Central composite experimental design for the investigation of the influence of various conditions of anodisation on the amount of antimicrobial agent loaded on TiO_2 nanotubes

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TiO_2 nanotubes on Ti substrate were prepared by electrochemical anodisation method. Central composite experimental design was used to design the experiments to investigate the influence of some variables on the loading of antibiotic gentamicin. A full quadratic model was used in order to find out the influence of the variables and their possible interactions and finally to estimate the optimum conditions. Titanium rods were anodised in ethylene glycol containing different amounts of ammonium fluoride and water at different voltages and different anodising times. Antibacterial activity of the nanotubes prepared under the optimum condition was evaluated by a culture test with *Staphylococcus aureus* bacteria.

1. Introduction: Orthopaedic implant surgeries often cause bacterial infections that can lead to failure of the surgery with an accompanying high mortality rate [1, 2]. Bacterial strains, such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are usually the causative organisms for these infections [3] and they can be observed even one year after surgery [4]. For treatment of these infections, either an implant replacement or antibiotic therapy is adopted. Administration of antibiotic either orally or by injection after implant surgery is not efficient as the antibiotic has to be distributed throughout the body, resulting in the delivery of lower effective doses at the infection site [2, 3, 5, 6]. Therefore, local drug delivery with implants is a very interesting research area that can overcome these difficulties. Various studies have been conducted to develop systems based on either antibiotic-loaded implants [7–11], or coating of implant surfaces with biodegradable polymers loaded with drugs [12–14].

Titanium (Ti) and its alloys are extensively used as biomaterials for implantation because of their good mechanical properties, high corrosion resistance and high biocompatibility [15]. Among them, titania nanotube (TNT) arrays, generated on Ti surfaces by electrochemical anodisation in fluoride containing electrolyte, are recognised as a more sophisticated approach [16, 17]. Bone cells, that is, osteoblasts, can grow faster on nanoscale surfaces, such as TNT and therefore the ability for new bone formation on them is higher than on other materials [18–22]. The increased surface area of these tube formations can be loaded with bioactive or medical agents and hence can serve as in situ drug delivery systems [23–26]. This approach has significant advantages compared to systemic medical treatment.

Conditions for anodisation of Ti, such as anodisation time, applied voltage, temperature, Ti foil roughness and calcination parameters and electrolyte composition including fluoride concentration, type of solvent, water content, pH, viscosity, conductivity and organic additives, are effective for varying the morphological structure of anodised TiO_2 nanotubes including diameter and height of the nanotubes [27–29]. Since the morphological structure of the nanotubes influences the amount of drug loading and release, one can change the condition of anodisation to create nanotubes that can be loaded with the maximum amount of drugs. This study focuses on the preparation of Ti nanotube arrays possessing good capacity for loading of gentamicin (as an illustrative antibiotic) using experimental design methodology. In this Letter, the anodisation of Ti rods has been studied in ethylene glycol electrolyte by varying the amount of ammonium fluoride and water content under different voltages and anodisation time. Conditions for the preparation of TiO_2 nanotube arrays with maximum ability to load gentamicin were optimised by using central composite design (CCD). The nanotube array was characterised by scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis.

2. Material and methods

2.1. Materials and apparatus: Ti rods (Ti 99.9%; height = 1 cm; diameter = 6 mm) were purchased from Atra Orthoped, Iran. All samples were mechanically polished prior to anodisation and sonicated sequentially in acetone, ethanol and distilled water for 15 min each, etched in an $HF/HNO_3/H_2O$ solution (volumetric ratio 1:4:5, respectively), washed in distilled water and finally dried using nitrogen gas. The reagent, *o*-phthalaldehyde, was purchased from Sigma-Aldrich and all other chemicals were of analytical grade.

DC power supply system (Agilent Technologies N5752A, 600 V/1.3 A, 780 W) was used for the anodisation of Ti rods. Determination of gentamicin was done with a WPA/Biowave II spectrophotometer.

