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Biological Dressings Based on Natural Polymers

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Abstract

The article presents a method for producing a IV generation hemostatic dressing in the form of powder consisted of fibrous, which ensures a high level of security in application. The dressing material was developed on the basis of natural polymers of the polysaccharide group such as chitosan and calcium sodium alginate in the form of micro- and nanofibrils. The dressing structure, utility properties and biocompatibility (cytotoxicity, irritation and sensitization) were studied.

Key words: wound dressing, polysaccharides, micro-fibrils, nano-fibrils, biocompatibility.

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Introduction

Hemorrhage and neurological trauma are the main causes of death during the pre-hospital period in civilian and military trauma patients [1 - 3]. Approximately 20% of combat casualties are killed before reaching medical help or a medical facility [4]. Hemorrhage – uncontrolled bleeding, should be controlled as early and as effectively as possible. Competent hemorrhage control is very important and can theoretically save more human beings than any other measure. However, the best way to achieve this in challenging combat situations is not a simple task. The ideal bleeding control method should be appropriate for application by a layman, be efficient within minutes in relation to bleeding from a variety of sources, and be associated with a low complication rate. Wounds located in places that are not suitable for the use of clamps or compression dressings are characterised by a high degree of mortality [5].

Surgery is confronted with the hemorrhage problem on a daily basis. Blood loss leading to hypovolemic shock is not the only problem in the operating room. During the surgical procedure, bleeding from capillaries or small vessels can obscure the surgical field, causing operation time prolongation and increasing the risk factors [6]. Military medicine, emergency medicine and surgery show an increasing interest in the development of better hemostatic agents [5].

For years, the development of hemostatic materials has been conducted. Safe, effective and easy-to-use hemostatic dressing material is sought. The material is expected to stop bleeding quickly, not to

require compression and to be possible to use at home, in the operating room and in combat conditions.

It seems that biopolymers are a suitable base for the construction of a new generation of hemostatic dressings [7 - 10]. From the group of biopolymers, alginian, collagen, chitin and its derivatives deserve special attention.

Alginate dressings are present in the form of nonwovens, plates or cord to prevent deep wounds [11]. Hemostatic activity is associated with calcium ions, which are released to the wound and accelerate the activation of platelets [12]. There are also known dressings prepared on the basis of chitin, chitosan and their derivatives [13 - 15]. Chitin and chitosan dressings are an important group of hemostatic dressings [16 - 18]. The hemostatic effect is based on direct adhesion, causing the physical sealing of the bleeding site. In addition, these dressings activate platelets, which accelerates the incorporation of red blood cells forming a clot [16 - 18]. Forms of chitosan hemostatic dressings are mostly patches or sponge made for controlling bleeding from small vessels. Although the chitosan used to design the above-mentioned specialised dressings accelerates fibrin formation and platelet aggregation, these dressings are not universal. Their usage is limited for various reasons, and it is repeatedly recommended to use compression during dressing application. The need for usage compression can cause the prolongation of bleeding control procedures, increasing the risk of infection and patient suffering. Therefore presently research is being conducted on the development of hemostatic wound dressings of the IV generation. Material of the IV generation

means rapid bleeding control and a high degree of safety during application. Examples of such materials are produced by the American Company - Medical Sam - Celox™ - chitosan hemostyptic dressing [19,20]. The form of pellets or foam applied by the pressure applicator does not require the use of compression. This dressing is currently undergoing preclinical and clinical studies [21].

For many years, research on the use of natural polymers for the construction of various types of dressing materials has been carried out at the Institute of Biopolymers and Chemical Fibres, Lodz, Poland (IBWCh). The article presents a method for producing domestic, IV generation hemostatic dressing in the form of powder based on a new utility form of natural polymers - micro- and nanofibrils developed at IBWCh. The main objective of the study was to obtain a "first aid" dressing exhibiting appropriate biological (hemostatic, biocompatible) properties and ensuring a high level of security in application.

