Celal Bayar University Journal of Science

Prolonged Biomolecule Release from Titanium Surfaces via Titania Nanotube Arrays

Cem Bayram[1*]

[1]Hacettepe University, Graduate School of Science and Engineering, Nanotechnology and Nanomedicine Department, Beytepe, 06800, Ankara, Turkey
*cemb@hacettepe.edu.tr
* Orcid : 0000-0001-8717-4668

Received: 16 July 2021
Accepted: 20 January 2022
DOI: 10.18466/cbayarfbe.972316

Abstract

Surface modifications against the failure of titanium implants used in hard tissue repair has become a trend in recent years. In the last decade, it has been investigated that nanoscale tubular spaces on the titanium surface can be used as a local drug release reservoir without the need for any chemical binder or polymeric coating. It is possible to obtain one-dimensional structures that can be grown by electrochemical anodic oxidation by controlling the diameters of less than 100 nanometers on titanium metal surfaces. The major disadvantage of biomolecules released from titania nanotube structures to the environment is the hard control of release kinetics and more than half of the loading amount releases in the first few hours of interaction with the biological fluid. Although the studies on controlling the kinetics have been tried to overcome by covering the nanotube arrays with barriers such as polymer structures, the risk of delamination of the polymers from the surface during implantation brings additional problems. In this manuscript, vancomycin and bovine serum albumin were loaded into titania nanotubes formed by anodic oxidation technique on titanium metal plates and the tube ends has been narrowed by gold sputtering technique. With this narrowing at the tube-ends, the length of the release time and the change in diameter according to the hydrodynamic diameter of the released biomolecule were investigated. It is seen that the increased gold sputtering time prolongs the release rate of biomolecules and offers a promising approach for sustained local drug releasing implants.

Keywords: titanium, sustained release, drug loading, implant, anodic oxidation, nanotube

1. Introduction

Millions of hard tissue cases require surgical operations worldwide, including hip and knee replacements, fracture fixations or missing tooth implants due to implant failure. The major reasons of implant failure are aseptic loosening and insufficient osseointegration during the post operation process and drug therapy approach is often recommended to inhibit infections or inflammations that may develop in the post-op period after implantation, or to eliminate the need for revision surgery that results in implant failure. [1-3]. The oral or intravenous administration of drugs have well known disadvantages like systemic toxicity and low efficiency at the target area after elimination throughout the body. Localized delivery of active agents and therapeutics is one of the most efficiently validated approaches, which helps to reduce systemic disadvantages of the administrated drugs [4-6]. Additionally, it also maximizes the bioavailability of drugs preventing them to be eliminated by systemic circulation or gastrointestinal tract Although implants that release drugs or active substances through polymer or ceramic coatings have been used to eliminate the above-mentioned disadvantages, these conventional approaches are often subject to remove/delaminate from the surface as a result of interactions and frictions in the application area. [7].

Even though, titanium and its alloys are the materials of choice in the majority of hard tissue and dental implant applications, they are still open to many challenges arising from bacterial infections, inflammation or poor adhesion of osteoblast cells, leading to implant failure [8-10]. Although titanium has excellent bulk properties, implant success lies behind its surface morphology and chemistry. The success of a bone/dental implant is highly dependent on the properties of the surface of the implant.

Electrochemical anodic oxidation is a classical technique used on titanium, however the use of fluoride containing organic electrolytes in the last decade has provided the opportunity to control oxide layer produced on titanium surface in terms of both composition and morphology. Controlled oxide dissolution rate achieved by newer electrolyte systems allows to design the surface architecture precisely. In the anodic oxidation process, it is possible to achieve a high aspect ratio or to extend the nanotube size by creating a localized acidification at the bottom of the pore and maintaining the pH value in the pore wall and its surroundings by controlling the TiO_2 dissolution in a controlled manner [11].

In the past decade, research on the direct release of biologically active molecules and drugs from metal surfaces has been intensively studied. The usability of nanotubular structures with less than 100 nm in diameter and controllable length, which can be formed on titanium surfaces by anodic oxidation as local release reservoirs have been tested in these studies. It has been reported that these nanotube structures can release different drugs and biomolecules for several days to several weeks as a result of varying diameters and lengths.

