engineering proceedings

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Proceeding Paper

In Vivo Biocompatibility and Biodegradability of Bilayer Films Based on Hyaluronic Acid and Chitosan for ENT Surgery †

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† Presented at the 4th International Electronic Conference on Applied Sciences, 27 October–10 November 2023; Available online: https://asec2023.sciforum.net/.

Abstract: Septal cartilage defects and tympanic membrane perforations are one of the main challenging clinical problems in modern ENT (Ear, Nose, and Throat) surgery. Polymer films based on the biocompatible and biodegradable polymers seems to be represent the perspective materials for surgical reconstruction of such defects. In this study we present the results of the pilot in vivo experiments of biocompatibility and biodegradability of bilayer films obtained by casting method from hyaluronic acid (MW = 1300 kDa) and chitosan (500 and 900 kDa) polymer solutions. Total toxicity, pro-inflammatory activity, biodegradation rate and proliferative potential of the connective tissue of the dermis in the implantation area were evaluated on days 7, 14, 30, 50 after the implantation. Studied samples demonstrated neglidgable overall acute and chronic toxicity. The influence of the preparation technique, as well as the effect of chitosan MW on the biodegradation rate are also demonstrated. These bilayer polymer films can be recommended for the ENT surgery, in particular, for the reconstruction of nasal septum and tympanic membrane.

Keywords: bilayer polymer films; chitosan; hyaluronic acid; nasal septal perforation; tympanic membrane perforation

Citation: Naumenko, M.; Snetkov, P.; Gribinichenko, T.; Bervinova, A.; Morozkina, S.; Zhuravskii, S. In Vivo Biocompatibility and Biodegradability of Bilayer Films Based on Hyaluronic Acid and Chitosan for ENT Surgery. *Eng. Proc.* **2023**, *52*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Name

Published: date

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1. Introduction

One of the most topical problems belonging to the ENT (Ear, Nose, and Throat) surgery is related to the successful reconstruction of various defects (e.g., perforations) occurring under the influence of different factors. Thus, nasal septal perforations are a common defect, which is diagnosed in 0.9–2.1% of the global population [1]. Septal perforations may be caused by various factors: uncontrolled administration of nasal drops and sprays for breathing recovery containing alpha-adrenomimetics (naphazoline, xylometazoline, oxymetazoline, etc.), intranasal synthetic steroids (mometasone, budesonide, beclomethasone, fluticasone, etc.) [2–4], other topical drugs (e.g., desmopressin against diabetes insipidus [5]), prolonged recovery period after septoplasty (e.g., for crooked nasal septum treatment) [6], serious trauma with massive cartilage destruction [7]. Under these pathological conditions, the vessels become dilapidated and sclerotic, and the mucous membrane together with the cartilage tissue atrophies being also thin and eroded [8].

Defects of tympanic membrane usually resulted from the longtime and untreated inflammatory processes (e.g., chronic otopyosis), and traumas, especially baric and acoustic [9,10].

Above mentioned injuries cannot be reconstructed even with advanced ENT microsurgery because of the lack of biocompatible composite materials for ENT prosthetic devices. Such materials have to be applied for significant area of the missing/damaged body part and have to promote volumetric repair process of functional restoration of cartilage, mucosa and connective tissue matrix for ENT organs and its parts [11,12]. Moreover, apart from the exploitation properties, such materials should have antibacterial effect or be easy sterilized without the changing of properties, because the formation the bacterial or/and fungal biofilms on the material surface [13].

Film materials based on biocompatible and biodegradable polymers seem to be the suitable materials for ENT surgery due to its unique properties. Such, chitosan, being a natural polysaccharide obtained from chitin, has its own antibacterial activity against gram-negative and gram-positive bacteria [14]. Hyaluronic acid, another member of polysaccharides family, have unique set of properties and is recommended for biocompatible and biodegradable devices, such as reconstructive materials for ENT surgery [15]. Moreover, natural antibacterial agents could be loaded into hyaluronic acid matrixes to obtain materials with excellent antimicrobial efficacy with synergy of action [16].

The aim of this study is to evaluate the in vivo biocompatibility and biodegradability of bilayer cast films based on high molecular weight (MW) hyaluronic acid and chitosan with various MW for the potential use as modern materials for surgical reconstruction of the tympanic membrane and septal cartilage defects. The combination of chitosan and hyaluronic acid leads to the formation of polyelectrolyte complex [17] on the interface region between the two polymer layers, that results in the possibility of biodegradable rate regulation and combination of polymer properties.

