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Electrophoretic encapsulation for slow release of vancomycin from perpendicular TiO₂ nanotubes grown on Ti6Al4V electrodes

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Abstract

Ordered perpendicular TiO₂ nanotubes (TNT) with 405 to 952 nm length and 60 to 90 nm diameter were grown via 40 to 120 min anodization of Ti6Al4V flat substrates. The samples were called TNT-40, −60, −80, −100, and −120. Vancomycin was loaded on the bare and anodized electrodes by separate immersion and electrophoretic (EP) deposition procedures. EP loading resulted in storage capacity of 5221.86 µg cm⁻² for TNT-80 which was much higher than 1036.75 µg cm⁻² of immersed sample. Drug release comprised of three stages: (i) burst release (78% for the bare, and 23% for the TNT-80 sample), (ii) gradual transport (21% for the bare, and 64% for the TNT-80 sample), and (iii) equilibrium. Transfer from all electrophoretically loaded TNT samples obeyed semi-infinite diffusion mechanism with a diffusion coefficient of 1.5 × 10⁻¹⁵ cm² s⁻¹. However, for bare specimens, external transfer prevailed. Anti-bacterial tests showed a bacteria-free region of 318 mm² on the drug-loaded anodized samples during the initial stage of drug release; while 254 mm² of bacteria-free region existed on the drug-loaded bare plates. Drug loading capacity of the anodized samples was enough for most biomedical applications. Ti6Al4V anodization proved a viable strategy for prosthesis drug loading and release.

1. Introduction

Eager interest in utilization of Ti base implants for hard tissue replacement has flourished during recent years [1–4]. Appropriateness of titanium base alloys has been ascertained by their biocompatibility, mechanical strength, low density and suitable electrochemical performance [2, 5]. Inadequate bioactivity has, however, been considered as a deplorable weakness in titanium and most other metallic alloys [6, 7]. Although the implants can provide many benefits, they may incur some problems such as infections and hence increase in the after treatment costs. A matter of concern has been local inflammation, bacterial infection (osteomyelitis) and bone destruction which impair bone healing [8, 9]. Compensation of these complications has been a challenging treatment of infections which has involved prolonged courses of high-dose intravenous and oral antibiotics for infection clearance [10].

Alternative methods should be considered so as to overcome the infection problem [11]. An appropriate strategy to conquer the limitations involved in the applications of implants has been the localized drug delivery. Thus, the flexibility during the drug releasing process and also the optimized parameters must be accounted for in the design of the TNTs-based drug-delivery systems [12].

In order to control release of the drugs, different TNTs-based systems’ strategies have been utilized [12–17]. Dip coating has been used as the simplest way of antibiotic loading on the manufactured implants [13–16]. Slow charging, long procedure, uncontrollable loading and poor drug penetration which result in unregulated release are some drawbacks of this procedure. To attain long-term drug delivery, Ordikhani et al [17] have electrodeposited drug-chitosan composite onto Ti substrate. They have announced the electrophoretic method as a single-step, facile and repeatable technique for loading glycopeptide antibiotic on implant.

Conventional drug usage have encountered many limitations like low drug solubility, weak selectivity, poor biodistribution, malicious side effects and uncontrolled pharmacokinetics. A remedy for these problems is local
delivery of drugs at implantation sites, often proposed as antibiotic-loaded bone cements which prevent implant-associated infections by decreasing the concentration of bacteria in the disturbed spots [18]. Nevertheless, unpredictable release of loaded medicine from bone cements has limited their usages in recent drug delivery systems.