It is known to use small molecules such as antibiotics and macromolecules like growth hormone and therapeutic protein in implants that release bioactive and biological agents. For this reason, an antibiotic and a protein were included in the study as a model compound to test the capability of nanotubes as active agent reservoirs. Vancomycin and BSA molecules were selected in the study. The molecular weight difference was considered as the first criterion in the selection of these two biomolecules. By loading the drugs into the nanotubular cavities produced by anodic oxidation, release profiles with various periods of several hours to a couple of weeks were reported [12-15]. The parameters affecting this diversity in release duration include differences in hydrodynamic diameter of molecule, nanotube length, nanotube diameter and loading procedures of the active agent. Popat et al used gentamicin antibiotics with a simple pipetting on the nanotubular layer and the colonization of S. epidermidis bacteria reduced by 40% compared to the control group [12]. In the loading procedures, drug release duration can reach several weeks by coating the nanotubular surfaces with polymeric or ceramic layers [13,16, 17]. As a result of loading operations performed without any additional coating on the surface, it was reported that the release from metal surface was completed within a few days [15,18]. Although the biocompatibility and in vitro stability of polymeric or ceramic coatings to prolong the release time are satisfactory, there is also the possibility of delamination and / or disintegration of these layers having micrometer thicknesses from the surface of the material during implantation or in a dynamic environment.

The inability to control the release kinetics and the burst release of titanium dioxide nanotubes as a local release element is one of the most important problems. It is inevitable for biomolecules, which stand out as much smaller compared to their nanotube diameters, to exhibit sudden release within the first few hours. In this study, nanotube diameters obtained after anodic oxidation were narrowed by gold, which is known by biocompatible and inert material for implant applications, after the biomolecule loading. The effect of sputtering time to the releasing time behavior were investigated in terms of diameter and the hydrodynamic radius of the biomolecule.

2. Materials and Methods

2.1. Nanotube Formation on Titanium Plates

A two-step anodic oxidation process was performed on the surface of titanium metal plates with a purity of 99.7% (Strem Chemicals, USA). In the process, titanium plates were subjected to the first step of the anodic oxidation for 2 hours by applying 80 V potential voltage in ethylene glycol electrolyte containing 1% of ammonium fluoride (NH_4F) by weight. A platinum mesh electrode was used as counter electrode. After the first step of anodic oxidation, titanium plates were cleaned in an ultrasonic bath for 2 hours for the removal of titanium dioxide nanotubes with irregular orientation. In the second step, titanium dioxide nanotube structures with controllable aspect ratio were created. In the second anodic oxidation step, 30 V potential applied for 120 min.

2.2. Active Agent Loading into Nanotube Structures

Bovine serum albumin (BSA) and vancomycin.HCl were used as model molecules in protein (large molecule) and drug (small molecule) studies to be loaded onto titanium surfaces. BSA and vancomycin.HCl at a concentration of 5 mg/mL were pipetted onto 10 mm x 10 mm cut, nanotube array produced sample surfaces in a volume of 30 µL and subjected to low-speed radial movement on the orbital shaker to diffuse into the nanotubes at room temperature. The samples were dipped in deionized water to wash away the adsorbed protein/drug to the surface and dried at 37 °C overnight.

2.3. Tube-end Narrowing by Gold Sputter

Tube-ends of the protein/drug loaded nanotubes were subjected to gold sputtering in order to reduce the diameter. In a typical process, samples having different diameters were placed in sputtering chamber and gold was coated on the top of the nanotube layer with a rate

of 2.5 nm per minute. Sputtering time was differed as 2, 5 and 10 mins to evaluate the effect of the narrowing on active agent release. The narrowing ratio of diameters were investigated by microscopic methods. Nanotube morphology and diameter measurements were analysed by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

2.4 Spectroscopic and Structural Characterizations

X-Ray Diffraction (XRD) pattern and Attenuated Total Reflection - Fourier Transform Infrared Spectroscopy (ATR-FTIR) data were obtained in order to characterize the crystal structure of surfaces and functional groups, respectively. 1x2 cm samples were analysed in XRD (Rigaku D Max-B, Japan) between 20 – 60 2θ degrees and 2000 – 600 cm^{-1} wavenumbers in ATR-FTIR (Thermo, Nicolet iS50, USA).

2.5. Drug Release Studies

Each sample placed separately in 1 mL PBS (pH = 7.2) containing closed vials and the total protein and drug amount released from titanium plates were analyzed with the Nanodrop UV spectrophotometer device (Thermo Scientific, USA) in A280 total protein amount and UV-Vis spectroscopy measurement mode, respectively. Release studies continued until the release curve reach to plateau. Tested samples were summarized in Table 1.