In this study total toxicity, pro-inflammatory activity, biodegradation rate and proliferative potential of the connective tissue of the dermis in the implantation area were analyzed on days 7, 14, 30, 50 after the surgical implantation of the samples using 20 Wistar rats (weight 220–240 g). Studied polymer samples demonstrated neglidgable overall acute and chronic toxicity. Moreover, the influence of the preparation technique, as well as the effect of chitosan MW on the biodegradation rate are demonstrated. These bilayer polymer films can be recommended for the reconstruction of nasal septum and tympanic membrane.

2. Materials and Methods

2.1. Materials

Hyaluronic acid sodium salt HA-T from *Streptococcus equi* with molecular weight (MW) equal to 1300 kDa was obtained from Bloomage Freda Biopharm Corporation Limited (Jinan, China). Chitosan with MW equal to 500 kDa and 900 kDa was purchased from LLC BioProgress (Moscow Region, Russian Federation). Glacial acetic acid (99.5% ACS, MW = 60.052 g/mol) was obtained from JSC EKOS-1 (Moscow, Russian Federation). Distilled water was prepared using laboratory distiller apparatus. All materials were used without additional purification.

2.2. Polymer Solutions Preparation

Individual polymer solutions were prepared as follows:

- Hyaluronic acid was dissolved in the required volume of distilled water to obtain 2.0 wt. % polymer solution.
- Chitosan was dissolved in the 1.0% *v*/*v* aqueous acetic acid solution to obtain the polymer concentration equal to 2.0 wt. %.

Each solution was prepared overnight using a magnetic stirrer at ambient temperature and relative humidity. After the preparation, the solutions were kept for stabilization and deaeration.

2.3. Film Casting

Polymer bilayer films based on hyaluronic acid and chitosan were prepared by layer-by-layer (LbL) casting of polymer solutions. The detailed methodology and the key properties of films prepared was demonstrated earlier [18]. Briefly, chitosan solution was transferred into the sterilized Petri dishes and dried at ambient conditions during 24 h. Hyaluronic acid solution was transferred on the chitosan film and dried during 120 h in the ambient conditions. After the drying half of bilayer polymer films were heated at 100°C in the chamber drier for 5 min, and washed by distilled water for 30 min. Non-heat-treated films were washed by distilled water immediately after the drying. After the washing the bilayer films were dehumidified at the ambient conditions during 24 h.

The scheme of film preparation is demonstrated in Figure 1.

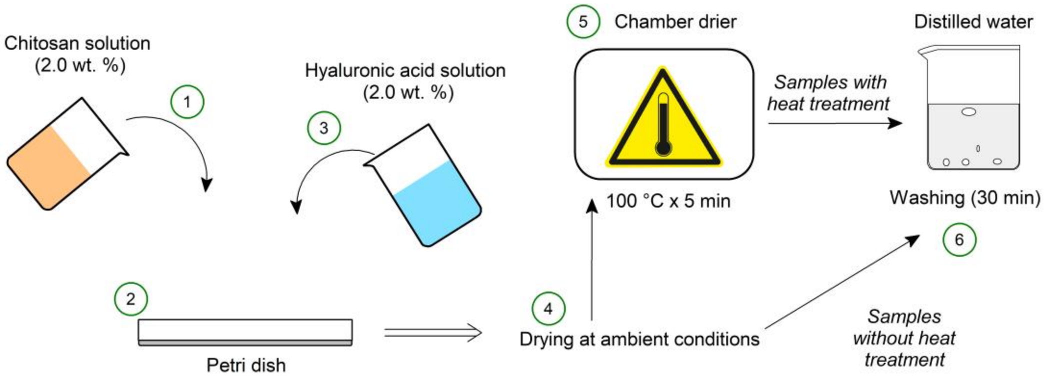


Figure 1. The scheme of bilayer film preparation: (1) chitosan solution casting, (2) chitosan film drying (24 h), (3) hyaluronic acid solution casting, (4) the drying of bilayer film at ambient conditions (120 h), (5) heat treatment (100°C × 5 min), (6) washing by distilled water.

2.4. In Vivo Pilot Experiments

For the in vivo assay 30 Wistar rats (weight 220–240 g) were used. Total toxicity, pro-inflammatory activity, biodegradation rate and proliferative potential of the connective tissue of the dermis in the implantation area were evaluated on days 7, 14, 30, 50 after the subcutaneous implantation.

