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Loading Drug on Nanostructured Ti6Al4V-HA for Implant Applications

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ABSTRACT

Arrayed Ti6Al4V nanotubes (TNT) coated with hydroxyapatite (HA) were synthesized via electrochemical anodization method. Paracetamol was loaded onto TNT-HA electrode. Effects of anodization, nanotube formation and hydroxyapatite deposition on sorption and release of the drug were investigated. Saturation time of paracetamol on the anodized samples was 30% shorter than the hydroxyapatite-coated samples. Release behavior of the loaded drug was studied by (a) plunging the probe into phosphate buffered saline (PBS), (b) sampling the drug-loaded PBS at different times and (c) analyzing the solution via ultraviolet-visible (UV-vis) spectroscopy. Results showed that HA electrodes hold higher amounts of paracetamol than the anodized samples at longer times. Scanning electron microscopy (SEM), MTT assay, and nanoindentation tests were used to characterize the produced electrodes.

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1. INTRODUCTION

Drug loading of implants aiming to enhance the biological properties is appealing to many medical areas of interest. To increase drug capacity, surface modifications leading to spacious structures are desirable. Titanium-based implants have been commonly used in implantology due to their excellent biological performance and excellent mechanical properties. They suffer; however, from some clinical problems like bacterial infection, local inflammation, stress shielding and repeated administrations. Drug and device combination can address such cases via regional drug therapy approach. Implants loaded by drug present themselves as a promising biomedical technology to provide essential improvements in the biological performance of the implants.

Controlling drug release from implants is of importance to biomedical applications. Nanostructured medical devices have provided drug-loading possibility. In recent studies, titanium nanostructures such as nanotubes [1-11], nanoparticles [12,13], nanorods [14,15] and nanowires [16] have been considered as the drug carriers. However, among them, nanotubes due to their reservoir shape seem to be an appealing choice to encapsulate and retain the bioactivity of biomolecules, including drugs. In this study, we aimed to investigate the possibility of loading drug into these nanotubular structures.

Zwilling et al. [17] and Grime et al. [18] were the first researchers who developed titanium nanotubes via electrochemical anodization. Later, titanium nanotubes attracted much attention for such applications as photocatalysis [17-21], solar cell sensitization [22,23] and drug delivery [24,1-11]. The nanotubular structures of TNTs have presented them as an auspicious candidate for drug storage and conveyance. Following this, Yao and coworkers [1] have been the pioneer in using titanium nanotubular structures as drug release carriers. Controlling drug release; however, has been an important issue to be considered. Few techniques like coating nanotubes with polymeric layers [5,25] and composite hydrogels [3] have been thought to control the extent of the drug release from the titanium nanotubes.

In this study, we used hydroxyapatite (HA) to cover the titanium nanotubes for greater bioactivity and controllability of the drug release. The objective of the study was to determine the release behavior of the loaded drug on the anodized HA-coated Ti-6Al-4V

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sheets. An analgesic drug; paracetamol, (N acetyl p aminophenol) was used as a model drug to ensure its positive effect when used with HA-coated TNT samples.

2. MATERIALS AND METHODS

2. 1. Substrate Preparation Titanium substrates were categorized into three groups: untreated titanium (Ti), titanium substrates with nanostructured tubes grown by anodization (TNT), and nanostructured titanium with HA coating (TNT-HA). Ti-6wt.%Al-4wt.%V (Ti-6-4) was utilized as a substrate. The substrates were polished with emery papers of 400, 600, 800, 1000, and 1200 grits; etched with Fluoric acid (HF) for 10s, and then dried in air.

2. 2. Formation of TNT on Titanium Substrate Electrochemical cell with two electrodes including Ti-6-4 sheet as anode and ST316 as a cathode was part of our anodic oxidation process. The electrolyte was 1M ammonium sulfate $(NH_4)_2SO_4$ and 5wt% ammonium fluoride (NH_4F) solution, and the electrodes were parallel, with 4cm distance from each other. Potential, temperature and time-duration of anodization were 25V, 25°C, and 60min, respectively.