Table 1. Samples tested in release studies.

Sputtering time	Drug/protein	Sample code
Without gold sputter	Vancomycin.HCl	30V_vanco
	BSA	30V_BSA
2 min gold sputter	Vancomycin.HCl	30V_2min_vanco
	BSA	30V_2min_BSA
5 min gold sputter	Vancomycin.HCl	30V_5min_vanco
	BSA	30V_5min_BSA
10 min gold sputter	Vancomycin.HCl	30V_10min_vanco
	BSA	30V_10min_BSA

3. Results and Discussion

3.5. Morphologic Evaluations

The surfaces obtained by the first step of anodic oxidation of are shown in Figure 1a. After the first treatment, the surface character is composed of nanotubes having inner diameters of approximately 100 nm. As mentioned in general information, these tubes are mechanically unstable as a result of the random alignment on the metal surface by different orientations, which makes them easily affected by external forces. The surface shown in Figure 1a was cleaned from nanotubes in the ultrasonic bath for 2 hours by applying low frequency (37 Hz) and high power (100 W) agitation to obtain the surface of Figure 1b. These concave homogeneous regions served as guides for the structures to be formed on the surface in the second step.

In the second step anodic oxidation process, 30 V potential was applied for 2 hours. Inner growth of new nanotubes was obtained, and the diameters of these nanotubes were found narrower than the template as the anodic oxidation voltage is in proportion with the tube diameter. By applying field-enhanced dissolution on the nanotube template-formed surfaces, the orientation of the nanotube formations on the entire surface was produced in the same direction. According to this, inward growing nanotubes through the template after anodic oxidation with a potential voltage of 30 V were achieved (Figures 1c).

After the gold sputtering step, it has been observed that the tube-endings start to get narrower. Although the narrowing of the tube diameters performed using 2 minutes of coating was not evident in the images, a significant restriction of the outer tube mouth diameters occurred, especially after 5 and 10 minutes of spraying. Especially after 10 minutes of sputtering, the decrease in outer diameter is evident (Figure 2b-d).

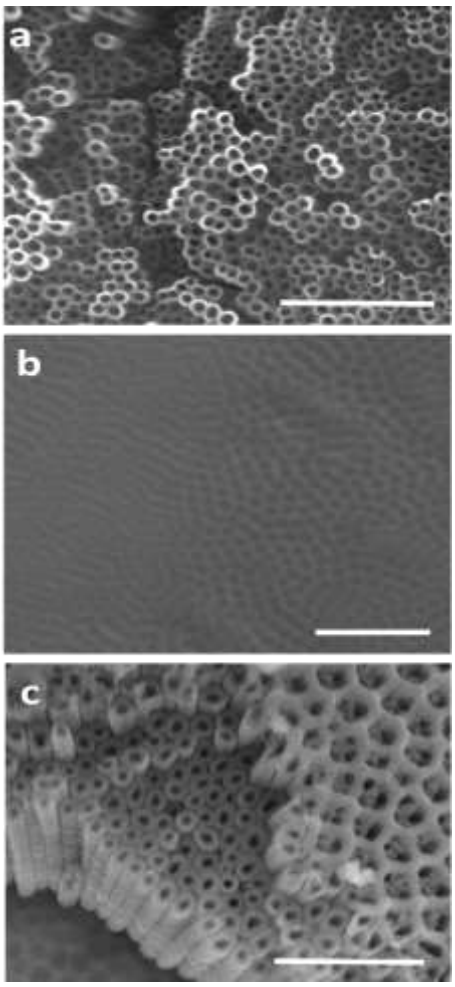

Figure 1. Titanium surfaces after first anodic oxidation process (a) after sonic treatment (b) and after second anodic oxidation (c). White bar indicates 1 µm.

Celal Bayar University Journal of Science
Volume 18, Issue 1, 2022, p 1-7
Doi: 10.18466/cbayarfbe.972316

Figure 3 also indicates that the narrowing of the tube-ends, especially after 5 and 10 minutes of sputtering. Images were obtained by an AFM system (Asensis, Turkey). Particularly after 10 minutes of spraying, the outer tube diameter of around 100 nm has been reduced to approximately 50-70 nm and the internal diameter shrinkage can be observed from the images (Figure 3).

