Visualization of morphological features of chitosan microtubes during biodegradation

T S Babicheva, N O Gegel and A B Shipovskaya
Educational and Research Institute of Nanostructures and Biosystems, Saratov State University 83 Astrakhanskaya St., Saratov 410012, Russian Federation

Abstract. Morphological features were visualized and the weight loss of chitosan microtubes during in vitro biodegradation was evaluated in two model media (an enzyme medium of lysozyme and the phosphate buffer) by means of scanning electron microscopy and gravimetry. It was observed that during incubation in the enzymatic medium, the relief and morphology of the microtube surface were changing, as expressed in the appearance of micropores, cracks and bulk defects. When keeping the samples in the phosphate buffer, no substantial defects were formed, only few micropores appeared. The weight loss of the samples after 180 days of storage in the lysozyme medium and phosphate buffer was 30 and 7%, respectively.

1. Introduction
Biodegradable polymeric materials in the form of films, pins, and complex 3D structures attract increasing interest in reconstructive medicine [1-4]. Such materials are used as drainage-separation systems and connecting elements. Topical is the design of bioengineering structures, wherein a polymer serves as a matrix for cell adhesion and proliferation. To obtain such materials, biodegradable synthetic [4, 5] or natural [6-8] polymers are used. For example, in the case of synthetic polymers, linear aliphatic polyesters (polyglycolic acid, polylactic acid) [5] are most often used. In the case of natural polymers, proteins, such as collagen [6] and fibrin [7], are used, as well as polysaccharides, among which the use of the aminopolysaccharide chitosan [8, 9] is most common. For example, the use of bioreabsorbable microtubes can avoid their further remodeling. In previous papers [10, 11] we described the preparation and formation mechanism of microtubes from chitosan solutions. The results of our study of the physicochemical, mechanical and biological properties of such microtubes have shown that they may be a promising material for designing biodegradable vascular prosthesis [12]. In this case, a microtube would act as a hollow frame, whose surface the endothelial cells would adhere to and proliferate on, thereby forming a new living organ. The polymer backbone would bioreabsorb in a natural way under the influence of host enzymes. In this context, further research should involve studying of the biodegradation process of chitosan microtubes. Scanning electron microscopy (SEM) is known to be an informative tool of research of synthetic and biological polymer objects. This method allows obtaining information of the surface morphology and topography of the material examined with sufficient accuracy. SEM has been successfully used to study structural features of chitosan-containing materials, such as composite films [13], nanoparticles [14], hydrogels [15], the transformation of films into hollow cylindrical structures [16], etc. It provides a basis for the possible use of SEM for visualizing structural changes occurring in biodegradable materials.

In this paper, SEM was used to visualize morphological changes on chitosan microtube walls during their in vitro biodegradation in two modeling media, namely: an enzymatic lysozyme medium and phosphate buffer. Chitosan-based materials are known to biodegrade in the presence of non-specific enzymes of hydrolase class, among which lysozyme is most common [17, 18]. Therefore, our
choice of a hydrolytic enzyme was due to the ability of lysozyme to break the $\beta-(1, 4)$-glycosidic bonds between the glucopyranose cycles of macromolecular chains [19].

2. Methodology
Microtubes were obtained from a chitosan solution in glycolic acid by an interfacial reaction of salting out chitosan glycolate into the form of a polybase. Chitosan with a molecular mass of 700 kDa, a deacetylation degree of 80 mol. % (Bioprogress Ltd., Russian Federation) and 1.5% aqueous solution of glycolic acid (Sigma-Aldrich, England) were used. A 4 wt. % chitosan solution was prepared by dissolving an air-dry weighed sample of the polymer powder in the presence of an acid while stirring with a magnetic stirrer at room temperature for 7 h. The finished solution was left for 24 h to remove air bubbles and used to form microtubes. NaOH was used as the salting-out agent. To obtain microtubes, a rod with a layer of the chitosan solution was first held in the salting-out medium for 1 min, then dried at a temperature of 50°C until the solvent completely evaporated and re-immersed into the salting-out solution for 3 h. The process of obtaining microtubes with a detailed description of all the stages is described elsewhere [10, 11].

To simulate the biodegradation process, chitosan microtubes were placed into a solution of the immobilized enzyme lysozyme (Medigen Ltd., Russian Federation) with a concentration of 2 mg/ml in phosphate buffer (pH = 7.4). A phosphate buffer solution with no enzyme (pH = 7.4) was used as a control. All solutions used in our experiments were sterilized by autoclaving at 120°C under 0.11 MPa for 45 min. The enzymatic and control model systems were incubated in a thermostat at 37°C throughout the experiment.

