail.com

Aleksei Kalinkevich,
Oksana Kalinkevich
Institute of Applied Physics
Sumy, Ukraine
kalinkevich@gmail.com

***Abstract*—Blood loss during surgery and combat trauma are still main complications leading patient death. Topical hemostasis in case of diffuse bleeding over past 2 decades have resulted in low mortality and morbidity rates but relatively high cost limited their application in low- and middle-income countries, that stimulate investigation of effective and low cost materials.**

In recent paper chitosan sponges with different solvents (acetic, ascorbic, lactic and oxalic acids) with/without tranexamic acid were made as a hemostatic sponges. SEM and Degradation test used for physical characterization and Cell Toxicity test and Blood Clotting Test – for biological evaluation.

Based on structural features and in-vitro studies, chitosan-acetate sponge with TXA is the best choice for development of high efficient hemostatic materials.

Keywords—chitosan; hemostatic sponges, parenchimal bleeding

I. INTRODUCTION

The fundamental principle of good surgical technique is minimization of blood loss, thus hemostasis is of critical importance during all surgical procedures. It is known that volume of blood loss in surgery is an established predictor of morbidity and mortality [Imamura H, 2003]. Reduced blood loss and ensuing reduction in blood transfusions has been demonstrated to result in superior surgical outcomes, including reduction of operative time and improvement quality of surgical tissue management [1].

Major bleeding from large vessels is initially managed by primary surgical methods (i.e., sutures, stapling ligatures, argon beam coagulation, and electrocautery). Diffuse bleeding may be managed by argon beam coagulation, electrocautery, and/or hemostatic agents [2]. Large number of reports shown different safety and effectiveness of hemostatic materials in liver, pancreatic and renal surgery. Topical hemostasis in case of diffuse bleeding over past 2 decades have resulted in low mortality (1-5%) and morbidity rates (4 to 20%) [3]. Due to relatively high cost (ranges from $15.00 to $650.00) [4] application of hemostatic agents limited in low- and middle-income countries, that stimulate investigation of effective and low cost materials.

Chitosan, an amino polysaccharide (poly 1, 4-D-glucoamine), has been known as a biological material in promoting the healing process of soft and hard connective tissues. It is important that the hemostatic mechanism of chitosan is independent of innate clotting mechanisms and can therefore act in the presence of anticoagulants [5] There are some topical hemostatic materials (HemCon, Celox) available for medical application, but they limited to use for external bleeding (in civil and combat traumas) [6]. There are some modification have been applied to increase effectiveness of hemostasis of chitosan-based materials. Tze-Wen Chung et all. used adenosine diphosphate and fibrinogen to fabricate hemostatic chitosan nanoparticle and shown only adenosine diphosphate reduced blood clotting times and provide effective hemostasis [7]. In other research PolySTAT-Modified Chitosan Gauzes are able to improve blood clotting with the addition of a fibrin cross-linking hemostatic mechanism [8].

It is known, that molecular weight of chitosan and type of solvent influence to the final biological properties of materials, such as physical characteristics, biocompatibility, antibacterial effect etc. [9-10]. But there are no publications about influence of different solvents to hemostatic properties of chitosan-based materials. By other hand, application of antifibrinolytic agent can increase effectiveness of topical hemostatic material with reduction of postoperative complications. Tranexamic acid (TXA) is most effective antifibrinolytic agent and Jinqian Liang used TXA-soaked absorbable gelatin sponge in posterior lumbar spine surgery to reduce post-operative blood loss and blood transfusion requirements [11]. But there are no data about possible combination of chitosan-based sponges and TXA for development of hemostatic materials.

In the present work, we aim to assess influence of different solvents and TXA to biocompatibility, blood clotting ability of chitosan sponges and develop effective chitosan-based hemostatic material for parenchymal bleeding management.