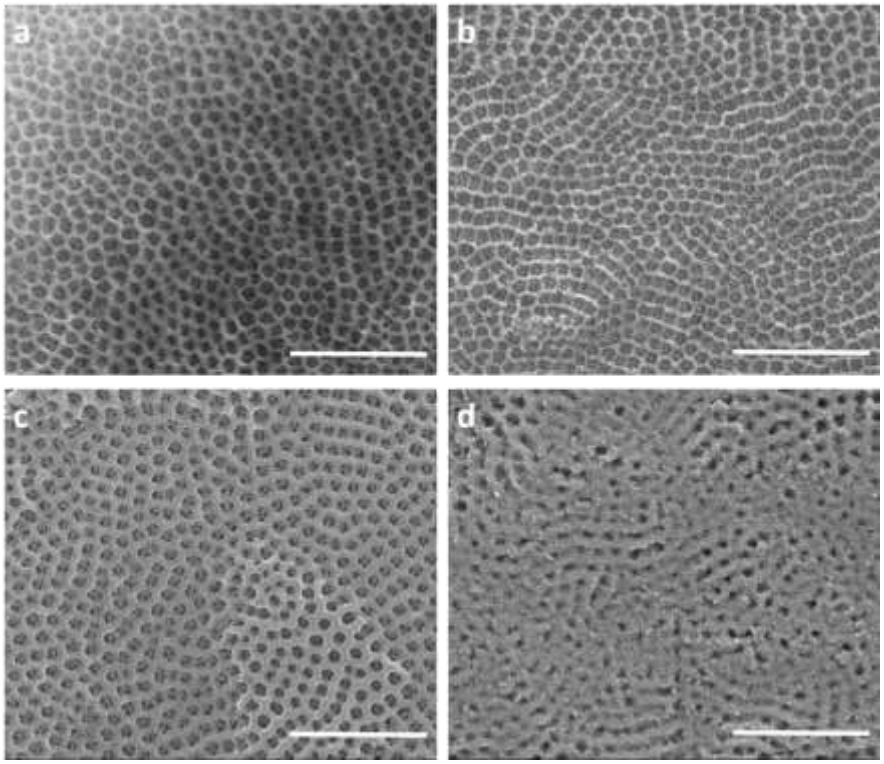

Figure 2. SEM images of titanium surfaces after 2nd anodization (a) and after subsequent 2 (b), 5 (c) and 10 mins (d) of Au sputtering.

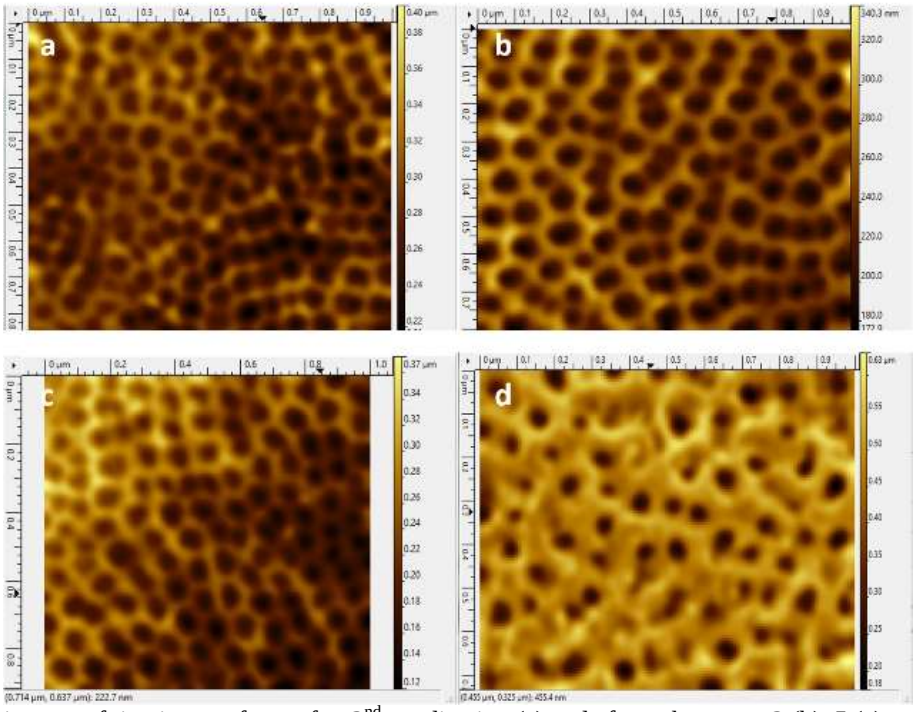

Figure 3. AFM images of titanium surfaces after 2nd anodization (a) and after subsequent 2 (b), 5 (c) and 10 mins (d) of Au sputtering.

