



# Effect of Surface Modification on Viability of L929 Cells on Zirconia Nanocomposite Substrat

Moluk Aivazi<sup>1\*</sup>, Mohammadhossein Fathi<sup>2</sup>, Farahnaz Nejatidanesh<sup>2</sup>, Vajihesadat Mortazavi<sup>3</sup>, Batoul Hashemi Beni<sup>4</sup>, Jukka Pekka Matinlinna<sup>5</sup>

<sup>1</sup>Biomaterials Research Group, Department of Materials Engineering, Isfahan University of Technology, Isfahan, Iran

<sup>2</sup>Dental Materials Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

<sup>3</sup>Torbinejad Dental Research Center, Department of Operative Dentistry, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran

<sup>4</sup>Department of Anatomical Sciences and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

<sup>5</sup>Dental Material Science, Head of Unit, Faculty of Dentistry, The University of Hong Kong, 4/FDental Material Science, The Prince Philip Dental Hospital, 34Hospital Road, Sai Ying Pun, Hong Kong

## \*Correspondence to

Moluk Aivazi, Biomaterials Research Group, Department of Materials Engineering, Isfahan University of Technology, Isfahan, Iran  
Tel: +983133912750;  
Fax: +983133912752  
Email: Moluk.Aivazi@gmail.com

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## Abstract

**Introduction:** Zirconia bioceramic can be considered for metallic replacement in dental implant applications. A proper method of surface modification may promote better osseointegration.

**Methods:** In study evaluated viability of fibroblast cell following surface treatment. Therefore, viability L929 cells were characterized using MTT assay and scanning electron microscopy.

**Results:** The viability assessment determined significant differences A-Y-TZP20 without surface treatment as compared to laser surface treatment (B), laser surface treatment + hydroxyapatite-yttrium stabilized tetragonal zirconia nanocomposite coat (C) and control. This study demonstrated that L929 cells approximately proliferated and spread on A-Y-TZP20 nanocomposite disk in laser surface treatment(B), Laser surface treatment + hydroxiapatite-yttrium stabilized tetragonal zirconia nanocomposite coat (C) groups similar to control group.

**Conclusion:** Laser surface treatment showed positive effect on the viability of L929 cells.

**Keywords:** Alumina-yttrium stabilized tetragonal zirconia nanocomposite; Femtosecond laser; MTT assay; Endosseous dental implant.

## Introduction

Titanium and its alloys as a dental implant have shown a high successful rate.<sup>1</sup> Most metals or alloys are prone to corrosion process. Therefore, management and control of corrosion is a crucial point from biological aspects and may not limit to a local problem because the produced particles may migrate to far sites from the implant. Also it is important in terms of biocompatibility.<sup>2</sup> So, the inert ceramic materials were suggested as alternatives to metals.<sup>3</sup> Zirconia ceramic has been included in dentistry for different applications. In the design of implant, type of material and surface conditions have an impact on reliable osseointegration.<sup>5</sup> Also, the degree of roughness is important. The different methods have been used to design the surface roughness.<sup>6</sup> Beside effects of micro and nanoscale topography on different cells functions have examined in numerous studies.<sup>7-13</sup> This result has been explained by good cellular behavior.<sup>7,10,13,14</sup> In

vitro and animal studies on zirconia have confirmed good biocompatibility and mechanical strength.<sup>15,16</sup> The osteoblastic response to different surface topographies of zirconia showed the higher cell proliferation in rough surfaces as compared to the smooth surface.<sup>17</sup> Therefore the type of modification on morphology and viability L929 on A-Y-TZP20 substrate was evaluated by an in-vitro method.

## Methods

### Deposition of Hydroxyapatite Coating on A-Y-TZP20 Nanocomposite Substrate

The Y-TZP-HA coats were deposited onto A-Y-TZP20 substrates by a dip coating process.

