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Full Length Research Paper

The use of factorial design for proliferation assay of hFOB cells on different TiO_2 nanotube arrays

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The ordered TiO_2 nanotubes on titanium substrate were fabricated by anodization. The nanotubes are well aligned and organized into uniform arrays. The effects of anodization voltage on the oxide layer of morphology were investigated. The average diameter pore size is 20 and 88 nm at 5 and 20 V, respectively. In surface wettability, these TiO_2 nanotubes showed hydrophilic behavior by sessile drop technique. In the cell responses, cell proliferation was increasing time with decreasing nanotube diameter by methylthiazoletetrazolium assay (MTT assay).

Key words: Titanium, nanotubes, anodization.

INTRODUCTION

The skeletal system is integral for all physical function in the body. When bone is damaged, an implant is required to fasten, support or replace bone. Titanium and its alloy are widely used in dental and orthopedic filed because of their high mechanical strength, low weight and low toxicity. However, due to the bio-inert properties of a natural oxide layer, it is difficult to achieve a chemical bond with bone tissue and to form a new bond on the surface (Zhao et al., 2007). To improve biocompatibility, surface treatments are often used to modify the chemical and morphological properties of metal. Among the modification methods, the anodization is an effective method with simple equipment and low cost. The nano-tubes were prepared by anodization technique.

These nanotubes could increase the roughness of titanium surfaces and was similar to the natural roughness of human bone. The TiO_2 nanotube arrays have been fabricated in phosphoric acid solution with the addition of hydrofluoric acid by anodization (Yao et al., 2005). The surface wettability, which could be able to attract cells is an important factor. Hence, the cells tend to attach to hydrophilic surface (Zhao et al., 2007). Balaur et al. indicated that TiO2 nanotubes showed hydrophilic wetting behavior (Balaur et al., 2005).

In this study, we used anodization method to manufacture the nanotubes with different pore size and evaluated the cell response. Furthermore, we use the factorial design of experiments to observed TiO_2 nanotubes surface and cell responses changes in the different situations.

MATERIALS AND METHODS

Preparation of specimens

The TiO_2 nanotube arrays were prepared by anodized method. Medical grade titanium (ASTM F-67) was selected as substrates. The dimension of titanium substrate was 12.7 mm in diameter and 2 mm in thickness. The substrates were mechanically ground by silicon carbide papers and polished, then ultrasonically cleaned in acetone, ethanol and de-ionized water prior to anodization. The titanium nanotubes were constructed in a two-electrode electro-chemical cell with a DC power supply at 5 and 20 V, respectively. In electrochemical cell, the specimen was used as the anode and the platinum foil was used as the cathode. The electrolyte solution was phosphoric acid (H_3PO_4) solution with the addition of hydrofluoric acid (HF) solution. All experiments were carried out at 20°C. The surface morphologies were observed by field emission scanning electron microscope (FE-SEM). The nanotubes phase was identified by thin-film X-ray diffraction (TF-

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Table 1. Factorial design and observed values of process parameters.

Factors			Time (days)			
	Limits		7		14	
	Indicator variables		-1		+1	
Pore sizes (nm)	20	-1	0.1710 0.1590	0.1470	0.2800 0.2800	0.2690
	88	+1	0.1090 0.1310	0.1160	0.1770 0.2050	0.1950