With the advent of nanotechnology, there has been an increasing interest in application of biomedical nanomaterials [19]. Numerous studies have focused on the surface modification of Ti-implants for enhancement of their biological response to the bone-implant interface [20–23]. Recent studies have indicated that TNT coatings could improve bone integration due to nanotubular pores which open pathways to body fluids, nutrients, cellular anchoring and bone regeneration. As a result, tissue stabilization and long-term healing has been attained [24–27]. We have thought out vertically aligned TiO2 nanotubes fabricated on Ti-based substrate as an apt strategy for drug loading/delivering purposes [28, 29]. In this study, we focused on the TNT covered titanium samples loaded with vancomycin antibiotic in order to control possible infections. Well controlled TNT arrays of proper chemistry and specific area can show excellent biocompatibility plus bioactivity for simultaneous implantation and drug delivery/release strategy. We used electrophoretic deposition to force drug into vertical array holes.

No scheme has so far been developed on proficiencies in carrier storage-capacity manipulation concurrent with speeds of drug-loading and drug-release. Most previous works have focused on sole capacity enlargement of the carriers. The anodization/electrophoretic method used here proves reliable for three fold improvement of capacity enhancement, prime property preservation and long-term gradual release. We expect storage enhancement and release rate controllability be considered as a potential improvement in future drug delivery studies. Aligned vertical TNT arrays show good potential for holding vancomycin antibiotic as well as proffering timely release rates.

2. Experimental procedure

2.1. Sample preparation

Ti6Al4V substrates of 1 mm thickness were polished by various SiC emery papers and alumina powder (~200 nm) suspension. The shiny plates were sonicated in acetone and ethanol for ~10 min and subsequently rinsed in deionized water. The substrates were then dried in a stream of nitrogen.

2.2. Production of TNT arrays

Before anodization, the shiny Ti6Al4V sheets washed using H2O containing 1 Vol% HF solution for 10 s to clean and activate the polished samples. Then, the samples were washed by deionized water and kept for the next step. Ti6Al4V substrates were positioned in anode of a two electrode electrochemical cell. A stainless steel (SS 316L) plate was applied as cathode. The electrolyte contained 0.5 wt% NH4F and 1 M (NH4)2SO4 and a cell potential of 25 V was applied by a DC power supplier (SL 2/25 PCS, Iran). Anodization was carried out at room temperature for 40, 60, 80, 100 and 120 min. Current signals were recorded during the anodization by a digital ammeter. The anodized plates were dried at 120 °C and heated up to 480 °C with the rate of 3 °C min−1. For transformation of amorphous TiO2 to anatase, the samples were held at 480 °C for 2 h based on the information obtained from the literature [30, 31].

2.3. Microstructure and phase analysis

Microstructural investigation, thickness measurements and phase analysis of the nanotube arrays were performed by field emission scanning electron microscope (FE-SEM, Mira 3-XMU, Česká Republika) and x-ray diffractometer (XRD; D6792, Philips, Netherlands) using Cu–Kα radiation (λ = 1.54 Å) in the diffraction angle range of 20–90° (scan speed: 1 s/step; increment: 0.10°).

2.4. Drug loading procedure

2.4.1. Standardization of drug measurement method

For calculating the amount of drug release, a standard absorbance/concentration curve was needed. Different concentrations of vancomycin in PBS solutions were, therefore, prepared. A UV-Visible array spectrophotometer (PhotonixAr 2015, made of Iran) measured the quantity of vancomycin when released in PBS. A wavelength range of 200 nm to 400 nm was used. Figure 1(a) illustrates the absorbance spectrum for vancomycin in PBS. The absorbance peak of vancomycin occurred at 280 nm, which demonstrates the soundness of the UV-Vis method to some extent. Different concentration measurements resulted in a standard curve. Each sample was analyzed by UV-Vis two times per day. Three different analyses were taken for each sample every time. The validation experiments were continued for three days. Each sample was analyzed 18 times in three days. Finally, the average of the obtained results was reported. The linearity of absorbance values