II. Materials and Methods

A. Materials

We have used chitosan with molecular weight 300 kDa and degree deacetylation 82% was purchased from *YuDa Chemicals*, Qindao, PRC, organic acid (acetic, ascorbic, lactic and oxalic: chemical or analytical grade) and TXA (pharmaceutical grade) to produce different type of hemostatic sponges (Table 1). Chitosan solution was prepared by dissolving the polymer in different organic acids with different concentrations, the solution was stirred for 6-24 h until it becomes homogeneous. TXA was added into solutions which were then mixed vigorously. The polymer/TXA/solvent solution was cast into the plastic mold, and the resulting samples were freezed at -5 - -20°C and then dried in vacuum chamber (0.1 Pa, 24 h). The preparation process of all hemostatic sponges was similar.

TABLE I. Composition of hemostatic sponges

Components	Sponges			
	AcCh	**AscCh**	**LacCh**	**OxCh**
Solvent, g/L	Acetic acid 10	Ascorbic acid 20	Lactic acid 10	Oxalic acid 50
Chitosan, g/L	20	20	20	20
TXA, g/L	10	10	10	10

B. Scanning electron microscopy

Scanning electron microscopy was performed using the electron microscope Hitachi TM-3000 (accelerating voltage - 5 kV) to obtain information about structural features of pure sponges and after blood clotting test. To avoid surface charge accumulation in the electron-probe experiment all samples were covered with the thin (30–50 nm) layer of gold in the vacuum set-up.

C. Degradation test

For the degradation test, samples were immersed in SBF (pH 7.4) solution (10 mg of samples in 3 ml of SBF) and incubated at 37 °C. Samples were taken out after 5, 15, 30, 60 minutes and 1, 7, 14, 21 days of incubation with complete solution refreshment. Degradation rate D was calculated as the change in mass divided by original mass.

$$D = \frac{M_0 - M}{M_0} \cdot 100\%$$

Remaining weight (Fig. 2) was determined

$$R = \frac{M}{M_0} \cdot 100\%$$

D. Cell toxicity experiment

Adipose-derived stem cells (ADSc) were isolated via enzymatic digestion of subcutaneous fat and derma fragments, then propagated in alpha-MEM medium supplemented with 10% FBS and 1 ng/ml bFGF under 5% O_2 (Sigma-Aldrich, USA). Cultured cells sown over the chitosan sponges in triples (10^4 cells per each type sample) in 24 well cell culture plates and incubated for 24 hours. Cell viability and proliferation rate were assured on 24h by combined staining with FDA/PI followed by fluorescent microscopy visualization.

E. Blood clotting test

The strips of chitosan sponges with weight 100 mg was placed to Becton Dickinson Vacutainers® with fresh human blood (obtained from 4 human subjects volunteers) and incubated in thermostat in temperature 36 0C during 10 minutes. All samples were removed and blood transported to the hematology tests. Untreated blood was used as a control. Sorption of blood, haemoglobin level (HGB, g/L), Red Blood Cells (RBC, T/L), Mean Corpuscular Volume (MCV, fL), RBC Distribution Width (RDW, %), Platelets (PTL, G/L), Mean Platelet Volume (MPV, fL), and Platelet Distribution Width (PDW, %) were evaluated after blood clotting test.

F. Statistics

Data were expressed as means ± standard deviation. Student's t-test on unpaired data was used to assess the statistical significance of the difference between the results obtained from the tested specimens. Statistical significance was assumed at a confidence level of 95% ($p < 0.05$)

III. Results and Discussion

A. Structural evaluation of chitosan sponges

All sponges have interconnected pores that allow sorption of liquid part of blood during hemostatic process. Pore distribution and morphology strongly depends on solvent that used for material synthesis. AcCh and LacCh have large pores 60-200 μm in diameter while pore size in AscCh and OxCh ranges from 10 to 100 μm (*figure 1*).