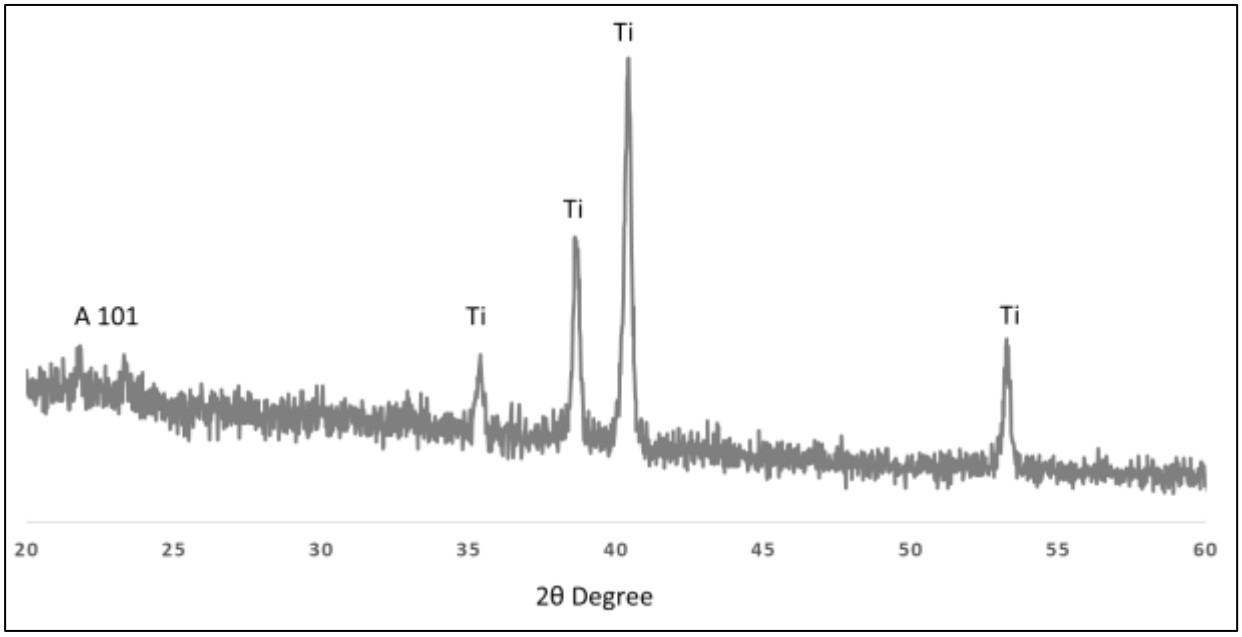

Figure 4. XRD diffraction pattern of anodized TiO_2 surfaces.