2. 3. Cathodic Deposition of HA on the Anodized Substrate Calcium phosphate coatings were deposited onto titanium alloy substrates via electro-deposition method. Titanium alloy Ti-6Al-4V sheets were etched in a mixture of nitric acid (HNO3 50%, 6 mL), Hydrofluoric acid (HF 40%, 3 ml), and water. After that, the samples were water-cleaned and dried. The experimental set-up was a two-electrode cell configuration. The working electrode (cathode) was a titanium alloy sheet and the counter electrode (anode) was ST316. Their distance from each other was about 4cm. A power-supply provided a pulsed direct current. Electrodeposition of calcium phosphate was conducted at room temperature. Both electrodes were immersed in an electrolyte containing 0.04M $Ca(NO_3)_2$, 0.1M $NaNO_3$ and 0.027 mol/L $(NH_4)_2HPO_4$, and water. The molar ratio of Ca to P in this solution was 1.67, and the electrolyte was buffered at 5.5, by using ammonia and nitric acid. The electrolyte was thermostated at 60-80°C, and stirred. The solution was stirred during electro-deposition to achieve a uniform electrolyte concentration. A direct current power generator was connected to titanium alloy and ST316 electrodes. The current densities of 0.5-1mA/cm^2 were applied to the electrodes. Titanium substrates, after anodic oxidation were reused as cathodes for further electrodeposition. The time-period for this experiment was 60min. After electrodeposition, the coated cathodes were rinsed with distilled water and then dried.

2. 4 Drug Loading Paracetamol, an analgesic drug, was selected as a working pharmaceutic in this study. Solution of paracetamol was prepared on the magnetic stirrer for usage as a model drug. To adsorb analgesic drug on the surface of the prepared titanium alloy probes, the samples were plunged into a test tube filled with the paracetamol aqueous solution and capped with a tight polyethylene lid preventing air. Then, through each selected times, 1μL of the solution was picked by a sampler to read the absorbance of each sample by NanoDrop 1000 spectrophotometer. Drug's maximum absorption peak was investigated using UV-Vis array spectrophotometer (PhotonixAr 2015) which was around 280nm. The samples were dried in air for some days after plunging in the drug solution. After that, by placing each drug loaded samples into 10ml of phosphate buffered saline (PBS) in a test tube, drug release was investigated by picking 1 μL of the solution to read the solution's absorbance by NanoDrop 1000 spectrophotometer.

2. 5. Structural Characterizations Scanning Electron Microscopy determined the surface morphology of the HA on the HA-coated samples. Nanoindentation tests were applied to the samples to evaluate the mechanical properties of the materials including hardness and reduced elastic modulus. During the test, load and displacements are continuously monitored. Nanoindentation tests were conducted using TriboScope system (Hysitron Inc., USA), equipped with a Berkovich type indenter tip, according to the method described in literature [26].

2. 6. Cell Culturing An indirect cytotoxicity test utilizing MTT; a colorimetric assay for assessing cell viability, was carried out in this research. Samples were exposed to gamma radiation and incubation at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) for a period of 3, 7 and 14 days. MG-63 cells were added to a 96-well culture plate and kept in an incubator for 24h at 37°C to let the cells stick to the bottom of the plate. Then, the extracts from each sample were added to the cell-culture well, and the cells were kept together with the extracts for another 24 hours. Afterwards, the cell cultures were removed, and 100μL 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added to each well. Optical density was calculated at the wavelength of 545nm. The wells with more cells revealed higher optical density (OD). Hence, cells-viability could be calculated by the following equations:

$$\text{Toxicity } \% = \frac{1\text{- mean OD of sample}}{1\text{- mean OD of control}} \times 100 \quad (1)$$

$$\text{Viability } \% = 100 - \text{Toxicity } \% \quad (2)$$

3. RESULTS AND DISCUSSIONS

In this work, we fabricated titanium nanotube arrays using simple anodization method. The nanoarchitecture of titanium nanotube arrays was examined for uniformity and repeatability using SEM summarized in Figure 1.

A nanoindentation apparatus explored the mechanical properties of the samples. The typical load-displacement curves (Force-Displacement) for TNT, HA-Ti and HA-TNT are presented in Figure 2, which provide the nano-mechanical characterization results. According to the figure, three regions are identified. The indentation depth varies from 300nm for TNT to 40nm for HA-TNT. As can be seen, the various coatings may change material properties including hardness and strength. Scientists count these values as apparent modulus of elasticity and hardness owing to the effect of substrate on the coating properties [27]. The hardness and reduced elastic modulus of TNT and HA-TNT are higher than that of HA-Ti because of the presence of nanotubular structure, whereas HA-TNT presented lower hardness and reduced elastic modulus than TNT (Table 1).