2.4.2. Monitoring and measuring the quantity of drug release

The drug release from the TNT coatings was monitored for a period of 10 and 20 days using UV-Visible array spectrophotometer. For that purpose, the samples were immersed in PBS for three days. After each day, the samples were removed and the absorbance of released vancomycin was measured at 280 nm. The average of the obtained absorbance was reported. The percentage of drug release in each day was calculated using the following equation:

\[
\text{Percentage of drug release} = \frac{\text{Absorbance of released drug}}{\text{Absorbance of initial drug}} \times 100\%
\]
versus the standard prepared PBS-based solutions containing different vancomycin concentrations gave a standard curve. The absorbance was plotted against the concentration. The regression line function and correlation coefficient ($R^2$) were calculated. Figure 1(b) (newly added in the manuscript) shows the obtained standard curve. According to figure 1(b) (newly added in the manuscript), vancomycin concentration (drug release amount) could be calculated considering the obtained absorbance peak from UV-Vis spectroscopy.

### 2.4.2. Electrophoretic drug deposition

Magnetic stirring of $2 \text{ g l}^{-1}$ vancomycin (Dana Tabriz; Iran) with 1% (v/v) glacial acetic acid (Merck, Germany) for 30 min resulted in a well dispersed aqueous solution ready for loading on TNT arrays by electrophoretic deposition (EPD). Metrohm® 827 pH Lab Meter (Metrohm, Switzerland) recorded $2.5 \pm 0.05 \text{ pH}$. Two types of cathodes were used: the untreated and the anodized Ti6Al4V plates. The counter electrode was another Ti6Al4V plate. The EPD process was conducted for 10 min at potential of $10 \text{ V cm}^{-2}$ by a power supply device (Payapajooehsh Co., Iran) to achieve deep penetration. Drug loaded samples were dried and placed in a desiccator for further investigations.

### 2.5. Drug release measurements

Drug loaded implants were contacted with phosphate-buffered saline (PBS) inside a well-controlled autoclave of 7.4 pH and 37 °C. Released drug was determined by ultraviolet-visible spectroscopy (Pishropajoohesh Co., Iran) analysis of the saline. Measurements were taken at specific intervals in order to monitor the prolonged release into PBS. An aliquot of 3 ml was placed in a quartz cuvette. Absorbance was measured at 280 nm and the released amount of drug concentration was calculated based on pre-constructed calibration curves (figure 1(b)). Measurements were repeated for three times and the average was used as result of the test. Percent release was calculated by dividing the amount of vancomycin entering into the buffer by the total release to the end of the test. The release profile of each experimental set was plotted against time with indication of both burst and delayed discharges.

### 2.6. Antibacterial activity assessment

The culture medium used in this study was Mueller Hinton Agar (MHA) at adjusted pH of 7.2–7.4. The solution was sterilized and spread onto the plates. First, sterile normal saline was added to the culture tube and two colonies were brought from new cultured bacteria of blood agar. To match a 0.5 McFarland turbidity standard, the culture was diluted by saline ($0.5 \text{ McFarland} = 1.5 \times 10^8 \text{ CFU/ml}$). After that, swabs were placed into the bacterial suspension and cultured on the plate having Mueller Hinton Agar. Using swab, Mueller–Hinton Agar plate was streaked to create a bacterial lawn. Plates were maintained under hood for 10 min. The metallic samples coated with vancomycin were kept under UV for 30 min. After that, the metallic samples were set 2 cm apart from each other and 1.5 cm away from walls of the plates and incubated at 37 °C. Afterwards, the plates were removed and the diameter of the zone was measured by a ruler. At the last step, plates were again moved to incubation at 37 °C and after 18 h the results were observed.
3. 3. Results and discussion

3.1. Phase analysis, morphology and microstructure of TNT arrays

FE-SEM micrographs of the nanotubular TiO2 arrays formed on Ti6Al4V samples are illustrated in Figure 2. According to the micrographs, porous oxide layers produced by 100 and 120 min anodization exhibit irregular islands scattered on the TNT-covered surfaces. Presence of irregular islands indicates possible variation of the electrolyte conditions (i.e. pH and temperature) at too long anodization trials.