2.2. Preparation of TiO_2 nanotubes: Ti rods were anodised in a two-electrode electrochemical set-up, using Ti as the cathode in ethylene glycol electrolyte with different amounts of water and NH_4F. Each parameter for the preparation had an effect on the size of the nanotubes; for example, the length of the TNT arrays could be controlled by using different anodisation times [30]. Different voltages and preparation times were therefore used for the preparation of TNTs with various sizes. The samples obtained were thoroughly washed with distilled water, followed by gentle ultrasonication for 10 s, then dried in air and calcinated at 450°C for 2 h to transform amorphous titania to anatase titania [31].

2.3. Experimental design and method of analysis: An MS Excel add-in software named Essential Regression and Experimental Design for Chemists and Engineers (EREGRESS), [32, 33] was

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Table 1 Independent variables at five levels

Variable name		Coded factor levels				
		−1	−0.5	0	+0.5	+1
F_1	water, %v/v	0.3	10.22	20.15	30.08	40
F_2	NH_4F, %w/v	0.024	0.318	0.612	0.906	1.2
F_3	voltage, v	5	41.25	77.5	113.75	150
F_4	time, h	0.25	3.44	6.62	9.81	13

used to design the experiments, obtain the optimum conditions and to determine the effects of independent factors on the response. The four factors used for determining the optimum conditions were the amount of water (F1) and NH_4F (F2) in electrolyte, voltage (F3) and anodisation time (F4). These four independent variables were studied at five levels with four repeats at the central point. For each of the four studied variables, high coded values and low coded values were selected as +1 and −1, respectively, to construct an orthogonal design (Table 1).

Using EREGRESS and polynomial equations, the response and central design for a particular response were obtained. For the four factorial model investigated in this Letter, the second-order polynomial equation including linear, quadratic and cross-terms can be presented as

$$\begin{aligned}\text{Response} = {} & b_0 + b_1 f_1 + b_2 f_2 + b_3 f_3 + b_4 f_4 \\ & + b_{11} f_1^2 + b_{22} f_2^2 + b_{33} f_3^2 + b_{44} f_4^2 + b_{12} f_1 f_2 \\ & + b_{13} f_1 f_3 + b_{14} f_1 f_4 + b_{23} f_2 f_3 + b_{24} f_2 f_4 + b_{34} f_3 f_4 \end{aligned} \quad (1)$$

where F1–F4 are the variable parameters and b_0–b_{34} are the coefficient values obtained through multiple linear regressions. Response surface methodologies graphically illustrate relationships between parameters and response and are used to obtain an exact optimum [33, 34–38].

2.4. Filling of nanotubes: A simplified lyophilisation method was used for filling of TNTs [38–40]. TNT surfaces were cleaned using deionised water prior to loading of the antibiotic. A measure of 2.5 ml of gentamicin solution (1% gentamicin in phosphate-buffered saline (PBS)) was pipetted onto the nanotube surface and gently spread. The surface was then allowed to dry under vacuum (100 mPa) at room temperature for 2 h. This step was repeated 8 times to load 200 µg of the antibiotic. After the final drying step, the surface was rinsed quickly with 1 ml of PBS to remove any excess drug. The rinse solutions were analysed to determine the amount of drug loaded onto TNTs.

2.5. Release of antibiotic from nanotubes: The TNTs were immersed in 1 ml of PBS at room temperature with orbital shaking at 70 rpm to release gentamicin from the nanotubes. Samples were taken after specific intervals of time to determine the release kinetics and replaced with fresh PBS (1 ml) every time. Samples were collected periodically for up to 6 h. The samples were analysed to determine the antibiotic content in solution using a colourimetric assay described previously [38]. In brief, the *o*-phthalaldehyde reagent was prepared by adding 2.5 g of *o*-phthalaldehyde, 62.5 ml of methanol and 3 ml of 2-mercaptoethanol to 560 ml of sodium borate (0.04 M) in distilled water solution. The reagent was kept in a brown bottle in darkness for at least 24 h before use. This reagent was used for only 3 days after preparation, as it degrades after that. The collected gentamicin solution, *o*-phthalaldehyde reagent and isopropanol (to avoid precipitation of the products formed) were mixed in the same proportion and stored for 30 min at room temperature before UV measurement. The amino groups of gentamicin react with *o*-phthalaldehyde and the absorbance of the chromophoric product was measured at 332 nm. A standard curve with known concentrations of gentamicin was used to determine the unknown concentrations.