Experimental

Materials

- Chitosan, Initial chitosan ChitoClear hqg 95, Mv=373kDa, DD=81%, Ash content=0.31%, Primex ehf Company, Iceland.
- Sodium alginate, Protanal LF 10/60FT, (FMC Company, England)

Methodology

Preparation of dressing material in powder form

For the preparation of dressing material in powder form, a chitosan/Na/Ca alginate compound was used in the form of micro- and nanofibrils. The compound contained 80 wt.% chitosan and 20 wt.% Na/Ca alginate. The fibrils were prepared by the wet forming method using an in-line homogenizer - Dispax Reactor Labor-Pilot 2000/4 [22]. The polymer forms obtained were dried by either freeze-drying or the spray-drying method.

The freeze-drying process was carried out using a laboratory lyophilizer - ALFA 2-4, (Martin Christ, Germany), in a time span of 20 - 22 h and vacuum range of 0.01 - 0.042 mPa. The dried polymer was ground using a M-20 (IKA, Germany) universal mill.

Table 1. Dependence between the angle of repose, compressibility index and Hausner ratio*, *European Pharmacopoeia 8.0, Chapter 2.9.36.

Flow property	Angle of repose, degrees	Compressibility index, %	Hausner ratio
Excellent	25 - 30	1 - 10	1.00 - 1.11
Good	31 - 35	11 - 15	1.12 - 1.18
Fair (aid not needed)	36 - 40	16 - 20	1.19 - 1.25
Passable (may hang up)	41 - 45	21 - 25	1.26 - 1.34
Poor (must agitate, vibrate)	46 - 55	26 - 31	1.35 - 1.45
Very poor	56 - 65	32 - 37	1.46 - 1.59
Very, very poor	> 66	> 38	> 1.60

Table 2. Qualitative morphological grading of cytotoxicity of extracts*, * ISO 10993-5: 2009 standard.

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

The spray-drying process of micro- and nanofibril suspension was carried out using a B-290 Mini Spray Dryer (Büchi, Switzerland). A spray nozzle of $\varnothing = 0,7$ mm was used and the process parameters were as follows: feed rate 5 - 7 ml/min, inlet air temperature 210 °C, outlet air temperature 94 - 96 °C, air flow rate in the aspirator 37 m³/h, and spray flow rate 357 l/h.

Evaluation of dressing absorbency

The evaluation of dressing absorbency was carried out in accordance with the standard PN-EN 13726-1: 2005 "Test methods for wound dressings. Part 1: Aspects of absorbency. The research was carried out at the Institute of Security Technology MORATEX, Lodz, Poland.

Structure assessment of dressing in powder form

The structure of the dressing in powder form was assessed using a scanning electron microscope - Quanta 200 (FEI Co., USA). Measurement of the powder particle dimensions was made using ANALYSIS Docu software (Soft Imaging System) adapted to the Quanta environment.

Bulk and tapped density of dressing in powder form

Analyses were carried out according to European Pharmacopoeia 8.0, Chapter 2.9.34 and 2.9.36. Analyses of the powders, consistent with the classification by Carr, included bulk density, tapped densi-

ty, the compressibility index and Hausner ratio. The dependence between the angle of repose, the compressibility index and Hausner ratio is presented in **Table 1**.

Sterilisation of dressing

Sterilisation was made at the Institute of Applied Radiation Chemistry, Lodz University of Technology by irradiating the samples with a 25 kGy dose of γ rays.

Cytotoxicity testing of the dressing

The study was carried out according to procedures described in the following standards: ISO 10993-5: 2009 Biological evaluation of medical devices - Part 5: Tests in vitro cytotoxicity and ISO 10993-12: 2009 Biological evaluation of medical devices - Part 12: Preparation sample and reference materials. Research was performed using extracts from tested and control materials. The research was carried out at the Department of Experimental Surgery and Biomaterials Research, Medical University, Wroclaw, Poland.

Table 3. Primary irritation index (PII)*, Part - 10: Tests for irritation and skin sensitisation. *Standard: ISO 10993-10.