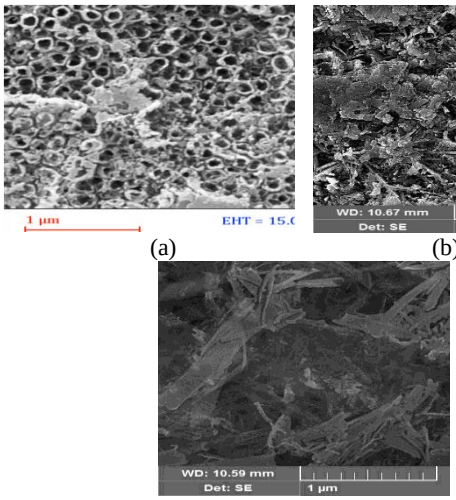

Figure 1. Scanning electron micrographs of (a) TNT, (b) HA-Ti and (c) HA-TNT.

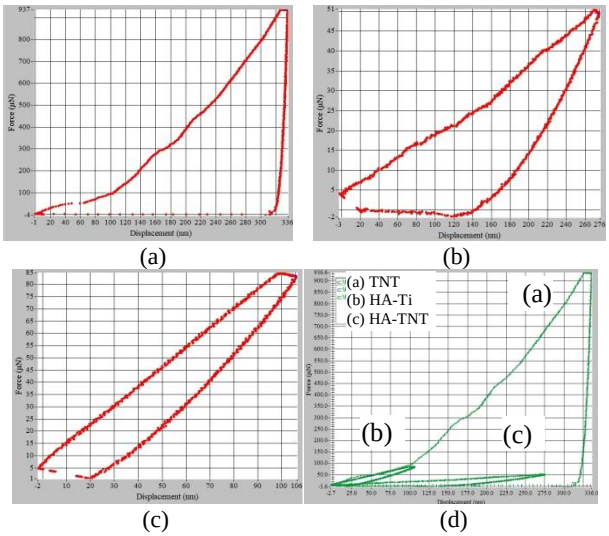

Figure 2. Characterization load vs. nanoindentation depth for samples (a) TNT, (b) HA-Ti, (c) HA-TNT and (d) Comparative exhibition of the three

TABLE 1. Mechanical properties; hardness and the reduced modulus (E_r) of TNT, HA-Ti and HA-TNT

Samples	Hardness	E_r (GPa)
TNT	0.59 ± 0.1737814	52.5
HA-Ti	0.03 ± 0.0178885	0.45
HA-TNT	0.12 ± 0.1387872	2.4

The scratch tests were performed on TNT, HA-Ti and HA-TNT to investigate the adhesion strength of the coatings to the substrate (Figure 3). These data exhibited that HA had not any effect on enhancement of the adhesion strength of the coating to the substrate. The failure points occurred at approximately 670nm, 50nm and 60nm for TNT, HA-Ti and HA-TNT, respectively. This result represents a better bonding between the nanotubular structures with the substrate. According to Zalnezhad and coworkers [27], since the TNT nanotubes are created from the body of titanium coating, they are bounded strongly to the substrate and cannot be detached easily. Poor adhesion of titanium nanotube arrays to the substrate (non-anodized titanium) has been reported as a reason for their application restrictions. Consequently, a number of researches have focused on the method of control of the stripping behavior of the tubular structures from the substrate [27,28].

The analgesic water-soluble drug paracetamol was chosen as a test-drug to be loaded inside the nanotubes.