The experimental groups of rats were divided as demonstrated in Table 1.

Table 1. Experimental groups of rats.

Group Number	Polymers, Its MW, and Sample Preparation Methodology	Sample Name
Intact Group 1	-	-
Control Group 2 (False operated)	-	-
Group 3	Chitosan (900 kDa) + Hyaluronic acid (1300 kDa) LbL polymer casting + heat treatment 100 °C × 5 min	CT900HA(t)
Group 4	Chitosan (500 kDa) + Hyaluronic acid (1300 kDa) LbL polymer casting + heat treatment 100 °C × 5 min	CT500HA(t)
Group 5	Chitosan (900 kDa) + Hyaluronic acid (1300 kDa) LbL polymer casting without heat treatment	CT900HA
Group 6	Chitosan (500 kDa) + Hyaluronic acid (1300 kDa) LbL polymer casting without heat treatment	CT500HA

The film samples were sterilized by UV irradiation for 1 h. An incision of 10 mm in length in the dorsal region along the middle line in the line of sight was introduced in the aseptic system (Zoletil 100) on the operating field. With the help of a raspator, soft tissues were separated with the formation of a bed between the dermis and the sterno-lumbar fascia. Flavored material (Prolene 6.0) was added to the ranunculus.

During the experiment, the weight of the animals, the condition of the postoperative wound, the severity of local edema, the size, the condition and density of the encapsulated implant, the histological picture of the surrounding tissues were evaluated.

Histological analysis was performed on paraffin sections stained with hematoxylin and eosin, using the Mallory method and toluidine blue. Macrophages were detected using primary monoclonal mouse antibodies Anti-CD68 antibody (ab 31630) (Abcam) and a multimeric biotin-free system (D&A, Reveal-Biotin-Free Polyvalent DAB, Spring Bioscience Corporation, USA). Nonparametric statistical methods (Mann–Whitney U test) was used to assess the reliability of the differences between the groups.

The differences between the averages were considered significant at $p < 0.05$.

3. Results and Discussion

The absence of mortality, the increase of animals' weight in the experimental groups indicate about the absence of acute, subacute toxicity of the studied materials. The absence of suture failure and purulent complications in the postoperative period illustrates its good biocompatibility.

In the acute period after the implantation, tissue edema was observed in all groups, persisting up to 7–8 days after the surgery (Figure 2).

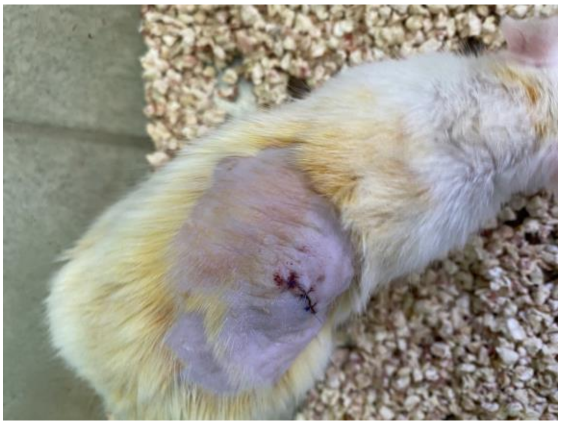
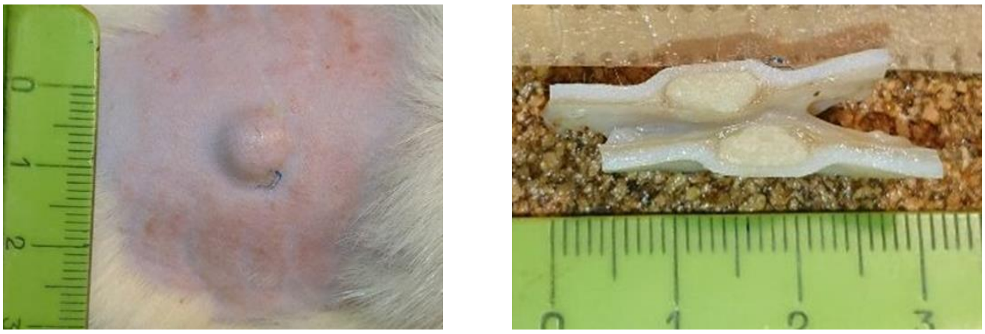


Figure 2. The photo of animal with the implantation of a polymer film on the 3rd day of the postoperative period.