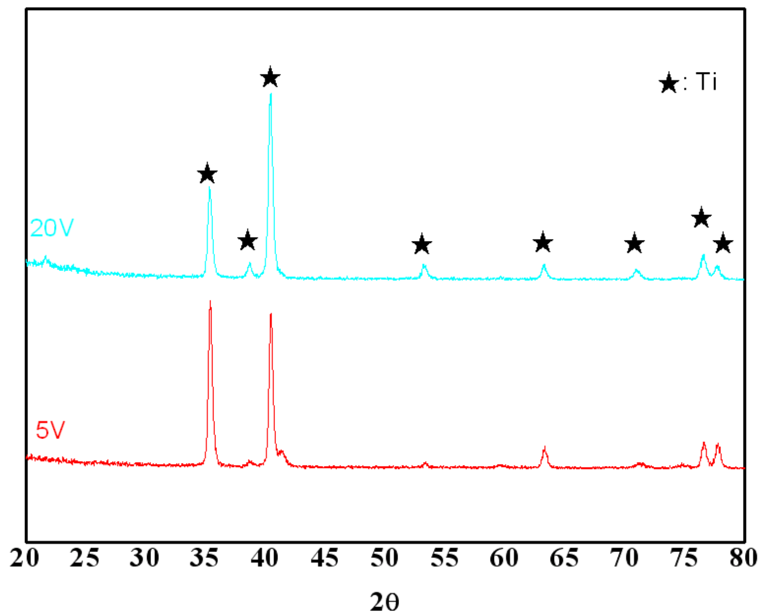


Figure 1. The TF-XRD pattern of the nanotube arrays on the titanium substrate.

XRD).

Cell responses by MTT assay

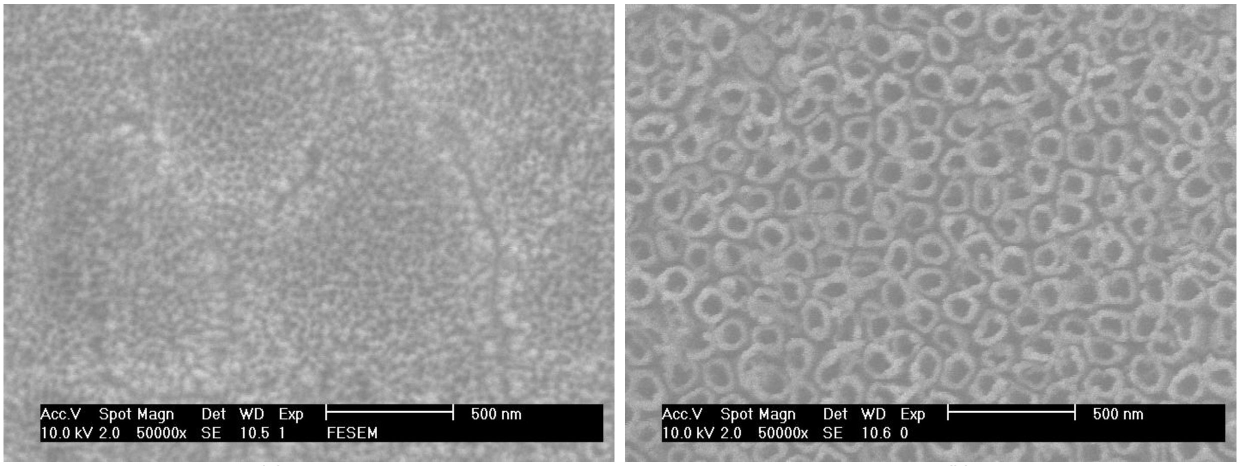
In the cell responses, the proliferation of hFOB cells was determined by methylthiazoletetrazolium assay (MTT assay). Each specimen put into culture well of the 24-well plate, then seeded with cells. The cells were cultured in a 37°C incubator with 5% CO_2. After 7 and 14 days of cell culture, the cell proliferation rate was determined by MTT assay.

Design of the experiment

Design of experiments is a structured and organized statistical method that is used to estimate the main effects and interaction factors and the response variables affect the outcome of modeling tools. This method could assess the feasibility study of biomedical for the implant surface nanostructures application. In the experiment, it was investigated that the cell responses with different cell growth time on the different diameters of TiO_2 nanotube arrays by the electrochemical anodic oxidation treatment. To accomplish this study, a model consisting of a 2^2 design (Montgomery, 2006) was selected and 3 times of replicates for corner points. Forming a test matrix with two factors each taking two levels which are high (1) and low (-1) level, as can be observed in Table 1.