Mean score	Response category
0.0 - 0.4	negligible
0.5 - 1.9	slight
2.0 - 4.9	moderate
5.0 - 8.0	severe

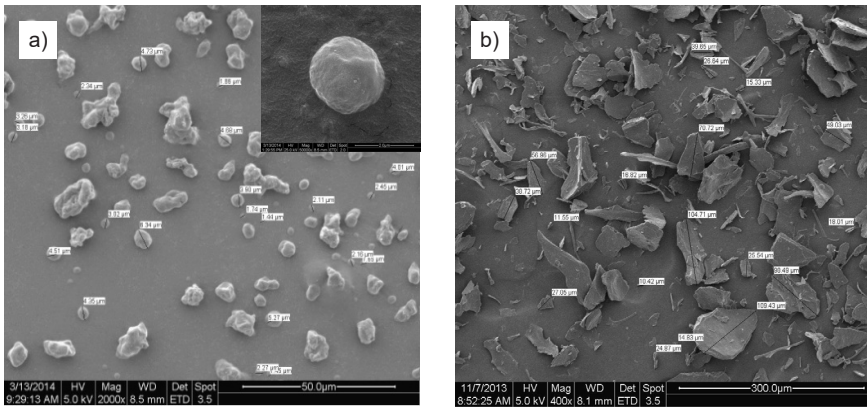


Figure 1. Structure and particle size assessment of models of hemostatic dressings developed; a) SEM of P/R/Chit/AlgNa-Ca dressing in powder after spray-drying, b) SEM of P/L/Chit/Alg/Na-Ca dressing in powder after freeze-drying.

Table 4. Assessment of bulk density of dressings in powder form; P/L- powder obtained by freeze-drying, P/R - powder obtained by spray-drying, S – dressing sterilized by irradiation.

Material	Bulk density, kg/m ²	Tapped density, kg/m ²	Hausner ratio
P/L/Chit/Alg Na-Ca	85	117	1.38
P/L/Chit/Alg Na-Ca/S	89	126	1.43
P/R/Chit/Alg Na-Ca	132	188	1.42
P/R/Chit/Alg Na-Ca/S	142	188	1.66

Morphological changes in cells were evaluated under an inverted phase contrast microscope according to the criteria given in **Table 2**.

Assessment of irritant properties

A test was performed according to Standard: ISO 10993-10:2011 Biological evaluation of medical devices – Part 10: Tests for irritation and skin sensitisation. The research was carried out at the Department of Experimental Surgery and Biomaterials Research, Medical University Wroclaw, Poland.

A test of irritant properties was conducted by the single exposure method on albino rabbits of the New Zealand White breed, of both sexes, weighing 3.3 - 3.9 kg. Dressing samples were put on the skin for a period of 24 hours. For each test sample and control sample the primary irritation index was determined using results at 24, 48 and 72 h (**Table 3**).

Table 5. Assessment of utility properties of dressings in powder form; L - freeze-drying, R - spray-drying.

Dressing	Liquid absorbency, g/per 1 g sample
P/L/Chit/Alg Na-Ca	8.7
P/R/Chit/AlgNa-Ca	8.1
Celox TM	17.7
QuikClot ACS+ TM	0.2

Assessment of allergenic properties

A test was performed according to Standard: ISO 10993-10 Biological evaluation of medical devices - 10: Tests for irritation and skin sensitisation. The assessment of skin sensitisation was carried out by the closed patch - Buehler method, chosen due to the future place of dressing contact. The research was carried out at the Department of Experimental Surgery and Biomaterials Research, Medical University, Wroclaw, Poland.

Evaluation of allergenic properties was carried out on albino guinea-pigs of both sexes, with an initial body weight of 340 - 400 g.

Results and discussion

Structure assessment of dressings in powder form

The structure assessment of the dressing material showed that the method of drying selected made it possible to obtain polymeric materials in powder form with a high surface area and dimensions of fibrils in the micro- and nanometric range. As a result of spray drying, a dressing material in powder form was obtained in which the fibrils were rolled up to form the shapes of pellets having a diameter of 0.9 - 6.0 µm. (**Figure 1**). The reason for this is believed to be in the specifics of spray drying, wherein the aqueous suspension of fibrils is sprayed into a hot

drying medium. In the case of freeze drying, a powder with fewer regular particles of 10-60 µm dimensions was obtained.