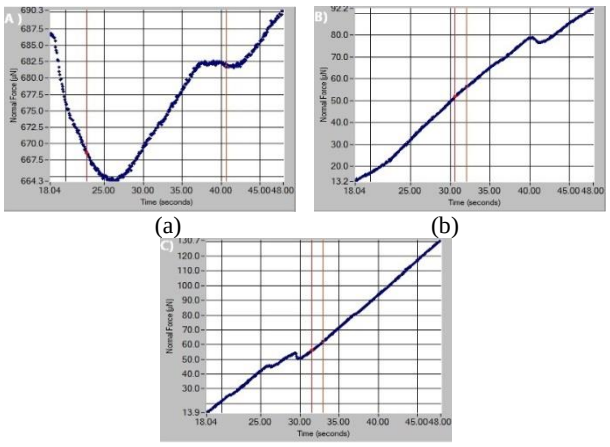

Figure 3. Scratch test results of (a) TNT, (b) HA-Ti and (c) HA-TNT

Herein, there is no limitation in choosing of the drug kind; any drug including the anti-bacterial as well as the anti-cancer drugs can also be loaded to the produced samples. The amount of drug was 0.2950gr equal to one tablet, which was an adequate doze of 650mg per 4h (i.e. 0.2mg per 1h), usually prescribed by physicians for no toxicity effects. It should be mentioned that the drugability of the nanotubes can be adjusted by their diameter and length (dimensions), based on the works of Çalışkan et al. and Rajesh et al. [7,8].

Besides nanotubes as a drug carrier, we can expect from curled flakes exhibited in SEM of HA-coated nanotube arrays to act as the carrier for the drug, too. Furthermore, nanopores and micropores can be influenced in the encapsulation of the drug. Also, through this surface modification technique, HA layer can play role as a barrier to drug release. In other words, HA layer is able to control the drug release kinetics from Ti nanotube arrays. In previous studies, using Chitosan [5], PLGA [5] and the composite hydrogels [3], as well as altering the dimensions of the nanotubes by the electrochemical parameters [7,8] have been considered as different tools of controlling drug release rate from the Ti nanotubes. In these studies, drug release has been assumed zero-order [5], first-order [1], and even a diffusion-controlled process with the assumption that diffusion of drug into the release-media follows one-dimensional Fick's law [4]. In the present study, we functionalized surface by the HA electrodeposition process with the aim of controlling the drug release rate. HA, a bio-ceramic having resemblance to the mineral bone was selected to allow (a) controlled drug release, (b) encapsulate more drugs with its flakes and micropores, and (c) to mimic the bone function and increase bone growth at later stages [29]. The advantage of this method is its simplicity and low cost. HA and other calcium-phosphate based ceramics have been used to improve the properties of the implant in the drug delivery [8] and several other aims that have been mentioned in the literature [30-34].

Comparative drug release profiles of Ti, TNT, TNT-HA are depicted in Figure 4. Both fast and slow release behaviors are seen in the figure. Percent drug release at various time intervals (15, 30, 45, 60, 75, 90, 105, 120, 135min) is shown in the figure. For TNT, the release kinetics can be delineated in two phases of initial burst followed by a small release rate at longer times of the measurement.

The burst-release can be described by the high concentration gradient across the pores [5], with the large diameter of (≈100nm) allowing rapid release of the drug. Although from previous studies, it can be said that by decreasing the nanotubes diameter, the burst release can be decreased. From these results, it is not clear where exactly the drugs were placed either in the top part of the nanotubes or deep in the pores.

As a result of deposition of HA, significant changes in the drug release profile appeared. The burst release was reduced from 95% for TNT to 43% for HA-TNT. From this dramatic decrease, it can be concluded that HA layer is acting as a barrier not letting the drug to release fast. Drug release from TNT during the first 135min of the experiment was approximately 95%, whereas this factor for HA-TNT seemed to be in the range of 30-40%.