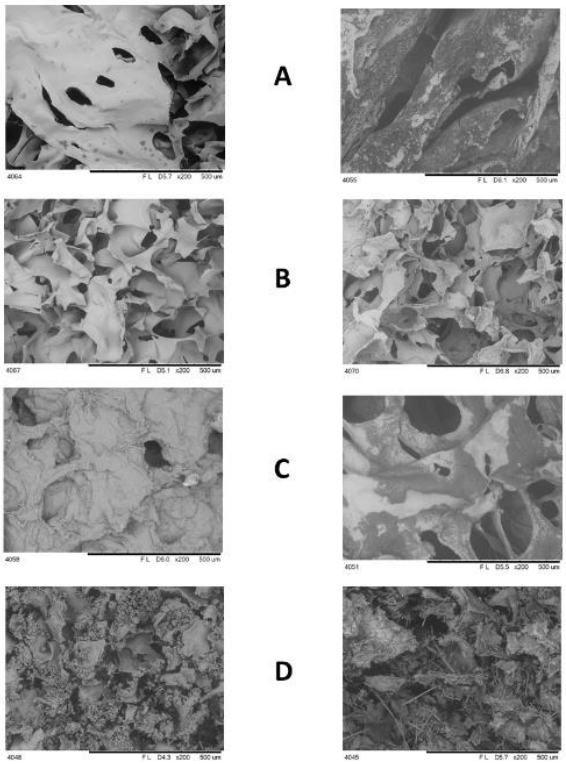

Fig. 1. SEM morphology of chitosan sponges made using acetate (A), ascorbate (B), lactate (C) and oxalate (D). Left column represent pure sponges, right – with TXA. Magnification 200x.

Addition of TXA to chitosan sponge does not affect their structure and pore size. It should be noted that OxCh has a loose structure and after TXA application crystal-shaped structures visualize inside pores that could affect hemostatic properties and complicate the application. The total volume of open pores, measured by the change in the volume of ethanol depends on solvent and ranges from 14.3 cm^3/g for LacCh to 40 cm^3/g for AcCh. Based on described structure and our previous results with chitosan coated gauze (12), these materials are available for application as a hemostatic agents.

Degradation test

OxCh dissolves completely in the first 5 min, giving turbid solution with pH 3.3, so it is not shown in the Fig. 2 and 3. AscCh within the first five minutes loses about 57% of the original mass. At the end of the incubation (21 days), mass loss was 80%. LacCh loses 40% within first 5 minutes, at the end of incubation about 55%. AcCh loses 63% of the mass during 15 min incubation, the largest mass loss of 74% is observed on the 7th day of incubation in SBF. Further mass loss is reversed (42% on 21st day), possibly due to the crystallization of salts present in SBF in the chitosan matrix (this fact requires further studies, since the initiation of chitosan mineralization in this case may lead to pathology). All sponges after incubation in SBF and drying do not retain their original shape (they dry up in the form of lumps or films). Changes in pH (compared with initial pH of SBF): 7.5 for AcCh and AscCh, 7.6 for LacCh.

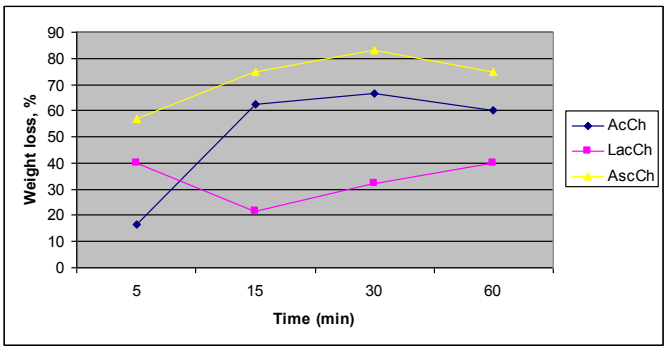

Fig. 2. The degradation of chitosan sponges in SBF, first 60 min.