During the experiment, a palpatory examination of the skin of the implantation zone revealed a different intensity of bioresorption of the studied samples. Thus, by day 30, complete resorption of the CT500HA film obtained without heat treatment was noted. Samples with 500 and 900 kDa chitosan subjected to the heat treatment continued to be detected throughout the observation period. It is noted that their biodegradation occurs through the swelling stage of the polymer matrix and with the formation of a stable reactive capsule of a foreign body. The CT900HA(t) sample subjected to heat treatment showed the longest duration of stay in the tissues, which indicates the minimum rate of the biodegradation of this material (Figure 3).

For all experimental groups, no visual formation of scar tissue deforming the thickness of the skin and subcutaneous tissue in the implantation zone was detected. Postoperative sutures at the end of the experiment did not differ from the group of sham-operated animals.

Thus, the rate of biodegradation of the studied samples of bilayer polymer films for the reconstructive purposes in ENT surgery is the following sequence: CT500HA ≥ CT900HA > CT500HA(t) > CT900HA(t) (Table 2).



(**a**) magnification × 2 (**b**) magnification × 4

Figure 3. Implantation zone (**a**) and the type of product during necropsy (**b**) by the 50th day of observation. Sample CT900HA(t). Macropreparations.

Thus, the rate of biodegradation of the studied samples of bilayer polymer films for the reconstructive purposes in ENT surgery is the following sequence: CT500HA ≥ CT900HA > CT500HA(t) > CT900HA(t) (Table 2).

Table 2. Diameter of polymer implants during necropsy by day 50 after implantation (M ± m).

Group Number	Sample Name	Implant Diameter, mm	Mann–Whitney U Test
3 (n = 5)	CT500HA	0.8 ± 0.45	
4 (n = 5)	CT900HA	1.2 ± 0.84	$U_{3–4} = 8.5$
5 (n = 5)	CT500HA(t)	5.0 ± 1.22	$U_{4–5} = 0$
6 (n = 5)	CT900HA(t)	9.0 ± 1.00	$U_{5–6} = 0$

Thus, the intensity of bioresorption of hybrid polymer systems depends on polymer MW and the use of heat treatment during the film preparation process.

Histological analysis of skin samples revealed the insignificant reactive proliferation of connective tissue and the absence of aseptic inflammation. The biodegradation of films passes through the stage of swelling of the polymer matrix associated with the hydrophilicity of the material and with persistent cellular infiltration mainly by tissue macrophages and migrating monocytes. The involvement of CD68+ cells mainly in the form of giant multinucleated cells of foreign bodies in the formation of the surrounding capsule and subsequent bioresorption is shown. The absence of eosinophils and signs of mast cell degranulation indicates the bioinertness of the matrix material (absence of allergenic properties).

4. Conclusion

For the development of a polymer-based implant for surgical reconstruction of tissue defects that require a long-term stay in the body, the molecular weight of polymers forming a bilayer hybrid film that is not more critical in comparison with the severity of the polyelectrolyte complex being created, stimulated, in particular, by heat treatment conditions. The chitosan/hyaluronic acid-based bilayer films obtained under these conditions demonstrate: the absence of acute toxicity, signs of reactive aseptic inflammation and allergenicity. The biocompatibility of these materials, the low rate of biodegradation, combined with the absence of scar deformation of the surrounding tissues, allows to consider them as perspective materials for reconstructive ENT surgery.

Author Contributions: Conceptualization, P.S., S.M. and S.Z.; methodology, M.N., P.S., T.G. and S.Z.; validation, M.N. and S.Z.; formal analysis, M.N.; investigation, M.N., P.S., T.G. and S.Z.; resources, P.S., S.M. and S.Z.; writing—original draft preparation, M.N., P.S., S.M. and S.Z.; writing—review and editing, S.M. and S.Z.; visualization, M.N., P.S. and S.Z.; supervision, A.B., S.M. and S.Z.; project administration, S.M. and S.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996) and the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The Institutional Animal Care and Use Committee at the Almazov National Medical Research Centre approved the study protocol (Protocol Number PZ_21-02#Zhuravskii S.G. V3. March 09, 2021). All efforts were made to protect the animals and minimize their suffering during the study. The experiments complied with the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE).

Informed Consent Statement:

Data Availability Statement:

Conflicts of Interest: The authors declare no conflict of interest.

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