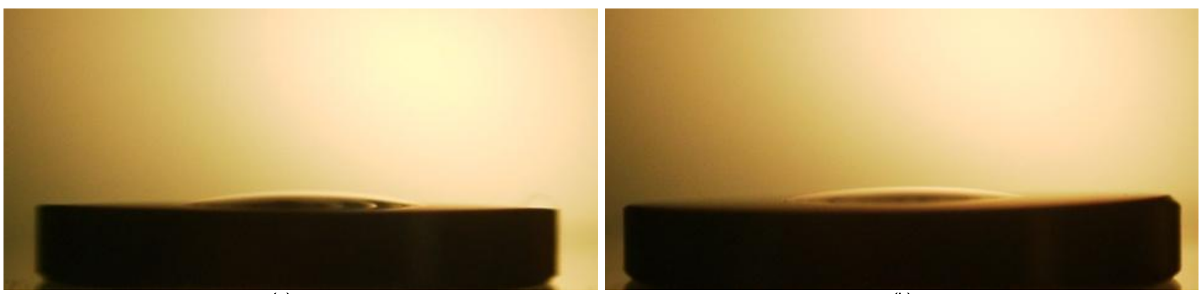
RESULTS AND DISCUSSION

The nanotube arrays on the titanium substrate grown at 5 and 20 V in the phosphoric acid solution with the addition of hydrofluoric acid. As shown in Figure 1, the phase of the nanotube arrays was identified by TF-XRD. The as-prepared nanotubes grown after the anodization processes were found to be composed of amorphous titanium oxide. Only Bragg from titanium substrate could



(a) (b)

Figure 2. The nanotube arrays of morphology: (a) 5 V and (b) 20 V.



(a) (b)

Figure 3. The contact angle of nanotube arrays surface: (a) 5 V and (b) 20 V.

be observed in the XRD. It indicated that the nanotube films were amorphous titanium oxide (TiO_2). This result is typical for the self-organized oxide films obtained after similar to anodization process at low voltages (Gong et al., 2001). Figure 2 shows SEM images of the self-organized nanotubes assay morphology. It is clear that indicated the tubes were homogeneous and high-ordered on the titanium substrate. The diameter average pore size of the tubes was about 20 nm at 5 V and 88 nm at 20 V. The current density of anodization process was similar fluctuations with Beranek and co-workers (Beranek et al., 2003). In wettability property, the contact angle of the tube films was very small by the sessile drop technique as shown in Figure 3. This phenomenon showed the nanotube films were hydrophilic surface. The cell likes hydrophilic surface (van Kooten et al., 1992). In the cell response, Table 1 shows the results of hFOB cells proliferation after 7 and 14 days of culturing by MTT assay as measured by counting cells. It is clear that indicated the highest cell numbers on the 20 nm diameter nanotubes and proliferation rates were increasing with decreasing nanotube diameter. The TiO_2 nanotubes of controlled diameter may provide a powerful tool to improve cell adhesion and cell proliferation (Park et al., 2007). By the way, the titanium nanotubes may serve as reservoir for such drugs for their controlled prolonged release.

The Aninwene et al. (2008) demonstrated that the drug-coated on titanium nanotubes surface can improve prolonged drug release. The titanium nanotubes was not only similar to the natural roughness of human bone but also store of drugs. Therefore, the titanium nanotubes are a multi-function structure. Equation 1 shows the results when fitting a polynomial model to describe the OD parameter, taking into account that variables with a P-value greater or equal to 0.05 are not statistically significant at the 95% or higher confidence level and Table 2 shows the variance analysis for the polynomial model:

$$OD = 0.18658 - 0.04775x\_1 - 0.03108x\_2 + 0.01092x\_1\,x\_2 \quad (1)$$

Where OD, x_1 and x_2 are normalised variables representing: optical displacement values, days and pore

Table 2. Analysis of variance for the process parameters [for OD (coded units)].

Source	SS	d.f.	MS	F-radio	P-value
Main effects	0.038955	2	0.0194774	152.17	0.000
2-Way Interactions	0.001430	1	0.0014301	11.17	0.010
Residual	0.000128	8	0.0001280		
Total	0.041409	11			