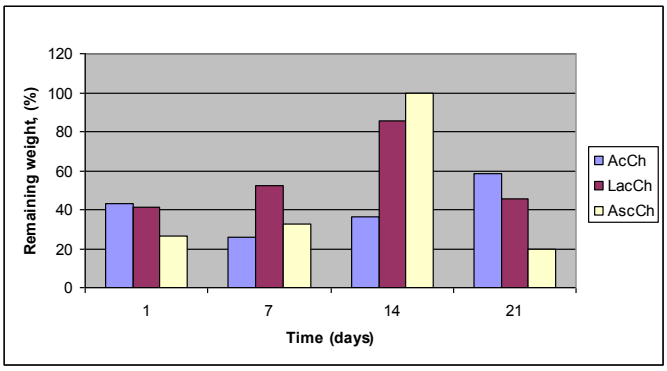

Fig. 3. The degradation of chitosan sponges in SBF, 21 days.

B. Cell toxicity test

24h culturing of adipose-derived stem cells with chitosan-based materials shown high toxicity and low adhesion rate for all pure chitosan sponges (*figure 4, left column*). There are controversial data about chitosan toxicity with different solvents and most authors pointed for obligatory residual removal from final product [13-14]. Our protocol did not include this step and probably during degradation, acid can release to culture media and affect cells. In fact, addition of TXA to chitosan sponge decrease cell toxicity for all materials. But cell adhesion and proliferation we can note for AcCh only (figure 4, right column, A). ADSc spread out the material's surface and ingrowth to open pores. Supposedly, TXA can bind with acid residuals that decrease material's toxicity and increase cells viability. Results of cell toxicity test shown potential clinical application of AcCh sponge with TXA only. For other composition it is need to provide thorough cleaning from acid residue to decrease toxicity.

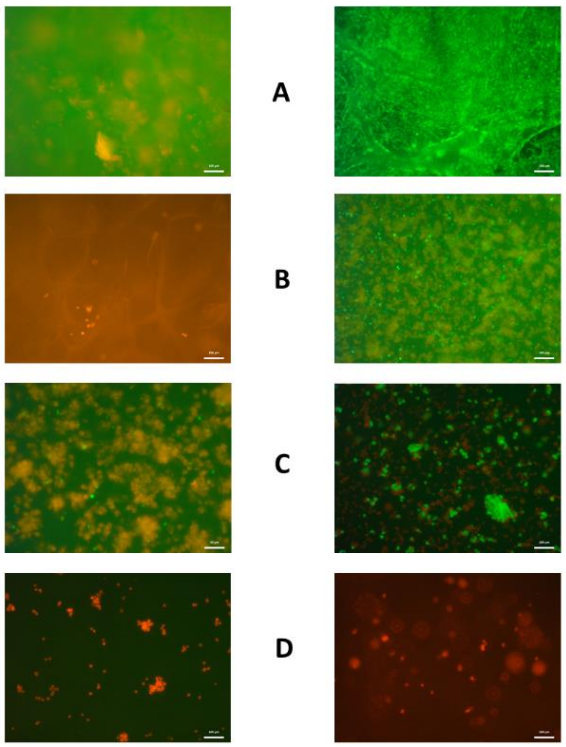

Fig. 4. The resulted FDA/PI stains of 24h incubation of chitosan sponges made using acetate (A), ascorbate (B), lactate (C) and oxalate (D) sown with ADSc. Left column represent pure sponges, right – with TXA. Fluorescent microscopy, magnification ×50.

C. Blood clotting test

Blood coagulation test did not show any changes of coagulogramm in our experiment. The RBC and hemoglobin level increased in all types of sponges, but the difference was not significant (*figure 5, A*). Pure AscCh and AcCh+TXA significantly decreased platelet count (*figure 5, B*) but only AcCh+TXA and AscCh+TXA significantly change Mean Platelet Volume and Platelet Distribution Width (*figure 5, C, D*).