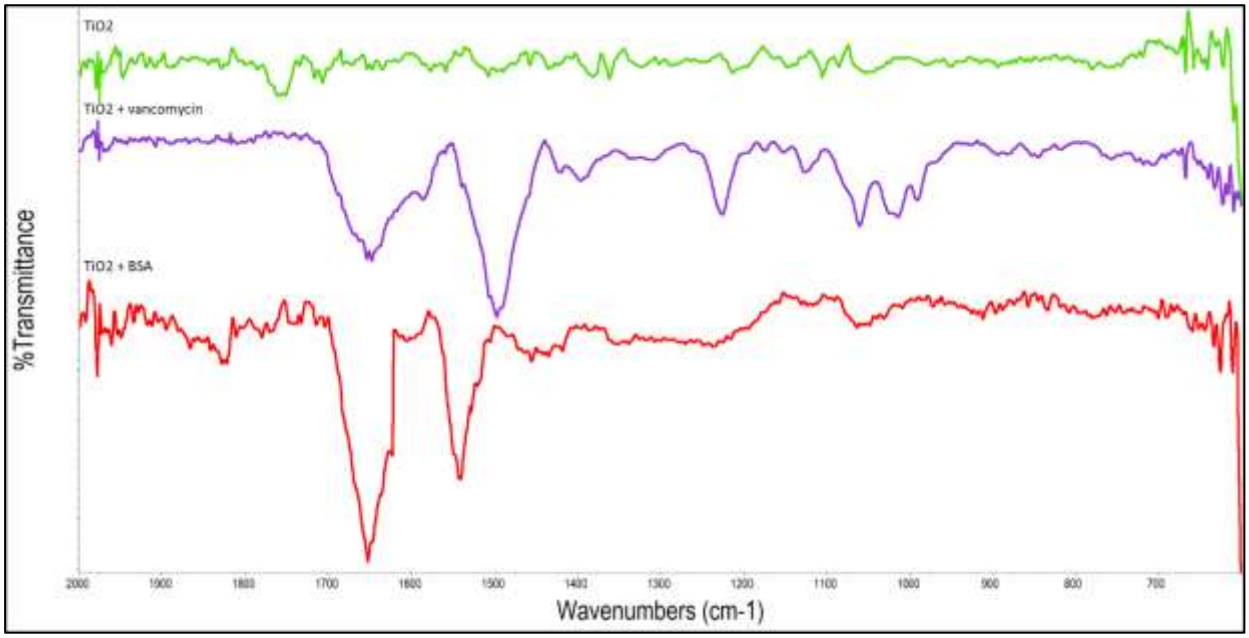

Figure 5. FTIR spectra of TiO_2, vancomycin loaded TiO_2 and BSA loaded TiO_2 surfaces.

3.6. Spectroscopic and Structural Characterizations

Figure 4 shows XRD patterns of prepared anodic TiO_2 nanotube array. The resulting surface exhibits clear Ti peaks as indicated on the graph along with the anatase (101) reflection peaks between 20-25 theta. Since the surface was not exposed to any heat treatment sharp crystal anatase structures were not observed.

The presence of vancomycin and BSA were analyzed with infrared spectroscopy. Vancomycin loaded sample exhibits a wide carbonyl stretching at 1650 cm^{-1}. The other indicators can be observed at 1500 and 1230 cm^{-1} for aromatic C=C and phenol ring, respectively. As for BSA loaded sample, The amide I and II bands are clear at 1650 and 1545 cm^{-1}, respectively (Figure 5).

3.7. Drug Release Studies

Two model compounds of bioactive substances with small and large hydrodynamic diameters were investigated in the release studies from titanium dioxide nanotube structures with different diameter openings. The release profiles of vancomycin.HCl (<1 nm) with

Celal Bayar University Journal of Science
Volume 18, Issue 1, 2022, p 1-7
Doi: 10.18466/cbayarfbe.972316

small hydrodynamic diameter and BSA (3.48 nm) molecules with large hydrodynamic diameter were investigated separately. The molecular weight difference was considered as the first criterion in the selection of these two biomolecules. It was desired to observe the trend of vancomycin with a weight of 1.4 kDa and BSA with a size of 65 kDa in the loading and release criteria. This molecular weight difference also affects the hydrodynamic diameters. Both components were selected as water soluble and the difference in hydrophilic-hydrophobic effect was eliminated in the release experiments.

Profiles for the release of small hydrodynamic diameter molecules vancomycin.HCl from titanium surfaces are given in Figure 6. The amount of drug released into the medium in all release trials reached the plateau at the end of the 4th day.

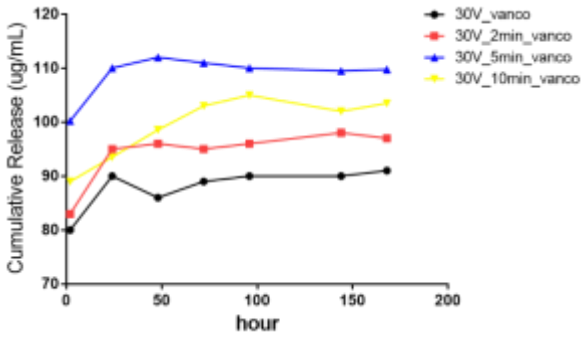

Figure 6. Release profile of vancomycin.HCl from titanium surfaces.

The drug released from the nanotubes reached the maximum level at the end of 24 hours on untreated surfaces, and the narrowing of the tube-ends obtained on the surface after 2 and 5 minutes of sputtering did not extend this period. The duration of release with narrowing in the tube diameters for a period of 5 minutes was one day longer than the first two sample groups; The release from the nanotube structures obtained after 10 minutes of sputtering continued for an additional period of 3 days. No further drug release from the tubes was observed in any sample group after day 4 (Figure 4).

BSA release from the surfaces was monitored up to one week. The release time was observed for 3 to 4 days on untreated and gold-sputtered surfaces for 2 min, respectively and extended to 6 days on 5-min and 10-min gold-sprayed surfaces (Figure 7). BSA release from titanium surfaces was longer in all sample groups compared to vancomycin.HCl release time, and the effect of gold sputtering was more effective than small hydrodynamic diameter molecular release.