The release curves were fitted to the Korsmeyer–Peppas model

$$\frac{M_t}{M_\infty} = kt^n$$

where M_t is the accumulated mass released at time t, M_∞ is the accumulated mass released at $t \to \infty$, K is the kinetic constant and n is the release exponent [38], which is characteristic for different release mechanisms. In the case of thin films, the release is diffusion-controlled when $n \le 0.5$ and swelling or degradation-controlled when $n \ge 1$. For $0.5 \le n \le 1$ the release kinetics is a combination of both mechanisms.

2.6. Characterisation: The morphology and crystallinity of the TNTs were characterised by field-emission scanning electron microscopy (FESEM, Mira 3-XMU, Razi Metallurgy Research center, Tehran, Iran) and energy-dispersive X-ray spectroscopy (EDX) (VEGA\\TESCAN-XMU, Razi Metallurgy Research Center, Tehran, Iran), respectively.

2.7. Antibacterial activity test: *S. aureus* bacteria (Gram-positive) was selected to evaluate the antibacterial activity of the nanotubes. A measure of 1 ml of bacterial solution in a culture environment was taken by a sampler and added to 10 ml of tris-buffered saline (TBS) culture environment. Then, this solution was incubated and shaken at 30°C for 24 h in a shaker incubator. A measure of 5 ml of this bacterial solution was added to 500 ml of TBS culture environment and again incubated at 30°C for another 24 h. The bacterial solution was then centrifuged for 10 min at 3000 rpm. The supernatant was taken out and the sediment was rinsed thrice with sterile physiological serum. Then McFarland bacteria solution (6×10^8 bacteria/ml) was prepared and the implant samples were dipped in this solution for 24 h at 30°C to calculate the number of bacterial colonies. The antibacterial rate was calculated according to the following equation

$$\text{Antibacterial rate} \frac{N_c - N_s}{N_c} \times 100$$

where N_c and N_s express the number of bacterial colonies on the control and on the sample, respectively.

3. Results and discussion

3.1. Experimental design: CCD was applied to (i) estimate the correlation of the four independent factors, with five levels for each factor; (ii) study the effect of concentration of water and ammonium fluoride along with voltage and time of anodisation on the amount of gentamicin loaded onto the TNTs and (iii) explore the variables that have a higher impact on the amount of gentamicin loaded onto the TNTs.

To find the important factors and build a model to optimise the procedure, we started with a full quadratic model including all terms as in (1). To obtain a simple but realistic model, the insignificant terms ($p > 0.05$) were eliminated from the model through 'backwards elimination' process. The regression equation coefficients were calculated and data were fitted to a second-order polynomial equation for the loading of gentamicin onto the TNTs

$$\begin{aligned} R = {} & b_0 + b_1 V + b_2(\text{NH}_4\text{F})^2 + b_3(\text{NH}_4\text{F})(\text{time}) \\ & + b_4(\text{NH}_4\text{F})(\text{water}) + b_5(V)^2 + b_6(\text{time})^2 + b_7(\text{water})^2 \end{aligned} \quad (2)$$

Table 2 Some characteristics of the constructed models

	Regression	Coefficient	Value
$\|R\|$	0.960	$b0$	25.43
R^2	0.922	$b1$	1.463
R^2 adjusted	0.902	$b2$	−175.20
standard error	10.02	$b3$	32.98
number of points	36	$b4$	9.605
PRESS	3817.83	$b5$	−0.01041
R^2 for prediction	0.894	$b6$	−1.124
		$b7$	−0.116

By the elimination of the insignificant terms of (1) from the constructed model, calibration R^2 decreased to 0.922 but adjusted R^2(R^2_{adj}), and R^2 of prediction (R^2_{pred}) increased to 0.902 and 0.894, respectively. The characteristics of this constructed model, including R^2 values, PRESS, standard error and significant linear, quadratic and interaction coefficients, are shown in Table 2.