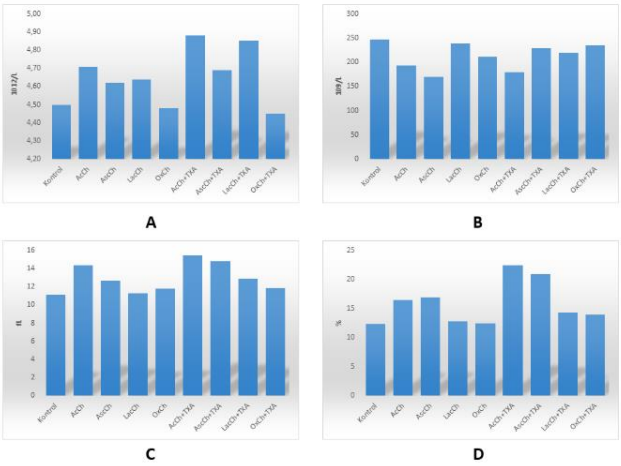

Fig. 5. RBC (A), Platelets (B), Mean Platelet Volume (C) and Platelets Distribution Width (D)after blood clotting test with different hemostatic sponges

Scanning electron microscopy after blood clotting test did not show principal difference between pure and TXA loaded sponges. Results shown that surface and pores of chitosan sponges covered with fibrin-like network that embedded with normal shaped erythrocytes and activated platelets. Some cells, spatially platelets, directly attached to surface of sponges (*figure 6*). It should be noted that chitosan-oxalate materials has pure fibrin-like network and preferential adhesion of erythrocytes compare other materials.

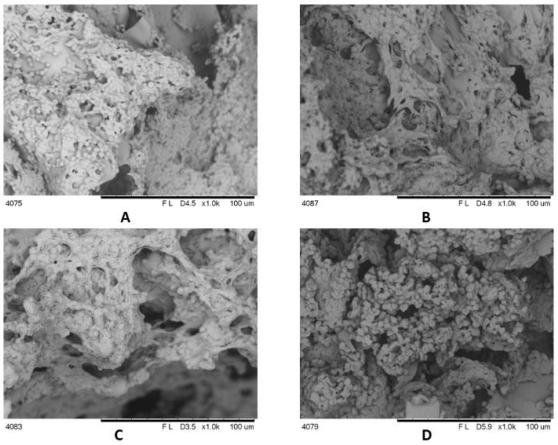

Fig. 6. SEM of chitosan sponges made using acetate (A), ascorbate (B), lactate (C) and oxalate (D) after 10 min blood clotting test. Magnification ×1000.

There are different mechanisms of hemostasis after chitosan application: initially it absorb a high amount of fluid (more than 20 times their dry weight) that lead to haemoconcentration, than electrostatic interaction with negatively-charged cell membranes of erythrocytes leading to erythrocyte agglutination and formation of a hemostatic plug at the site of injury [15]. TXA is an antifibrinolytic agent that reversibly inhibits the activation of plasminogen to plasmin by binding to a specific lysine site of plasminogen, thereby delaying clot degradation. Addition application of TXA to chitosan sponge can stabilize existing clot and prevent recurrent bleeding.

IV. CONCLUSION

Freeze-dried sponges from chitosan with different solvents (acetic, ascorbic, lactic and oxalic acids) has high porous structure. TXA did not influence to structure and degradation of hemostatic sponge but significant affect cell distributing after blood clotting test. Cell toxicity have shown high toxicity of all pure materials and confirmed cell viability and proliferation on TXA loaded materials.

Based on structural features and in-vitro studies, chitosan-acetate sponge with TXA is the best choice for development of high efficient hemostatic materials.

ACKNOWLEDGMENT

The authors are thanks to Prof. Wojcieh Simka (Department of Inorganic Chemistry, Analytical Chemistry and Electrochemistry, Faculty of Chemistry, Silesian University of Technology) for SEM investigation.