### Evaluation of the Surface of A-Y-TZP 20 Nanocomposite Substrates

The studied samples were (A) without any surface

treatment; (B) femtosecond laser treatment and design microgrooves with 30  $\mu\text{m}$  width and 30  $\mu\text{m}$  depth, (C) femtosecond laser treatment to design microgrooves with the width of 30  $\mu\text{m}$  and the depth of 30  $\mu\text{m}$ , that were coated using hydroxiapatite–yttrium stabilized tetragonal zirconia polycrystal (10% wt) nanocomposite to sol-gel method.<sup>18</sup> The evaluation of the surface of the nanocomposite disks was assessed.

### Cellular Morphology

The L929 cells were cultured for 5 days on A-Y-TZP20 nanocomposite specimens in control, A, B and C groups and were examined with a scanning electron microscope (Phillips, XL30, the Netherlands).

### Colorimetric MTT Assay

MTT assay was performed on four cultured A-Y-TZP 20 disk substrates (A: non-surface treat, B: femtosecond laser treat, C: laser surface treat +3Y-TZP-HA nanocomposite coat and D: control groups).

### Cell Culture

The A-Y-TZP 20 nanocomposite disks were sterilized using ultraviolet and ethanol alcohol (70%). Cell culture studies of L929 were conducted on four groups (A: non-surface treat, B: laser surface treat, C: laser surface treat + 3Y-TZP-HA nanocomposite coat and control group).

### Statistical Analysis

Kolmogorov–Smirnov test, analysis of variance (ANOVA) test and post hoc analysis were applied.  $P < 0.05$  was considered as significant.

## Results

### Topographic Assessment

In evaluation with scanning electron microscopy, A-Y-TZP20 with laser micro-texture had laser processing, whereas the A-Y-TZP20 with HA-Y-TZP coat and laser micro-texture surface (C) contained longitudinal and

parallel microgrooves that were covered with HA-Y-TZP nanocomposite coat.

### Assessment of Cellular Morphology

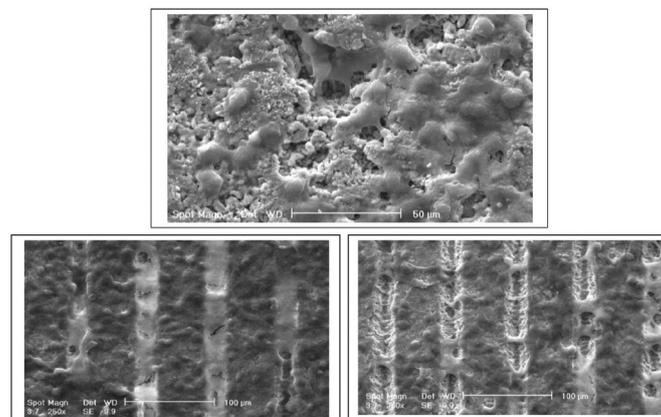
The A-Y-TZP20 substrates with 3 different surfaces (A: non-surface treat, B: laser surface treat, C: laser surface treat and HA-Y-TZP nanocomposite coat) supported continuous cellular growth for 5 days (Figure 1). After culture, the L929 cells were covered by the surfaces of substrates (A, B and C) and connected with each other (A, B and C).

### Cellular Viability

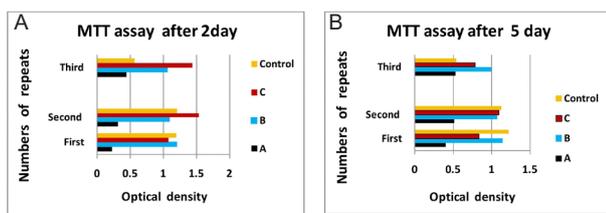
Figure 2 shows the optical density in A, B, C and control group in 2 and 5 days incubation. L929 were proliferated on B and C disks more than A disks. However, the cellular proliferation on B and C disks was almost same as with the control group. The degree of cellular proliferation on B disks group was the highest value. According to ANOVA analysis, and homogeneity of variances (Table 1), the variance of an optical density (2 days after incubation =  $OD_1$  and 5 days after incubation =  $OD_2$ ) variables were significant ( $OD_1$ :  $P \leq 0.018$  and  $OD_2$ :  $P \leq 0.036$ ). Also, normality distribution of means was significant (Table 2). Following significant difference in optical density means and optical density variances in 4 studied groups, post hoc (LSD) test was carried out for comparing of pairwise (Table 3 and Table 4).