Assessment of bulk density of dressings in powder form

The bulk density of the dressing materials was measured. In the case of dressings in powder form, this parameter is extremely important as it allows to determine the capacity of the powder to settle on a wound. Prototypes of dressings manufactured by freeze-drying (P/L/Chit/Alg Na-Ca) and spray-drying (P/R/Chit/Alg Na-Ca) were tested before and after irradiation sterilization.

The results obtained showed that the powders developed are characterised by a Hausner ratio of 1.38 - 1.66. These parameter values indicate a high ability of the powder to settle, which is a very advantageous feature in terms of dressing applicability since the powder is held in the place of the intended operation and is not easy to flow. The results obtained also showed that the sterilisation process did not affect, in a significant manner, the test parameters determining the bulk density of the powders investigated.

Assessment of utility properties of dressings in powder form

The medical devices designed must meet a number of requirements defined by the relevant Directive 93/42/EEC harmonised standards. One of the requirements to be met by dressings are the appropriate utility properties, including the ability to absorb liquids. Determination of this parameter in a laboratory enables the assessment of dressing applicability to abundantly or moderately exuding wounds. Powders developed by either spray-drying or freeze-drying were tested. As a comparison, medical dressings in the form of a powder under the trade names CeloxTM and QuikClot ACS+TM were used. Dressings subjected to radiation sterilisation were evaluated.

The study showed that the wound dressings prepared in the form of a powder obtained by freeze-drying (P/L/Chit/algNa-Ca) and spray-drying (P/R/Chit/algNa-Ca) show good absorption properties. The values of the parameter in both dressings were at a similar level, reaching approx. 8 g liquid/1 g dressing. The ability of the dressings tested to absorb liquids as compared to commercial dressing CeloxTM was twice lower, and

in comparison with QuikClot ACS⁺™ it was significantly higher (Table 5).

Assessment of biocompatibility of dressings in powder form

Dressings developed in the form of powders were subjected to *in vitro* and *in vivo* evaluation of biological properties. The studies included both an assessment of hemocompatibility and hemostatic properties of the dressing in vitro [23, 24] and an assessment of biocompatibility in vitro and in vivo (cytotoxicity, irritation and allergisation properties).

Cytotoxicity assessment

Evaluation of the cytotoxic effect of dressings obtained from the chit/AlgNa-Ca compound by freeze-drying and spray-drying was performed on L 929 mouse fibroblasts using the MTT test. The samples for tests were radiation sterilized. Evaluation of changes in the culture and cell viability was carried out after 24, 48 and 72 h of contact with the primary extracts of samples tested and their serial dilutions of 1:2, 1:4 and 1:8. The commercial dressing Celox™ was a reference sample.

The study showed that the primary extract of sterile dressing (P/L/Chit/AlgNa-Ca) in powder form obtained by freeze-drying showed no cytotoxic effect after 24 h. After 48 and 72 h the toxic effect culminated in the decreased viability of cells as compared to the blank (67% and 58%, respectively). Diluted (1:2, 1:4 and 1:8) extracts did not show any cytotoxic effect at any period of the study (Table 6). The primary extract of sterile dressing (P/R/Chit/AlgNa-Ca) in powder form obtained by spray-drying showed no cytotoxic effect after 24 and 48 h. After 72 h the toxic effect culminated in the decreased viability of cells as compared to the blank (70%). Diluted (1:2, 1:4 and 1:8) extracts did not show any cytotoxic effect at any period of the study (Table 6).

In the case of Celox™ dressing it was not possible to assess cell viability due to the fact that during dissolution the sample turned into gel. Toxicity of the primary extract and 1:2 diluted extract was carried out only to assess the changes in the cultures. The toxicity of Celox™ 1:4 and 1:8 diluted extracts was assessed on the basis of changes in the culture and cell viability.

Table 6. Assessment of cytotoxic effect of Chit/AlgNa-Ca powder dressings obtained by freeze-drying and spray-drying.