Reactions (1)–(3) indicate formation steps of TiO2 nanotube layer in presence of the fluoride containing electrolyte.

\[
\begin{align*}
\text{Ti} & \rightarrow \text{Ti}^{4+} + 4e^- \quad (1) \\
\text{Ti} + 2\text{H}_2\text{O} & \rightarrow \text{TiO}_2 + 4\text{H}^+ + 4e^- \quad (2A) \\
\text{Ti}^{4+} + 4\text{H}_2\text{O} & \rightarrow \text{Ti(OH)}_4 + 4\text{H}^+ \quad (2B) \\
\text{Ti(OH)}_4 & \rightarrow \text{TiO}_2 + 2\text{H}_2\text{O} \quad (3)
\end{align*}
\]

Hydrolysis rate increases with pH resulting in hydrous titanic oxide precipitation on the nanotube ends which reduces vertical nanotube growth rate. Formation of irregular nanotube islands start at this stage.

Figure 3 illustrates XRD pattern of the anodized Ti6Al4V plate. As is seen, the oxide layer consists of TiO2 anatase phase which seems resulted from amorphous oxide transformation at 480 °C heat treatment temperature (equation 4).

\[
\text{TiO}_2(\text{Amorphous}) \xrightarrow{480^\circ\text{C}} \text{TiO}_2(\text{Anatase}) \quad (4)
\]

The sharp peaks of titania layer could attribute to highly crystalline structure of the oxide phase. The appearance of the substrate peaks indicates the formation of a thin anodic oxide array.

3.2. Electrophoretic deposition

Vancomycin capacity of Ti6Al4V samples determined by UV-Vis spectroscopy is tabulated in Table 1. According to the table, the anodized samples can receive 1036.75 μg cm⁻² of the vancomycin by immersion. This value is almost quadrupled by electrophoretic method in TNT-60 sample. In order to compare the results of immersion with electrophoretic method, the amount of loaded drug was determined for two similar samples of TNT-80. It was seen that TNT-80 receives around 5 times greater drug than immersed sample.

Figure 4 illustrates a schematic representation of drug loading for TNT-coated Ti6Al4V by immersion route in comparison with EPD method. As is seen from the immersion method, drug molecules (left section) move downwards under gravitational force and deposit onto the TNTs. However, many forces such as drug aggregation barrier, neighbouring molecules and nanotube forces drive drug molecules upwards. Hence, drug molecules are constantly under opposite forces; many of them applied to the drug molecules in inappropriate directions. It is worth noting that if gravitational force assists molecules to overcome opposing forces and results...
in precipitation of drug molecules on the substrate surface, the nanotube walls reduce and prevent more drug movement and deposition. It could prevent entering the drug molecules into the nanotubes and exclusively lead to the accumulation of drug on the surface. This effect makes free-drug and empty nanotubes, which results in the reduction of drug loading.

Figure 3. XRD pattern of the anodized Ti6Al4V substrate showing TiO$_2$ anatase phase.

Table 1. Loading capacity of untreated and anodized samples by immersion method and electrophoretic deposition for vancomycin antibiotic. One sample was loaded by immersion.

| Surface treatment | — | Anodization |
|-------------------|---|-------------|
| Anodization time (min) | — | 40 | 60 | 80 | 100 | 120 |
| Drug loading method | EP | EP | EP | EP | Immersion | EP | EP |
| Sample name | Bare | TNT-40 | TNT-60 | TNT-80 | TNT-80-Im | TNT-100 | TNT-120 |
| Releasing Capacity (Effective Drug Loading amount) ($\mu g \ cm^{-2}$) | 854.75 | 3220.00 | 4392.98 | 5221.86 | 1036.75 | 4223.75 | 4085.33 |

Figure 4. Schematic representation of immersion (left side) and electrophoretic (right side) drug loading mechanisms.
Drug molecules are subject to different forces in the electrophoretic deposition method. The forces resulting from neighboring molecules as a barrier to the drug deposition affect drug molecules during the loading. The role of other forces could be different. There is a large electric force field to the inside of nanotubes (cathode) due to the applied potential. This force can easily overcome the opposing forces and causes the entering of a large amount of drug into the nanotubes. It is interesting to point out that the interaction result of wall barrier and gravity causes the drug molecules to separate from the nanotube walls and fall into the path of the electric field force. The molecules move to the cathode and fill the nanotubes.