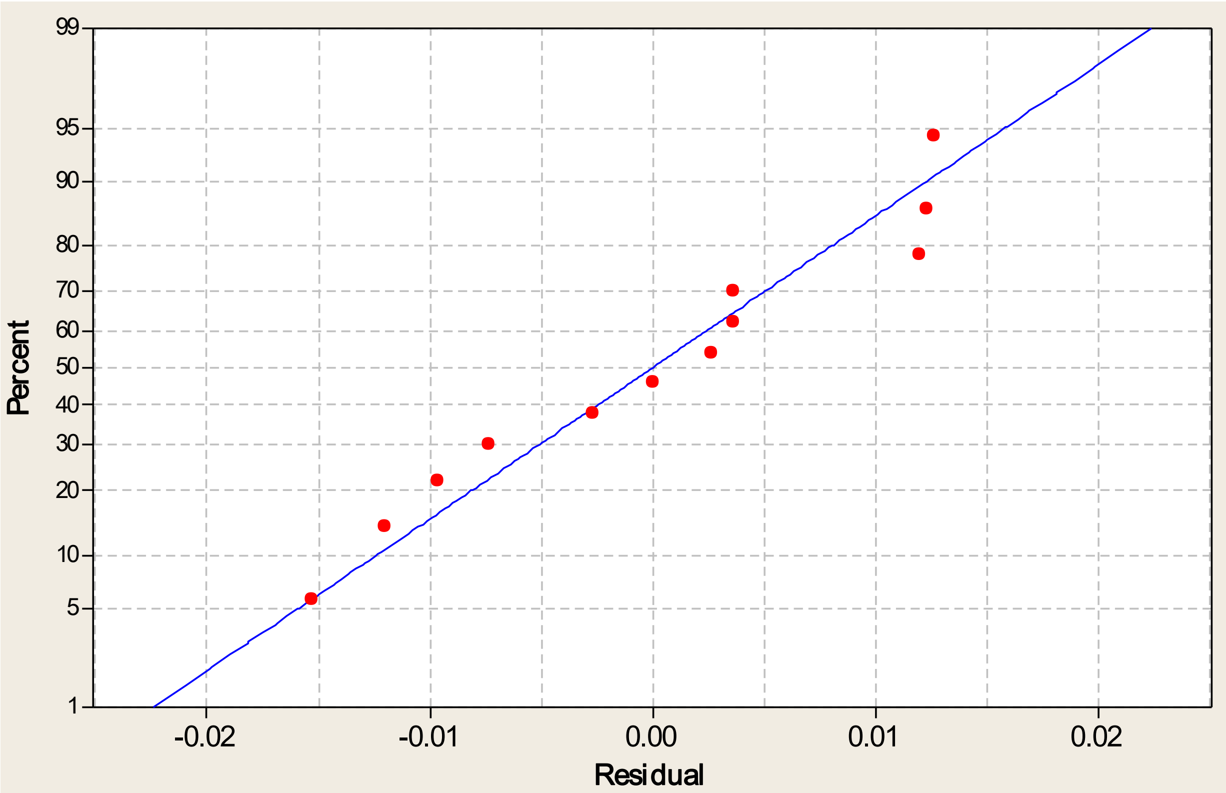


Figure 4. Normal probability plot for OD.

sizes.

The R-squared and adjusted R-squared statistic indicates that the polynomial model explains 94.44 and 96.60% of the variability in OD. Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level. Figures 4 and 5 represent the normal probability distribution plot and the residuals versus fits plot. Although, the high OD of Figure 5 is showed less variation, but these points of Figure 4 be showed normal probability distributions. Figure 6 is displayed in the significant level of 0.05, the two factors and interaction of both are significant. Figure 6 shows the estimated OD as a function of each experimental factor and Figure 8 shows the interaction plot for days and pore sizes. In each plot, the factor of concern is varied at its two levels, while all other factors remain unchanged at their central values. It can be observed that days and pore sizes negatively influence the OD parameter (Figure 7). Figure 9 shows the estimated OD parameter as a function of each of the normalised independent variables. Low of surface represents the value of the OD parameter. The other factors are held constant, where OD represents 'optical displacement' values, x_1 represents days and x_2 represents pore sizes. Figure 10 shows contours for each of the response surfaces. From this figure, it may be easily deduced that the OD parameter improves with the increase of days and pore sizes.

Conclusions

The properties of TiO_2 nanotube arrays were characterized by TF-XRD, SEM, wettability and factorial