## Discussion

A Y-TZP ceramic has suitable biocompatibility, high bending strength and white aesthetic, therefore favour for implant application.<sup>19-21</sup> Zirconia bioceramic can be considered a proper candidate for metallic replacement in endosseous dental implants. To, A-Y-TZP20 in vivo application, at first can be checked with an in vitro model. The biocompatibility of the materials depends on their physical, chemical and the first response of the cells on surface properties. Also, the interaction of the cells



**Figure 1.** The SEM images of L929 cells on the surface of A-3-Y-TZP nanocomposite disk in A: non-surface treat, B: femtosecond laser treat, C: femtosecond laser + HA-3Y-TZP nanocomposite coat treat and control (D) groups 5 days after cell culture.



**Figure 2.** The results of MTT assay in A: non-surface treat, B: femtosecond laser treat, C: femtosecond laser +HA-3Y-TZP nanocomposite coat treat and control (D) groups at 2 and 5 days after incubation.

**Table 1.** The Results of the Homogeneity of Variances Test at 2 (OD1) and 5 days (OD2) After Incubation

Optical Density (OD)	Levene Test	df <sub>1</sub>	df <sub>2</sub>	P
OD <sub>1</sub> : 2 d after incubation (n = 6)	4.694	3	8	0.036 <sup>a</sup>
OD <sub>2</sub> : 5 d after incubation (n = 6)	6.094	3	8	0.018 <sup>a</sup>

<sup>a</sup> P value is significant at the level of <0.05.

with the material surface is important for contributing to osseointegration.<sup>22</sup> On the other hand, an implant morphology affect bone metabolism: rougher surfaces motivate differentiation, growth and attachment of bone cells, and increase mineralization. The different methods were reported in the literature to design implant roughness. Recently, the surface modification of implant is considered using of femtosecond laser. The microtexture specially groovy pattern was more effective than pored patterns.<sup>23</sup> Therefore, the morphology and viability of L929 cells on three different surface of A-Y-TZP20 substrate was evaluated (A: non surface treat, B: laser surface treat, C: laser surface treat and HA-Y-TZP (10%wt) nano-composite coat). All the substrates in this study were A-Y-TZP20 nanocomposite disk but their surfaces were different. In microstructure analysis, dense nanocomposite (Figure 3A) was visible. In EDAX analysis (Figure 3B, 3C) and mapping of x-ray (Figure 3D) proved nanopowders in nanocomposite. Figure 5A show the modified surface with femtosecond laser. Longitudinal microgrooved pattern with specific dimension is evident in nano-composite surface. Figure 5B and Figure 5C show a view of an one groove that was designed using femtosecond laser on nano-composite surface but in Figure 5C, the microgroove was covered by nano-composite coat (hydroxyapatite-yttrium-stabilized tetragonal zirconia polycrystal) . As it is clear from Figure

**Table 2.** The Results of ANOVA Analysis at 2 (OD1) and 12 Hours (OD2) After Incubation

Optical Density (OD)		Sum of Squares	df	Mean Square	F	P
OD <sub>1</sub> : 2 d after incubation (n = 6)	Between groups	1.727	3	0.576	10.898	0.003 <sup>a</sup>
	Within groups	0.423	8	0.053		
OD <sub>2</sub> : 5 d after incubation (n = 6)	Between groups	0.602	3	201	4.640	0.037 <sup>a</sup>
	Within groups	0.346	8	0.43		

<sup>a</sup> P value is significant at the level of <0.05.