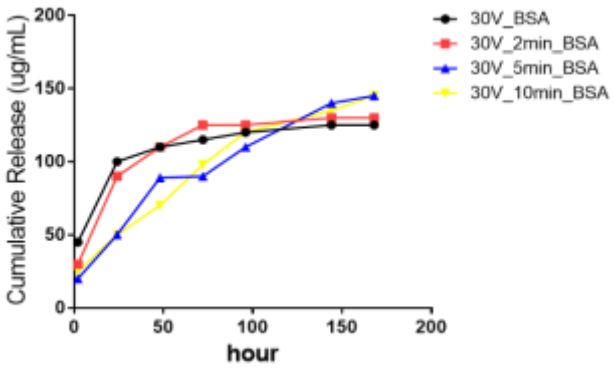

Figure 7. Release profile of BSA from titanium surfaces.

The prolongation of release time is much more evident in experiments with protein. There are two main reasons behind this. The first is that the hydrodynamic diameter of the protein is several times larger than the vancomycin.HCl molecule, as it is considered during the experimental design. Smaller molecules are known to diffuse more rapidly during diffusion. However, another factor slowing protein release can be considered as hydrophobic nature of the protein. Although BSA protein is a water soluble protein, the interaction of hydrophobic regions with titanium structure is much greater than vancomycin.HCl. This interaction is also thought to cause an additional delay in during release.

In the preliminary study conducted by Peng et al. [14] the length of the nanotubular structure was found as the most important parameter affecting the release time; whereas the diameter of the nanotube (when the length was held constant) was the most important parameter affecting the amount of drug loaded. In another study by Çalışkan et al.[19], Parallel findings were observed and parameters affecting drug loading amount and release time were also reported as nanotube diameter and length, respectively. By revealing the importance of the effect of nanotube aspect-ratio in barrier-free studies, this feature became controllable and became a priority for localized drug release studies.

4. Conclusion

Direct release of active biomolecules from metallic implants is a challenge for implant applications. Although titania nanotube layers offer an opportunity for drug releasing reservoirs, sustained release remains as a problem. In order to eliminate the risk of infection that may arise from the operation in the critical period after the surgical process, there is a need for implants that can release the antibacterial active substance locally or metal surfaces that will contain larger macromolecules such as growth hormone to accelerate osseointegration. For this reason, burst and rapid release should be avoided. Sputter coating of tube ends with inert metals may be a promising approach to overcome this drawback. In this study, titania nanotube structures with adjustable mouth widths on titanium metal plates

were obtained as a result of a two-step anodization process. Vancomycin and BSA as biomolecules with small and large hydrodynamic diameters were also used as model releasing compounds within these nanostructures. In order to prolong the release duration, the tube-ends were narrowed by gold sputter method and the effect of these narrowings on the release profiles was investigated separately for both molecules. It is seen that the increasing sputtering time slows down the release rate of BSA, which is selected as a large molecule. Within the scope of this study, it was planned to obtain a narrowing on the surfaces of titania nanotubes, which are intended to be used as local release reservoirs, with gold, a bioinert material that will not be affected by mechanical abrasion and friction in the local region after implantation. The findings obtained were promising results and the feasibility of the approach will be tried to be demonstrated with the studies to be carried out with bacteria and osteoblasts in the next steps.

Acknowledgement

This project was financially supported by Hacettepe University, Scientific Research Projects Coordination Unit with the project number FHD-6700.

Author's Contributions

Cem Bayram: The hypothesis of the study, experimental design, performance, result analysis and writing of the study.

Ethics

There are no ethical issues after the publication of this manuscript.

References

[1]. Dong, H, Liu, H, Zhou, N, Li, Q, Yang, G, Chen, L, Mou, Y. 2020. Surface Modified Techniques and Emerging Functional Coating of Dental Implants. *Coatings*, 10(11), 1012.

[2]. Palka, K, Pokrowiecki, R. 2018. Porous Titanium Implants: A Review. *Advanced Engineering Materials*. 20,1700648.

[3]. Nicholson, JW. 2020. Titanium Alloys for Dental Implants: A Review. *Prosthesis*, 2(2), 100-116.

[4]. Draghi, L, Preda, V, Moscatelli, M, Santin, M, Chiesa, R. 2020. Gentamicin-Loaded TiO2 Nanotubes as Improved Antimicrobial Surfaces for Orthopedic Implants. *Frontiers in Materials*, 7:233.