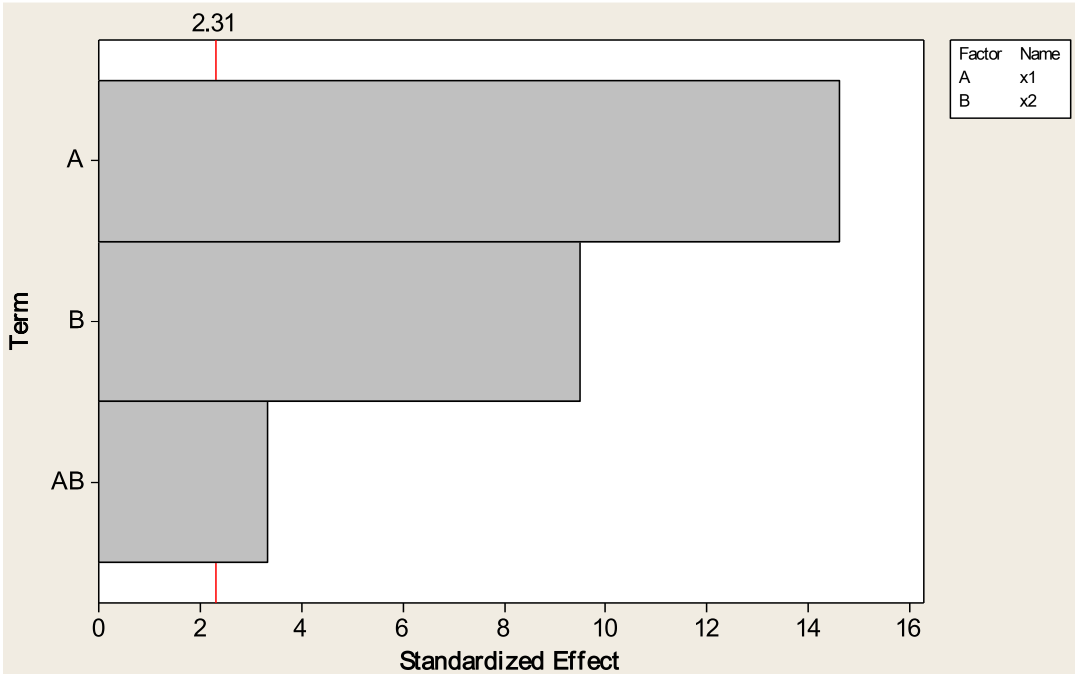


Figure 6. Pareto chart of the standardized effects for OD (Alpha = 0.05).

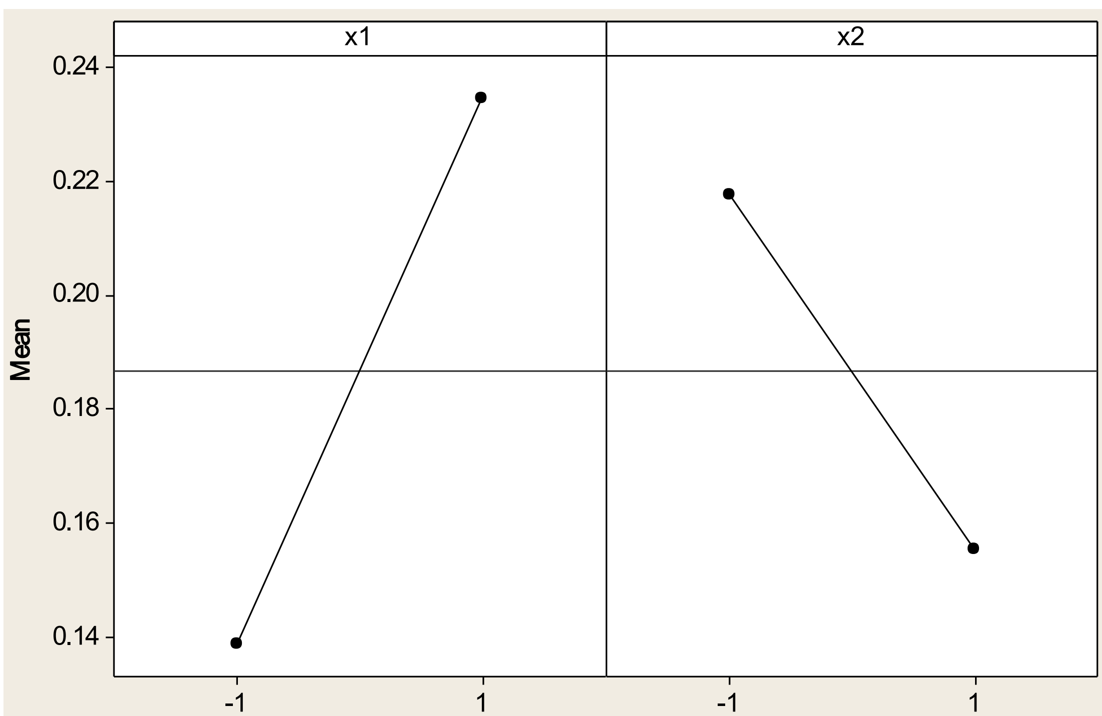


Figure 7. Main effects plot for OD.

design. The following experimental results were obtained.