**Table 3.** The Result of Post Hoc Analysis at 2 Days After Incubation Between Studied Groups<sup>b</sup>

Dependent Variable	(J) Group	(I) Group	P
OD <sub>1</sub> (after 2 days) No. of samples per each group = 6	A	B	0.003 <sup>a</sup>
		C	0.001 <sup>a</sup>
		D	0.008 <sup>a</sup>
		A	0.003 <sup>a</sup>
		C	0.239
		D	0.527
	B	C	0.001 <sup>a</sup>
		B	0.239
		D	0.089
		D	0.008 <sup>a</sup>
		B	0.527
		C	0.089

<sup>a</sup> P value is significant at the level of <0.05.

<sup>b</sup> A: non-surface treat, B: laser surface treat, C: laser surface treat + Y-TZP-HA nanocomposite coat and D: control.

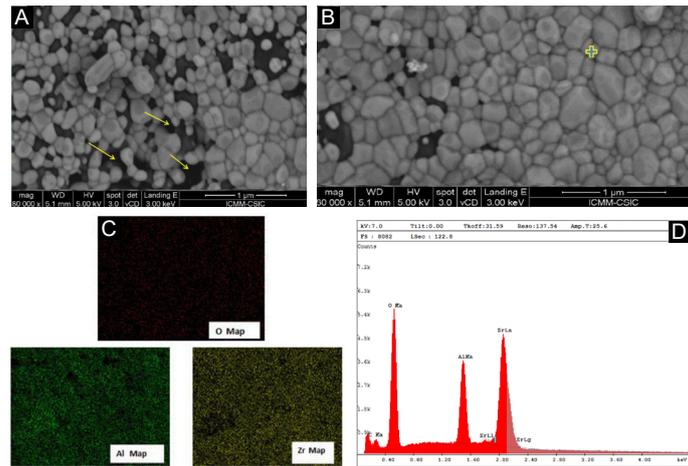
**Table 4.** The Result of Post Hoc Analysis at 5 Days After Incubation Between Studied Groups<sup>b</sup>

Dependent Variable	(J) Group	(I) Group	P
OD <sub>2</sub> (after 5 days) No. of samples per each group = 6	A	B	0.008 <sup>a</sup>
		C	0.035 <sup>a</sup>
		D	0.022 <sup>a</sup>
		A	0.008 <sup>a</sup>
		C	0.377
		D	0.540
	B	A	0.035 <sup>a</sup>
		B	0.377
		D	0.776
		A	0.022 <sup>a</sup>
		B	0.540
		C	0.776

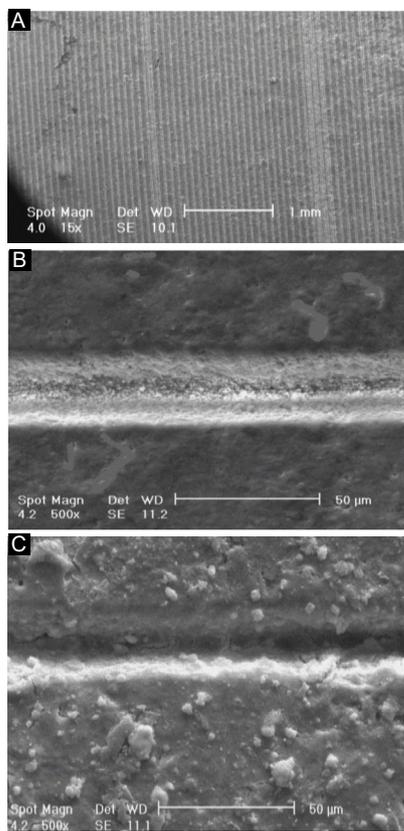
<sup>a</sup> P value is significant at the level of <0.05.

<sup>b</sup> A: non-surface treat, B: laser surface treat, C: laser surface treat + Y-TZP-HA nanocomposite coat and D: control.