On the other hand, it should be emphasized that the anodic TNT semiconductor coating is much thicker than the natural (2–5 nm) oxide layer formed on the Ti surface. Additionally, the surface of anodic titanium oxide (TNTs) consists of oxygen and titanium dangling bonds. Therefore, various ionic species such as H\(^+\) and OH\(^-\) ions of anodization electrolyte could be adsorbed/chemisorbed to the anodic TiO\(_2\) layer in a curvature-dependent route. Since the TiO\(_2\) layer connected to its metallic Ti substrate, conduction electrons transfer from the Ti substrate to the thick TiO\(_2\) coating layer. This electron transfer from the metallic Ti substrate to the TiO\(_2\) layer causes the accumulation of negative charges in the nanotube walls. Hence, an attractive force between negatively charged nanotube walls (inner green region of nanotubes in figure 4) and drug molecules occurs. Opposite charges attraction and electric field forces result in drug molecules flow near the wall and into the nanotube, which could be demonstrated by principle transfer phenomena in tubes. A maximum drug deposition amount is the result of the electrical-assisted drug loading process of electrophoretic. As has been indicated in table 1, the electrophoretic loading resulted in a storage capacity of 5221.86 \(\mu\)g cm\(^{-2}\) for TNT-80, which is much higher than 1036.75 \(\mu\)g cm\(^{-2}\) of the immersed sample.

3.3. Drug-release

Figure 5 shows cumulative drug release from anodized and bare samples which have been loaded with vancomycin. Three distinctive stages named (i) burst, (ii) gradual, and (iii) equilibrium can be recognized for drug release from all samples. Stage (i) occurs when drug enters the phosphate buffer solution immediately after immersion. Stage (ii) attributes to discharge of the drug embedded within the TNT tube arrays. Stage (iii) completes when release has come to the end (i.e. equilibrium state).

It should be noted that during the first hour of the release test, the TNT-80 sample discharged 84% of the drug it had received by immersion; while the same sample released only 23% of its electrophoretically loaded drug. Vancomycin release continued from the latter even after 28 days. Therefore, the electrophoretic loading not only controls the first burst drug release, but even supplies long-lasting gradual release from the TNT arrays. This behavior is attributed to the full loading of the lengthy nanotubes present on the anodized samples by the electromotive pressure exerted in the electrophoresis process figure 6 indicates a comparison of cumulative drug release from TNT-80 loaded by immersion and the electrophoretic method.
According to figure 3, about 78% of the electrophoretically loaded drug on the control sample \( (854.75 \, \mu\text{g} \, \text{cm}^{-2}) \) was released during the first hour. The remaining drug continued to diffuse into the PBS in four successive days. Burst release for TNT-80, TNT-60 and TNT-40 samples was much lower: 22, 27 and 38%, respectively. The difference seems due to the length difference of the nanotubes which allow various penetration depths for the vancomycin molecules into the TNT pores due to the electrophoretic forces. Penetration of vancomycin molecules into TNT-120 and TNT-100 pores retarded, however, because of titanium hydroxide blockage of the nanotube holes which also caused the irregular TNT morphology observed in figure 1. Release percentage in these cases reduced to 48 and 57%, respectively.