The biodegradation process was studied as follows. Samples were weighed and placed into the model medium: either the lysozyme solution or phosphate buffer (control). The fermentation medium was freshened every 30 days to avoid loss of the lysozyme activity. The experiment lasted 6 months. The total number of samples taken for this experiment was 48. Every 30 days, 3 samples were withdrawn from the experiment to estimate the weight loss, and one served to examine changes in the surface morphology. Microtubes were dried by standard methods. The weight loss was calculated by the formula:

$$\Delta m = \left(\frac{(m_0 - m)}{m_0}\right) \cdot 100\%,$$

where $m_0$ is the mass of the original microtube sample, considering its humidity (the moisture content was determined in preliminary experiments), g; $m$ the mass of the sample after exposure to the model medium and dried to constant weight, g.

In special experiments, the microtube was separated from the enzymatic and control systems with a cellulose dialysis membrane. Dialysis was performed against phosphate buffer. To analyze biodegradation products, the qualitative Rouemann reaction for free amino acid was used. Optical density was measured at $\lambda = 545$ nm on a semiautomatic flatbed photometer Stat Fax 4200 (USA). A calibration line for the glucosamine concentration evaluation was plotted using the amino acid glycine (chemically pure, Vekton, Russian Federation) and ninhydrin (chemically pure, Diaem Lyd., Russian Federation).

The surface morphology of chitosan microtubes was examined by SEM. Photos were obtained on a MIRA LMU microscope (Tescan, Czech Republic) at a voltage of 15 V and a conductive current of 400 pA. Sample preparation was performed as follows: a sample was removed from the model media and filled with chlorhexidine for 10 min. It was then washed with sterile water, cut along and kept in a 96% ethanol solution for 30 min. Then it was picked up from the alcoholic medium and dried in air at room temperature for 24 h. A gold layer was sputtered onto the resulting air-dried sample (a 5 nm thickness) on a K450X Carbon Coater device (Germany).

3. Results and discussion
Degradation is performed in microtubes two systems: in the enzyme lysozyme and the medium of phosphate buffer medium. It is known from the literature that lysozyme is contained in many human biological fluids and serves as a nonspecific antibacterial barrier [20]. In this connection, the enzyme solution was chosen as the model of wound, phosphate buffer - as a model of the blood plasma. By
assessing the kinetics of biodegradation experiment results microtubes samples held for 6 months are shown in the table.

**Table.** The kinetics of microtubule mass loss of the sample of chitosan in phosphate buffer and lysozyme fermentation medium.

| The holding time, day | Weight loss (Δm), % |
|-----------------------|---------------------|
|                       | Phosphate buffer (control) | Lysozyme solution |
| 30                    | 3                    | 12                 |
| 60                    | 3                    | 23                 |
| 90                    | 3                    | 24                 |
| 120                   | 3                    | 25                 |
| 150                   | 5                    | 27                 |
| 180                   | 7                    | 30                 |

As seen from the table, the highest weight loss was realized in the fermentation medium, namely: 30% after 180 days of incubation. Just in the first 60 days of incubation the sample loses 23% of its weight. In the next 120 days of incubation, the overall weight loss is 7%. In the phosphate buffer medium (control) the weight loss was only 7% for the entire duration of the experiment, probably due to dissolution of the low-molecular-weight polymer fractions. The higher weight loss of the microtube in the lysozyme medium is logically explained by hydrolytic chitosan degradation, which proceeds by rupture of the β–(1, 4)–glycosidic bonds in the macromolecules by the enzyme.

Carrying out the Reuemann qualitative reaction confirmed the course of the biodegradation process of our chitosan microtubes by the mechanism of breakage of the β–(1, 4)–glycosidic bond. In the enzymatic hydrolysis process, the samples are destroyed to chitosan n-mers and glucosamine. This is confirmed by the fact that a blue-violet complex was formed as a result of the interaction of the biodegradation products with ninhydrin, the color intensity being proportional to the free amino acid amount. It was established spectrophotometrically that the total concentration of the hydrolyzed chitosan products was 3 μg/ml for 10 days of the experiment.

At the next stage, the surface morphology of both source microtubes and those kept in the model media was evaluated by SEM. The outer and inner wall surfaces of the source samples taken for the experiment were visually smooth without any visible defects. In the process of keeping in the model media, the microtube wall surface morphology underwent changes. Typical SEM photos of the inner surface wall of the tested samples are shown in Figure. Let us analyze the morphological changes occurred in the samples after 6 months of storage in the lysozyme medium and phosphate buffer.

No occurrence of serious defects on the sample surface was observed when keeping a microtube in phosphate buffer during 180 days (Figure a–c). Only the formation of micropores was observed in the process of storage and even only under prolonged storage of the sample in the buffer medium (Figure c). For the sample kept in the fermentation lysozyme medium, the formation of micropores was also characteristic. However, the changes in the surface morphology of these samples were significantly different from the control ones. E.g., small bulk defects appeared along with micropores on the microtube surface after 120 days of storage (Figure d). At the 150th day of storage, both the pore size and their total number over the entire wall surface increased. The bulk defects acquired the view of extended cracks and depressions (Figure e). After 180 days of exposure in the lysozyme medium, the microtube surface micro-relief changed more and acquired a pronounced loose structure (Figure f).
Therefore, SEM has allowed visualization of the changes occurring in the surface topography and structure of our chitosan microtubes under the influence of the model media. The most significant changes in the sample surface were realized under the influence of the non-specific enzyme lysozyme (a hydrolytic enzyme).