1) We were fabricated the different diameter size nanotubes on the titanium substrate by electrochemical anodic oxidation method. The nanotubes surface was hydrophilic property by using sessile drop technique. In

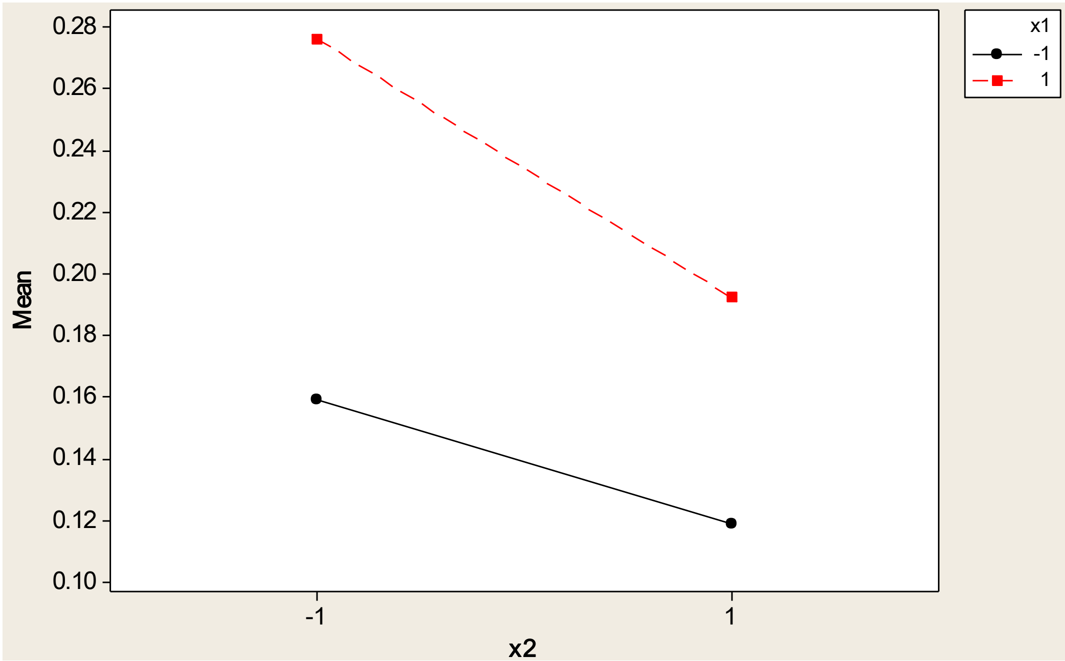


Figure 8. Interaction plots for OD.