REFERENCES

[1] W. D. Spotnitz and S. Burks, "Hemostats, sealants, and adhesives II: update as well as how and when to use the components of the surgical toolbox," Clinical and Applied Thrombosis/Hemostasis, vol. 16, no. 5, pp. 497–514, 2010.

[2] S. Kawasaki, H. Origasa, V. Tetens, and M. Kobayashi, "Comparison of TachoSil and TachoComb in patients undergoing liver resection—a randomized, double-blind, non-inferiority trial," Langenbecks Arch Surg, vol. 402, pp. 591–598, 2017.

[3] J. Briceno, A. Naranjo, and R. Ciria, "A prospective study of the efficacy of clinical application of a new carrier-bound fibrin sealant after liver resection," Archives of Surgery, vol. 145, no. 5, pp. 482–488, 2010.

[4] S. Vyas Krishna and P. Saha Sibu, "Comparison of hemostatic agents used in vascular surgery", Expert Opin Biol Ther., vol. 13(12), pp. 1663–1672, December 2013.

[5] O. Koksal, F. Ozdemir, B. C. Etoz, N. I. Buyukcoskun, and D. Sigirli, "Hemostatic effect of a chitosan linear polymer (Celox®) in a severe femoral artery bleeding rat model under hypothermia or warfarin therapy," Turkish J. Trauma Emerg. Surg., vol. 17, pp. 199–204, 2011.

[6] R. W. J. Millner, A. S. Lockhart, H. Bird, and C. Alexiou, "A new hemostatic agent: initial life-saving experience with Celox (chitosan) in cardiothoracic surgery," Ann. Thorac. Surg., vol. 87, pp. 13–14, 2009.

[7] Tze-Wen Chung, Pei-Yi Lin, Shoei-Shen Wang, and Yen-Fung Chen, "Adenosine diphosphate-decorated chitosan nanoparticles shorten blood clotting times, inflencing the structures and varying the mechanical properties of the clots," International Journal of Nanomedicine, vol. 9, pp. 1655–1664, 2014.

[8] W. Chana Leslie, Chae Hwa Kimb, Xu Wangc, Suzie H. Puna, Nathan J. Whitec, and Tae Hee Kim, "PolySTAT-Modified Chitosan Gauzes for Improved Hemostasis in External Hemorrhage," Acta Biomater., vol. 31, pp. 178–185, 2016.

[9] R. Spin-Neto et al., "Histological evaluation of chitosan-based biomaterials used for the correction of critical size defects in rat's calvaria," J. Biomed. Mater. Research, vol. A93(1), pp. 107-114, April 2010.

[10] O. V. Kalinkevich, M. V. Pogorelov, I. M. Babich, S. N. Danil'chenko, and G. F. Tkach, "In-vitro degradation of the chitosan membranes under various syntheses conditions," Biomeditsinskaia khimiia, 2014.

[11] J. Liang, et. al., "Using tranexamic acid soaked absorbable gelatin sponge following complex posterior lumbar spine surgery: A

randomized control trial,” Clinical Neurology and Neurosurgery, vol. 147, pp. 110–114, 2016.

[12] M. Pogorielov, O. Kalinkevich, V. Deineka, “Haemostatic chitosan coated gauze: In vitro interaction with human blood and in-vivo effectiveness,” Biomaterials Research, vol. 19, no. 1, p. 22, 2015.

[13] S. Xin et.al., “Cytotoxicity and antibacterial ability of scaffolds immobilized by polysaccharide/layered silicate composites,” Carbohydr. Polymers, vol. 92, no. 2, pp. 1880-1886, February 2013.

[14] A. Lagarto et al., “Safety evaluation of chitosan and chitosan acid salts from Panurilus argus lobster,” Int. J. Biol. Macromolecules, vol. 72, pp. 1343-1350, January 2015.

[15] B. L. Bennett, L. Littlejohn, “Review of new topical hemostatic dressings for combat casualty care,” Mil. Medicine, vol. 179, pp. 497–514, 2014.