5B the walls of groove is regular and without any crack. In present study and MTT assay after 2 day (Figure 2A) in laser surface treat group (B), laser surface treat+HA-Y-TZP nanocomposite coat (C) and control groups, the optical density was higher than non-surface treat (A). Also, in post hoc analysis and pairwise comparison of means (Table 3) were only significant non-surface treat (A group) with the other groups ( $P \leq 0.05$ ). Also, MTT assay after 5 day (Figure 2B) the optical density in non-surface treat (A) showed the lowest value between studied

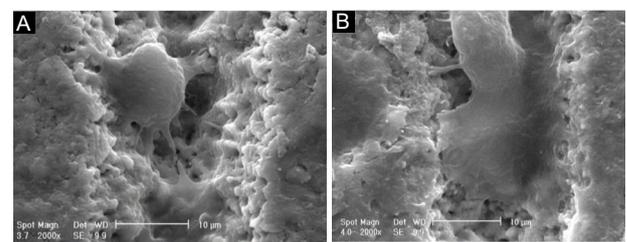


**Figure 3.** (A) The FESEM image from A-3Y-TZP20 nanocomposite. (B) The FESEM image from A-3Y-TZP20 nanocomposite sintered at 1270°C. (C) EDAX analysis of A-3Y-TZP20 nanocomposite sintered at 1270°C. (D) X- ray mapping from A-3Y-TZP20 nanocomposite sintered at 1270°C.



**Figure 4.** The SEM image of microgroove pattern on A-3Y-TZP20 nano-composite disk after laser processing disk and one microgroove without (B) and with (C) HA-3-Y-TZP nanocomposite coat.

groups. In post hoc analysis and multiple comparison of means (Table 4) merely the non-surface treat (A group) with the other groups were significant ( $P \leq 0.05$ ). But in comparison of B, C and control group no significant differences were observed. It seemed, therefore that, the surface treatment had significant effect on optical density.



**Figure 5.** The SEM image of femtosecond laser treatment group Band L929 cell morphology in the depth of microgroove (A) and in the surface of microgroove (B) 5 day after cell culture.

So that, the optical density in non-surface treat group at 2 different times repeated three times were the lowest value among the studied groups. Although, the significant difference was not observed between B and C group. It seems, more studies are needed on 2 different modified surface especially comparison of osteoblast cells with L929 cells is proposed. In present study, surface modification procedures had maximally influence the proliferative behavior of L929 cells. The cellular proliferation and density in B and C groups, were approximately similar to control group. Day 5 cultures showed a lower density of L929 cell in non-surface treat Figure 4A than B and C groups. These findings were confirmed via SEM image analysis. Based on SEM image analysis, at day 5, the coverage rate of the L929 cells in non-surface treatment was lower than B and C groups (Figure 1). It was suggested that these surfaces (B and C groups) provided good conditions for the L929 cells growth. In A and B substrate groups evident a more growth of fibroblasts. It seemed, the modification of substrate topography promoted cell spreading, this result in increased mitosis and cellular proliferation. Also, in microgroove region can be seen the cells morphology in the depth of microgroove round (Figure 5) but proximal edges (Figure 5) of microgroove

is flattened with the extended numerous filopodia. Based on this study L929 cells proliferated and spread approximately in laser surface treat (group B) and laser surface treat + HA-Y-TZP nanocomposite coat (group C) similar to control group.

### Conclusion

The cell proliferation of laser surface treatment (B) and laser surface treatment + hydroxyapatite-yttrium stabilized tetragonal zirconia nanocomposite coat (C) groups were comparable to the control group. The surface treatment of both laser surface treatment (B) and laser surface treatment + hydroxyapatite-yttrium stabilized tetragonal zirconia nanocomposite coat (C) was influenced the viability of the L929 cells, like to control group. The specimens with surface treatment (B and C groups) had better MTT result as compared to the non-surface treat (A group). Finally laser surface treatment showed a positive effect on the viability of L929 cells.

### Conflict of Interests

The authors declare no conflict of interest.

### Ethical Considerations

Not applicable.

### Acknowledgements

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