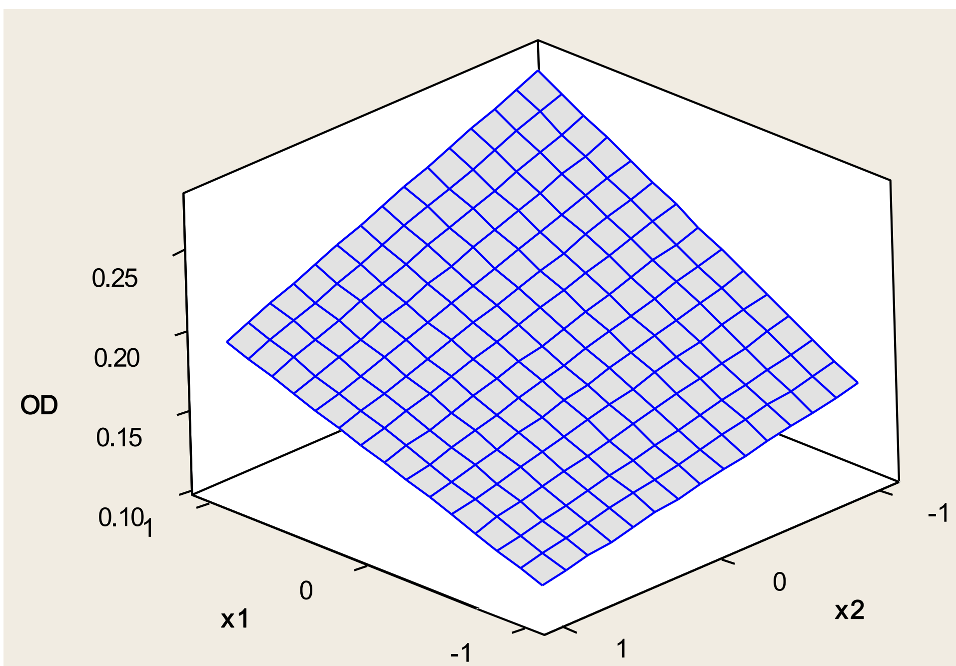


Figure 9. Estimated response for OD.

the cell responses, proliferation rates by MTT assay were increasing with decreasing nanotube diameters.
2) The days have a positive impact on the OD average as increasing this previous variable means increasing the OD parameter. In addition, there is an optimum value of days that provides a maximum value of OD.
3) The pore sizes have a negative impact on the OD average as increasing the previous variable means decreasing the OD parameter.
4) The polynomial model has been used. It has been

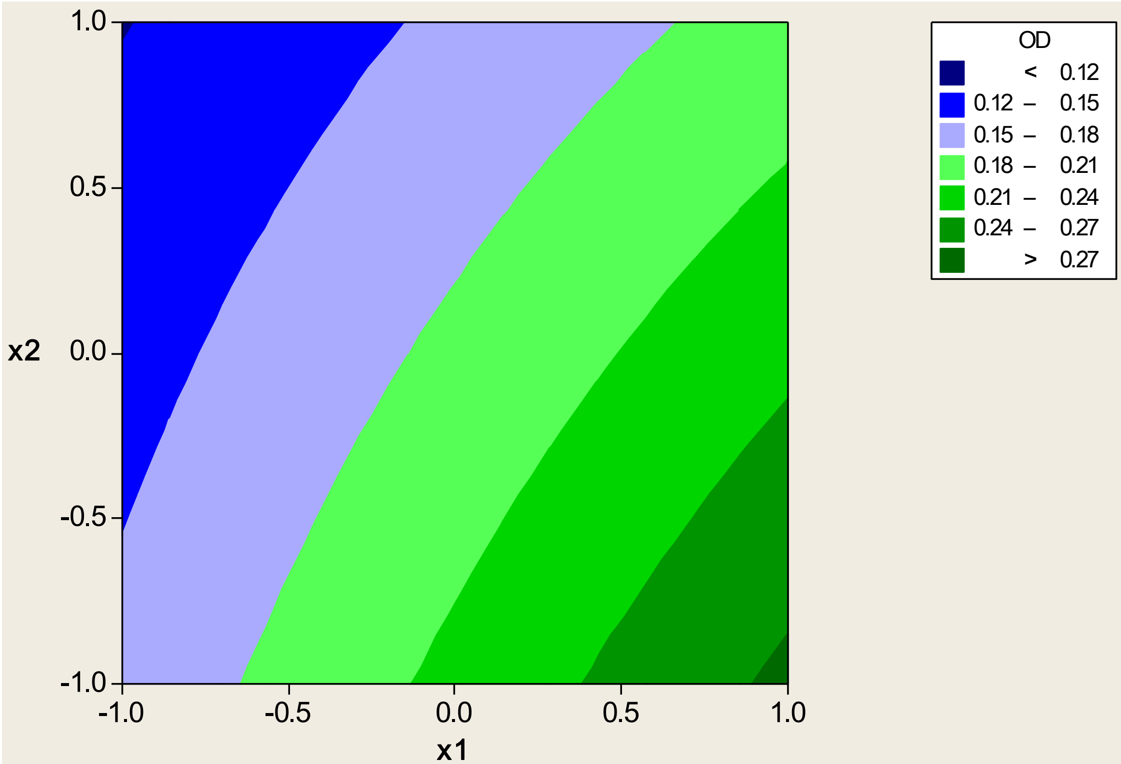


Figure 10. Contours of estimated response surface for x1 vs. x2.

shown to be combined with design and experimental techniques and could be applied to the modeling of the behavior depends on two variables. It was an effective method which was not to need a large number of experiments.

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