3.2. Response surface method and selection of optimum conditions: To gain insight into the effect of each variable, the three-dimensional (3D) plots for the predicted responses were also formed, based on the model function to analyse the change of response surface. Some of the response surface plots are shown in Fig. 1, which shows the 3D plots of loaded drug percentage against the pairs of variables whereas two other variables were kept at the centre levels. As shown in Fig. 1, there is a non-linear relationship between the response and the variables F1–F4. From the coefficients represented in Table 2, it is clear that several linear, squared and interaction parameters are statistically significant. Using the response surfaces, the optimum conditions were obtained and the respective values have been tabulated in Table 3.

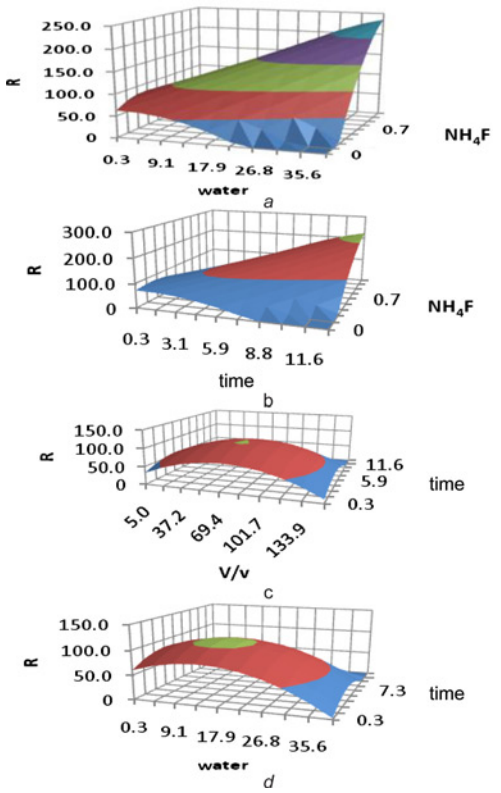

Figure 1 *Response surface plots (a–d)*

Table 3 Optimum conditions obtained by response surface modelling

Variable names	Optimum values	Selected values	
F_1	water, %v/v	9.1–20	10
F_2	NH_4F, %w/v	0.35–0.75	0.35
F_3	voltage, V	55–75	60
F_4	time, h	4.5–5.9	4.5

From the constructed models (the results of Table 2 and the response surfaces of Fig. 1), the following results can be concluded: voltage of anodisation significantly affects the proposed preconcentration method, both by linear and quadratic terms. In contrast, the effect of water and NH_4F percentage and time on the proposed method is quadratic. Moreover, the response surface shows that there are interactions between NH_4F percentage and time, as well as NH_4F percentage and water percentage.

3.3. Surface characterisation: After anodisation of the samples under different conditions, TiO_2 nanotubes were formed on most of the surfaces. As an example, Fig. 2 shows the surface of a sample that was anodised in ethylene glycol electrolyte under optimum conditions. The SEM image of the top surface of the TNT layer shows vertically aligned uniform nanotubes with a diameter of 140 ± 10 nm and wall thickness of 40 ± 5 nm. Since the cross-sectional images could not be taken because of the shape of the samples, the length of TNTs could not be determined.

EDX, used for element analysis, is shown in Fig. 3. As shown in the spectrum, there are Ti and O atoms on the surface of Ti samples. Data of EDX analysis are shown in Table 4.

3.4. Drug release: To release gentamicin from the nanotubes, the surfaces were immersed in 1 ml of PBS at room temperature with orbital shaking at 70 rpm. The PBS solution was taken out completely after specific intervals of time to determine the release kinetics and replaced with 1 ml of fresh PBS solution. Samples were collected periodically for up to 180 min and the concentration was determined using colorimetric assay. The anodised surfaces show a retarded release rate for the time-dependent release of gentamicin in relation to the total