Anodizing time was an important parameter controlling the drug release rate. Extended drug release could be achieved in samples having longer nanotube arrays. For control samples, almost all drug escaped in four days. While liberation time for TNT-100 and TNT-120 was about 28 days. Much greater release times were obtained with TNT-60 and TNT-80 samples. This change was because of both higher capacity and longer nanotube arrays. Longer nanotubes let greater drug loading and lower release rates. For TNT-100 and TNT-120, due to the irregular morphology (figure 2) and partial nanotubes blockage, the release could take no longer than 28 days. From FE-SEM images of figure 1 and the drug release curves of figure 3, it was concluded that uniformly grown perpendicular TNT arrays of 80 min anodized samples provided the looked-for long-time drug release condition.

### 3.4. Anti-bacterial tests

One of the most important steps in the evolution of orthopedic implants is the attachment of bacteria to their surfaces which is why many attempts have been focused on prevention of such adherence. In order to prevent infections related to the implant, vancomycin was selected as antibiotic to reduce the concentration of bacteria and to inhibit bacteria adherence. Drug loaded TNTs could release vancomycin to kill *Staphylococcus aureus* bacterial strains and to clear area around the specimens and form an inhibition zone. In the drug-free specimens, no antibacterial effect can be seen in Ti6Al4V specimen without any drug being loaded, as expected. It is noteworthy to mention that the percentage of the burst release in the untreated Ti6Al4V was considerably more than titanium oxide nanotubes. But the amount of released drug from the nanotube arrays in the initial hours \( (1201.02 \, \mu\text{g} \, \text{cm}^{-2}) \) was more than the total amount from the control sample \( (854.75 \, \mu\text{g} \, \text{cm}^{-2}) \). It is seen that the bacteria free area dramatically enhances with drug containing anodized samples.

![Figure 6. Comparison of cumulative drug release for the drug loaded TNT-80 processed using (∎) immersion and (●) electrophoretic method.](image-url)
The amount of drug needed in the initial hours for prevention of the bacteria adherence is more than 8 \( (\mu g \ cm^{-2}) \). The amount of released drug from TNT-80 on the first hour is 1201.02 \( (\mu g \ cm^{-2}) \). This means that by having 125 ml of fluid around an implant, drug concentration would be 9.6 \( (\mu g \ cm^{-2}) \) which is enough for prevention of bacteria adherence. This concentration is only 5.47 \( (\mu g \ cm^{-2}) \) for untreated sample. This shows a higher amount of burst release in the anodized samples which is in a full accordance with the results from drug release graphs. Consequently, it can be concluded that the identity of the drug remains unchanged during electrophoretic deposition. This indicates an effective antibacterial capability of the perpendicular TNT arrays which is introduced as a suitable choice for coating of the bone implants. Deposition of a hydroxyapatite layer on the TNT sample can further improve the gradual release of the loaded drug from the imbedded implant.

4. Conclusions

TiO\(_2\) nanotube coatings were successfully synthesized using anodizing process for biomedical applications. The length of the nanotubes increased from 400 to 1000 nm by the anodizing time enhancement from 40 to 120 min. Metal hydrolysis at long anodizing times produced uneven surface topography of the coatings which reduced drug capacity and vancomycin release duration to some extent.

Electrophoretic method was used to load vancomycin as an anti-bacterial drug into the nanotube arrays. The loading capacity and releasing rate of the vancomycin were determined by UV–vis spectroscopy. Electrophoretic method was proved capable for controlled large loading of drug onto perpendicular TNT covered Ti6Al4V samples. Drug capacity of TNT-80 on Ti6Al4V was six times greater than the control bare sample. Results exhibited considerable vancomycin chemical stability during electrophoretic procedure.

Effectiveness of the applied method was approved by the Agar diffusion assay against Gram-positive S. aureus bacteria. Nanotube arrays caused less burst at primary step of implantation and long-term (more than 28 days) drug release duration. Anti-bacterial in vitro tests demonstrated satisfactory drug performance.

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Figure 7. Antibacterial test for untreated Ti6Al4V alloy (a) with drug and (b) without drug load, and anodized surface containing TiO\(_2\) nanotubes (c) without and (d) with drug load.
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