[5]. Ion, R, Necula MG, Mazare A, Mitran V, Neacsu P, Schmuki P, Cimpean A, 2020. Drug Delivery Systems Based on Titania Nanotubes and Active Agents for Enhanced Osseointegration of Bone Implants. *Current Medicinal Chemistry*, 27(6), 854-902.

[6]. Losic, D. 2021. Advancing of titanium medical implants by surface engineering: recent progress and challenges, *Expert Opinion on Drug Delivery*, 10.1080/17425247.2021.1928071.

[7]. Zhao, L, Chu, PK, Zhang, Y, Wu, Z. 2009. Antibacterial coatings on titanium implants, *Journal of Biomedical Material Research B Applied Biomaterials*, 91, 470–480.

[8]. Turanli, AE, Sokullu, E, Nikolayev, A, Dagci, T, Öztarhan, A. 2016. Dental İmplant Materyallerin İyon İmplantasyon Yöntemiyle Modifikasyonlarının in vitro Hücre Tutunmasına Etkisi, *CBÜ Fen Bil. Dergi*, Cilt 12, Sayı 2, 243-251.

[9]. Sarkar, N, Bose, S. 2020. Controlled Delivery of Curcumin and Vitamin K2 from Hydroxyapatite-Coated Titanium Implant for Enhanced in Vitro Chemoprevention, Osteogenesis, and in Vivo Osseointegration. *ACS Applied Materials and Interfaces*, 12(12),13644-13656.

[10]. Zilberman, M, Elsner, JJ. 2008. Antibiotic-eluting medical devices for various applications. *Journal of Controlled Release*,130, 202–215.

[11]. Macak, JM, Tsuchiya, H, Schmuki, P. 2005. High-Aspect Ratio TiO2 Nanotubes by Anodization Titanium, *Angewandte Chemistry International Edition*, 44, 2100-2102.

[12]. Popat, KC, Eltgroth, M, LaTempa, TJ, Grimes, CA, Desai, TA. 2007. Decreased Staphylococcus epidermis adhesion and increased osteoblast functionality on antibiotic-loaded titania nanotubes, *Biomaterials*, 28, 4880-4888.

[13]. Yao, C. Webster, TJ. 2009. Prolonged Antibiotic Delivery from Anodized Nanotubular Titanium Using a Co-precipitation Drug Loading Method, *Journal of Biomedical Material Research B Applied Biomaterials* 91B: 587–595.

[14]. Peng, L, Mendelsohn, AD, LaTempa, TJ, Yoriya, S, Grimes, CA, Desai, TA. 2009 Long-Term Small Molecule and Protein Elution from TiO2 Nanotubes, *Nano Letters*, 9, 1932-1936.

[15]. Ma, M, Kazemzadeh-Narbat, M, Hui, Y, Lu, S, Ding, C, Chen, DDY, Hancock, REW, Wang, R. 2012. Local delivery of antimicrobial peptides using self-organized TiO2 nanotube arrays for peri-implant infections, *Journal of Biomedical Material Research* 100A: 278–285.

[16]. Sinn Aw, M, Kurian, M, Losic, D. 2014. Non-eroding drug-releasing implants with ordered nanoporous and nanotubular structures: Concepts for controlling drug release, *Biomaterials Science*, 9, 9243-9257.

17]. Gulati, K, Kant, K, Findlay D, Losic, D. 2015. et al., Periodically tailored titania nanotubes for enhanced drug loading and releasing performances. *Journal of Material Chemistry B*, 2015. 3: p. 2553-2559.

[18]. Gulati, K, Ramakrishnan, S, Aw, MS, Atkins, GJ, Findlay, DM, Losic, D. 2012. Biocompatible polymer coating of titania nanotube arrays for improved drug elution and osteoblast adhesion, *Acta Biomaterialia*, 8, 449-456.

[19]. Caliskan, N, Bayram, C, Erdal, E, Karahaliloglu, Z, Denkbas, EB. 2014. Titania nanotubes with adjustable dimensions for drug reservoir sites and enhanced cell adhesion. *Material Science and Engineering C*, 2014. 35: p. 100-105.

[20]. Chopra, D, Gulati, K, Ivanovski, S. 2021. Understanding and optimizing the antibacterial functions of anodized nano-engineered titanium implants. *Acta Biomaterialia*, 2021. 127:80-101.