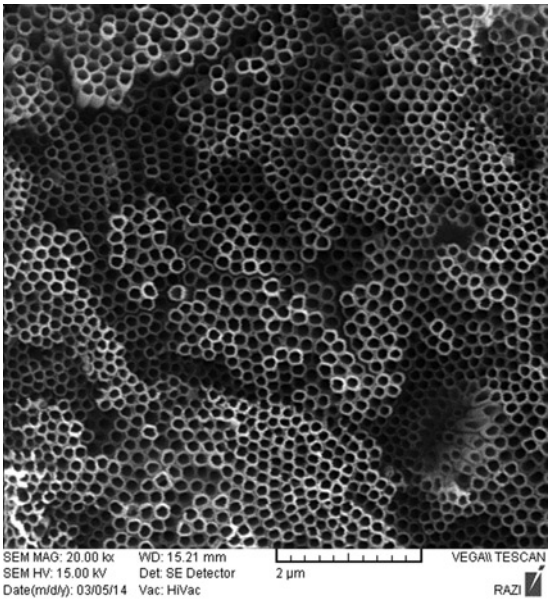

Figure 2 *SEM image of TNTs (conditions for anodisation: water 10%, NH_4F 0.35%, 60 V and 4.5 h)*

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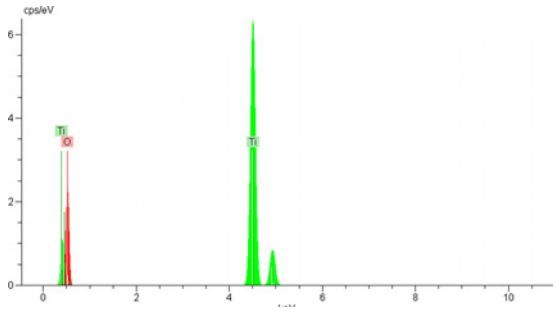

Figure 3 *EDX spectrum of TNTs*

Table 4 Data of EDX analysis of spectra: 1

Element	Series	Unn. C, wt%	Norm. C, wt%	Atom. C, at%
oxygen	*K*	32.62	38.07	64.79
Ti	*K*	53.05	61.93	35.21
total, %			85.7	

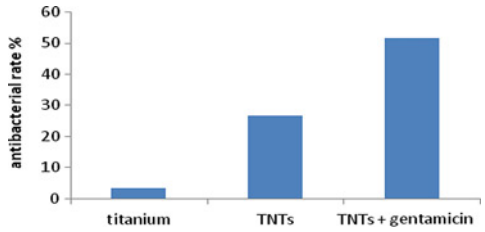

Figure 4 *Antibacterial rates of Ti, TiO_2 nanotubes (TNTs) and gentamicin-loaded TiO_2 nanotubes (TNTs + gentamicin)*

adsorbed amount; the kinetic constants *K* and the release exponent *n* obtained from the release curves of gentamicin from the nanotubes were 0.5213 and 0.1589, respectively.

3.5. Antibacterial activity: To evaluate the effects of gentamicin-loaded TiO_2 nanotubes on antibacterial activity, the antibacterial rates were measured for the nanotube samples with and without gentamicin and compared with Ti, as shown in Fig. 4. From Fig. 4, it can be seen that the antibiotic-loaded TiO_2 nanotube sample shows the highest antibacterial rate, about 51%, whereas the TiO_2 nanotube sample without the antibiotic shows about 26%, and the Ti sample shows the lowest.

4. Conclusion: The aims of this study were the preparation of nanotube arrays on Ti containing medical implant surfaces under different conditions (amount of water and NH_4F in electrolyte, voltage and time of anodisation) and the evaluation of their potential for storage and release of the model antibiotic, gentamicin. Results show that the voltage of anodisation significantly affects the proposed preconcentration method, both by linear and quadratic terms. In contrast, the effect of water and NH_4F percentage and time on the proposed method is quadratic. Moreover, the response surface shows that there are interactions between NH_4F percentage and time, as well as NH_4F percentage and water percentage.

In summary, the experiments show that nanotube arrays on medical implant materials fabricated under optimised conditions show a high potential for maximum loading with gentamicin and possess sustained antimicrobial activity against *S. aureus* bacteria.

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6 References

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