4. Conclusions
The biodegradation of our chitosan microtubules was studied in vitro in the fermentation lysozyme medium and phosphate buffer. It was established that after 6 months of enzymatic hydrolysis and storage in the buffer the sample weight decreased by 30 and 7%, respectively. In the fermentation medium, the maximum mass loss was realized during the first 60 days of storage. Further, the rate of destructive processes reduced. During enzymatic degradation, micropores, cracks, and other bulk defects appeared on the walls of the microtubular substrates, up to the formation of a pronounced loose structure. It is suggested that the degradation process in the lysozyme medium goes through the mechanism of rupture of chitosan macrochains by the $\beta$–(1, 4)–glycosidic bonds. The rate and degree of biodegradation of the examined samples of chitosan microtubes are comparable with the corresponding values of these parameters demanded for prosthetic blood vessels made of bioresorbable materials [21]. Our experiments allow considering chitosan microtubes as promising bioartificial materials with controlled duration and degree of biodegradation in vivo.

Acknowledgments
This work was supported by the Russian Foundation for Basic Research, project No 16-33-00953 «Scientific fundamentals of the preparation of 3D biodegradable structures from natural polysaccharides to create blood vessel analogs». 
References

[1] Teo A J, Mishra A, Park I, Kim Y J, Park W T and Yoon Y J 2016 ACS Biomater. Sci. Eng. 2 4 454-472
[2] Annabi N, Tamayol A, Uquillas J A, Akbari M, Bertassoni L E, Cha C and Khademhosseini A 2014 Advanced Mater. 26 1 85-124
[3] Moisenovich M M, Pustovalova O L, Arhipova A Yu, Vasiljeva T V, Sokolova O S and Bogush V G 2010 Mater. Research. 96A 1 125–131
[4] Pham Q P, Sharma U and Mikos A G 2006 Biomacromol. 7 2796–2805
[5] Uematsu K 2005 Biomaterial. 26 20 4273-79
[6] Pabbruwe M B, Esfandiari E, Kafienah W, Tarlton J F and Hollander A P 2009 Biomaterial. 30 26 4277-86
[7] Altman G H, Diaz F, Jakuba C, Calabro T, Horan R L, Chen J, Lu H, Richmond J and Kaplan D L 2003 Biomater. 24 401- 416
[8] Dmitriev Y A, Shipovskaya A B and Kossovich L Y 2011 Izvestiya Vysshikh Uchebnykh Zavedenii. Seriya Khimiya I Khimicheskaya Tekhnologiya 54 11
[9] Kashyap M, Archana D, Semwal A, Dutta J and Dutta P K 2016 Chitin and Chitosan for Regenerative Medicine 261-277
[10] Gegel N O, Shipovskaya A B, Vdovykh L S and Babicheva T S 2014 J. Soft Mat. Article ID 863096 p.9
[11] Babicheva T S, Gegel N O and Shipovskaya A B 2015 J. Nat. Sci. Sustainable Techn. 9 2 285-295
[12] Gegel N O, Babicheva T S and Shipovskaya A B 2015 Butlerov Comm. 41 3 44-53
[13] Cárdenas G, Anaya P, Del Rio R and Schrebler R 2010 J. Chil. Chem. Soc. 55 3 352-58
[14] Fazilova S A, Yugai S M and Rashidova S S 2010 Chem.of plant raw mat. 1 13-19
[15] Malaise S, Rami L, Montembault A, Alcouffe P, Burdin B, Bordenave L, Delmond S and David L 2014 Mater. Sci. Eng. Mater. Biol. Appl. 42C 5 374-384
[16] Gulati K, Johnson L, Karunagaran R, Findlay D and Losic D 2016 Biomacromolec. 17 4 1261-71
[17] Kean T and Thanou M 2010 Adv. Drug Delivery Rev. 62 1 3-11
[18] Kulish E I, Chernov V V, Volodin V P and Kolesov S V 2008 Bull. Bashkir University. 13 1 23-26
[19] Zhang L, Dou S, Li Y, Yuan Y, Ji Y, Wang Y and Yang Y 2013 Mater. Sci. Eng. C. 33 5 2626-31
[20] Cutting K F 2003 British j. of community nursing. 8 4-9
[21] Cleary M A, Geiger E, Grady C, Best C, Naito Y and Breuer C 2012 Trends in molecular medicine. 18 7 394-404