As Gulati and coworkers [9,16] have mentioned, one of the reasons for molecular transport of the drug in the coating layer is the permeability of the coating for drug molecule, which depends on the coating's chemical composition, structure, electric charge, surface tension and interface hydrophobicity and hydrophilicity. Herein, HA is hydrophilic, while Ti nanotubes are hydrophilic, too [35]. Since Paracetamol is hydrophilic [36] and water soluble, it is expected that solubility and diffusion of this drug through both phases (HA and TNT) will be possible. Gulati and coworkers [9,16] have attributed the permeation of the drug directly to the interfacial properties. However, as we mentioned here, due to similarity of this property among the two examined sample of TNT and HA-TNT, the higher permeability of drug in HA-TNT cannot be explained by this assumption. Furthermore, we consider other possible factors for prolongation of the release process to include large size of the drug particles [37], and positive electrostatic charge of the drug [35]. Popat and coworkers [24] confessed that vicinity of terminal hydroxyl groups on the surface of TNTs results in the formation of negative charge, which in turn leads to a retarded release of the positively charged drugs. In this study, the model drug, paracetamol, is neutral [38] with the small molecular weight of 151.163Da (Dalton). Therefore, it is assumed that encapsulation of the drug and its rate of release for TNT and HA-TNT is not affected by its electrostatic charge, whereas the small size of the drug molecules (molecular weight lower than 900Da are counted as small molecules) can be a reason of the fast release spotted in our study. Based on another assumption, this initial burst release can also be related to the accelerated diffusion of the drug molecules resulted from the high concentration gradients between the drug interface and the PBS solution.

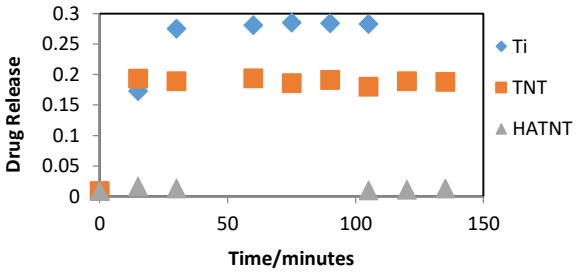

Figure 4. Release behavior of Ti , TNT and HA-TNT in PBS

Comparing the sorption and desorption profiles of HA-TNT and TNT specified in Figures 4 and 5, one can conclude that larger pores along with small pores are loaded with greater amount of drug. In other words, HA-TNT has the potential to have higher amount of drug and keep it for longer times in comparison with the TNT and Ti-6-4. Ti-6-4 and TNT show higher and faster burst release than HA-TNT. Gulati and coworkers' theory applies here for HA layer. In addition, in our case the size of the formed pores and drug are discussed as the reason for differences in drug release profiles of the two examined samples, whereas the mentioned literature tributes these differences to the hydrophobicity/ hydrophilicity behaviors.

A basic demand for the use of the implants in the human body is that they must be biocompatible with regard to the bone cells, and mainly the osteoblast cells. Thereby, the cellular behavior of the substrates was investigated. An experiment was built for cell culturing of the control, Ti-6-4, TNT, HA-TNT, and HA-Ti. The viability of the cells on these substrates is graphically presented in Figures 6 and 7.

The viability of control sample was taken as 100%. The number of the survived cells on the treated samples was higher than the untreated ones.

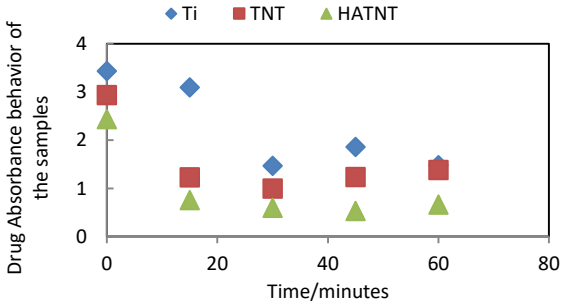

Figure 5. (a) Ti, (b) TNT and (c) HA-TN plunged into the drug solution; the sorption data were shifted 0.5 unit for Ti-6-4 and 1 unit for TNT