	Sample	Extract	Cell viability, %	Degree of change in culture	Cytotoxic effect
Evaluation of cytotoxicity after 24 h	Negative sample	100%	93.38	0	none
	Positive sample SLS	0.075 mg/ml	80.93	1	none
		0.1 mg/ml	3.3	3	+
		0.15 mg/ml	24.9	4	+
		0.2 mg/ml	17.9	4	+
	P/L/Chit/AlgNa-Ca	Primary	87.25	0	none
		50%	87.29	0	none
		25%	86.68	0	none
		12.5%	91.35	0	none
	P/R/Chit/AlgNa-Ca	Primary	88.83	0	none
		50%	89.99	0	none
		25%	94.09	0	none
		12.5%	103.6	0	none
	Celox™	Primary	Not assessed	2	+
		50%	Not assessed	2	+
25%		92.7	0	none	
12.5%		101.8	0	none	
Evaluation of cytotoxicity after 48 h	Negative sample	100%	107.27	0	none
	Positive sample SLS	0.075 mg/ml	66.28	2	none
		0.1 mg/ml	20.46	4	+
		0.15 mg/ml	14.6	4	+
		0.2 mg/ml	10.23	4	+
	P/L/Chit/AlgNa-Ca	Primary	66.58	0	+/-
		50%	76.15	0	none
		25%	94.2	0	none
		12.5%	95.56	0	none
	P/R/Chit/AlgNa-Ca	Primary	79.73	0	none
		50%	81.13	0	none
		25%	93.91	0	none
		12.5%	98.77	0	none
	Celox™	Primary	Not assessed	3	+
		50%	Not assessed	3	+
25%		89.1	2	none	
12.5%		94.2	2	none	
Evaluation of cytotoxicity after 72h	Negative sample	100%	94.8	0	none
	Positive sample SLS	0.075 mg/ml	56.39	3	+
		0.1 mg/ml	22.49	4	+
		0.15 mg/ml	13.69	4	+
		0.2 mg/ml	14.43	4	+
	P/L/Chit/AlgNa-Ca	Primary	57.57	1	+/-
		50%	70.66	0	none
		25%	86.62	0	none
		12.5%	90.77	0	none
	P/R/Chit/AlgNa-Ca	Primary	69.68	0	+/-
		50%	87.56	0	none
		25%	96.08	0	none
		12.5%	99.58	0	none
	Celox™	Primary	Not assessed	3	+
		50%	Not assessed	3	+
25%		68.56	2	+	
12.5%		81.92	2	none	

The primary extract of Celox™ dressing showed no cytotoxic effect after 24 h. After 48 and 72 h the toxic effect culminated in a change in the culture of the 3rd degree. Diluted (1:2, 1:4 and 1:8) extracts showed no cytotoxic effect after 24 h. For the 1:2 diluted extract after 48 h, and for 1:2 and 1:4 diluted extracts after 72 h, the cytotoxic effect culminated

in changes in the cultures of the 3rd degree and decreased cell viability as compared to the blank (Table 6).

Assessment of irritation and allergization

In vivo studies were performed only on Chit/AlgNa-Ca dressing in powder form obtained by spray-drying due to the fact

that the cytotoxicity tests showed no significant effect of the method of powder preparation on the cell culture. The commercial dressing Celox™ was a reference sample.

Irritation and allergisation tests were conducted through direct contact with the skin of animals. Based on the test results, it was found that dressings in the form of a powder made of the chitosan/alginate Na-Ca complex as well as commercial dressing Celox™ do not irritate the animals' skin. The difference between the average scores for tested and reference dressings was zero. Also in the case of allergization tests none of the test animals showed an allergic reaction. The animals' skin in the application site of both the examined dressing and reference dressing Celox™ had a normal appearance. The gender of the animals had no effect on the skin reaction. Both groups of treated and control animals showed normal weight gain.

Conclusions

1. Based on chitosan/alginate Na-Ca complex in the form of micro- and nanofibrils (chitosan-80% algNa-Ca-20%), the prototypes of dressing materials in the form of powder were prepared using freeze-drying and spray-drying methods.
2. Chit/algNa-Ca dressings developed in powder form obtained by freeze-drying and spray-drying show good absorption properties (approx. 8g liquid/1g dressing) and a high ability of the powder to settle in the application site.
3. Dressings developed in powder form showed no cytotoxicity in *in vitro* tests and no irritation and allergisation to the skin in *in vivo* tests, which proves that they meet all the requirements in terms of biocompatibility for this type of medical device.

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