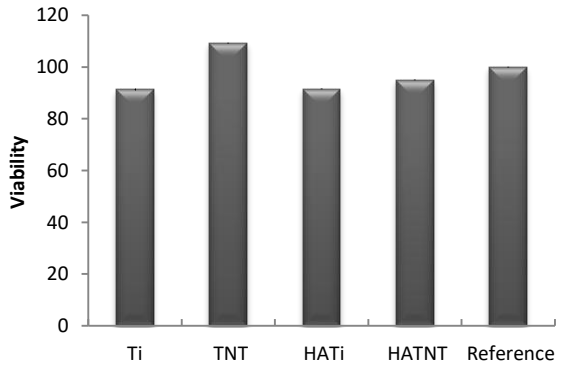

Figure 6. Viability of Ti-6-4, TNT, and HA-TNT

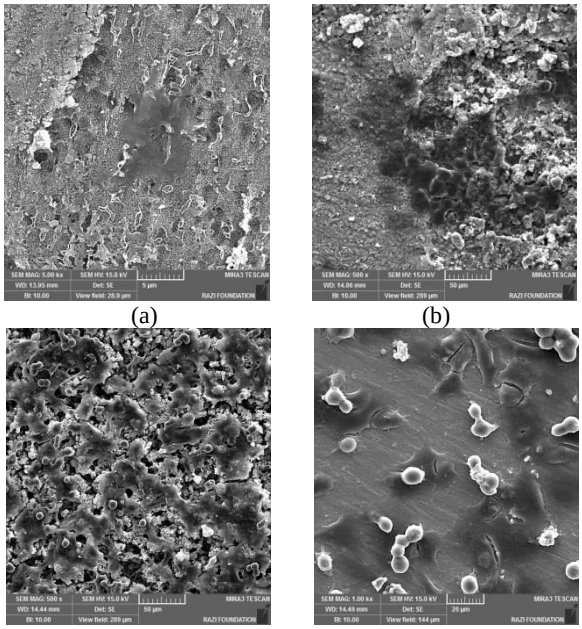

Figure 7. SEM images of (a) TNT, (b) HA-Ti, (c) HA-TNT and (d) control sample

Of these, the TNT sample depicted greater cell viability. We can refer this high viability to the substrate binding ability to the TNTs. According to Gongadze and coworkers [39], the osteoblasts can bind to the regions of nanostructured titanium with the high charge densities, like the sharp edges of the Ti nanotubes aving improved cell adhesion. For this reason, HA-TNT due to the HA frontier layer on the nanotubes and so a lower charge density on the surface as compared to the TNT shows lower cell viability. These results indicate that the modification of the Ti-6-4 surface with nanotubes and the HA enhances the biocompatibility properties of the Ti-6-4. This is indicative of the durability of the techniques used here for use in the biomedical devices.

4. CONCLUSIONS

Anodization and coating of the Ti-6-4 implants with a hydroxyapatite layer can improve their drug-release behavior, biocompatibility, human osteoblasting and analgesic properties. A simple electrochemical method is used for anodization, drug loading and deposition of HA. The loading and release of the analgesic paracetamol drug was achieved as a model to determine the adsorption and release characteristics of the system. The ability to tune the release rate of the drug over long terms, depending on the vicinity of the barrier layer of HA is aimed. We also tested the biocompatibility of the bioceramic coatings on the Ti-6Al-4V, using osteoblastic cells. It should be noted that, the devised system can be utilized for various implants and different

drugs whether painkiller, antibacterial and/or anticancer. This means that the produced method is applicable to a wide variety of prosthetic models. Although, detailed study of each specimen is definitely required for confirmation of the special clinical usage.

5. ACKNOWLEDGMENTS

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Loading Drug on Nanostructured Ti6Al4V-HA for Implant Applications

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چکیده

نانولوله های ردیفی Ti6Al4V (TNT) پوشش داده شده با هیدروکسی آپاتیت (HA) با استفاده از روش آندایزینگ الکتروشیمیایی سنتز شدند. پاراستامول بر روی الکترود TNT-HA بارگذاری شد. تاثیر آندایزینگ، تشکیل نانولوله و نشستن هیدروکسی آپاتیت بر جذب و واجذب دارو مورد بررسی قرار گرفت. زمان اشباع پاراستامول بر روی نمونه های آندایز شده 30 درصدکمتر از نمونه های پوشش داده شده با هیدروکسی آپاتیت بود. رفتار رهایش داروی بارگیری شده از طریق (a) وارد کردن پروب به داخل محلول نمکی فسفات بافرشده (PBS)، (b) نمونه برداری از محلول دارو دار PBS در زمان های مختلف، و (c) تجزیه محلول با استفاده از طیف سنجی با اشعه ماوراء بنفش (UV-vis) انجام شد. نتایج نشان داد که الکترودهای HA مقادیر بیشتری از پاراستامول را در مقایسه با نمونه های آندایز شده در زمان های طولانی نگهداری می کنند. برای مشخص کردن الکترودهای تولید شده از میکروسکوپ الکترونی روبسی (SEM)، آزمایش MTT و آزمون نانواینداننتیشن استفاده شد.

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