Research on Biomedical Engineering
rbejournal.org
Volume 32, Number 2, p. 144-152, 2016

Original Article
DOI: http://dx.doi.org/10.1590/2446-4740.03616

# Macrophages adhesion rate on Ti-6Al-4V substrates: polishing and DLC coating effects

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**Abstract** **Introduction:** Various works have shown that diamond-like carbon (DLC) coatings are able to improve the cells adhesion on prosthesis material and also cause protection against the physical wear. On the other hand there are reports about the effect of substrate polishing, in evidence of that roughness can enhance cell adhesion. In order to compare and quantify the joint effects of both factors, i.e, polishing and DLC coating, a commonly prosthesis material, the Ti-6Al-4V alloy, was used as raw material for substrates in our studies of macrophage cell adhesion rate on rough and polished samples, coated and uncoated with DLC. **Methods:** The films were produced by PECVD technique on Ti-6Al-4V substrates and characterized by optical profilometry, scanning electron microscopy and Raman spectroscopy. The amount of cells was measured by particle analysis in IMAGE J software. Cytotoxicity tests were also carried out to infer the biocompatibility of the samples. **Results**: The results showed that higher the surface roughness of the alloy, higher are the cells fixing on the samples surface, moreover group of samples with DLC favored the cell adhesion more than their respective uncoated groups. The cytotoxity tests confirmed that all samples were biocompatible independently of being polished or coated with DLC. **Conclusion:** From the observed results, it was found that the rougher substrate coated with DLC showed a higher cell adhesion than the polished samples, either coated or uncoated with the film. It is concluded that the roughness of the Ti-6Al-4V alloy and the DLC coating act complementary to enhance cell adhesion.

**Keywords:** DLC, RMS roughness, Cell adhesion, Ti-6Al-4V, Macrophages J774, Biomedical prosthesis.

## Introduction

According to the survey of World Health Organization (WHO) in 2014, the average life time expectancy has increased substantially in the last years. Consequently, the number of elders in the world has increased (World…, 2014). According to the report of the Geography and Statistics Brazilian Institute (IBGE), the proportion of elder people in the population was estimated to be 8.17% in 2015 and 6.22% in 2006 (Instituto…, 2016), an increase of about 30% of elders in the population of Brazil in the last 10 years.

Many health problems are likely to appear during the elder life stage, often requiring a considerable amount of money for heath treatments. Osteoarthritis is one of the most common diseases that cause joint pain and weakness during physical activity, especially with elder people (Kawtar et al., 2013). This disease is characterized by the degeneration of articular cartilage, overgrowth of bone cells and remodeling of the bone (Benz et al., 2015). Medicines are widely used for symptom relief in the osteoarthritis at the early stage, but they do not work very well in complicate cases. Major surgical interventions, such as total joint replacement, are the only effective options for people with severe osteoarthritis (Dieppe, 2005).

The medical expenses associated with surgery for placement of orthopedic implants grew significantly. In 2015, the Brazilian Ministry of Health (BMH) reported that many of the South American countries showed interest in the prosthetic production sector higher than in any other industrial sector. An increase of 15% in this sector is estimated until 2020 (Brasil, 2015). Though many kinds of implants are used, patients suffer many post-surgical complications related to the biocompatibility and wear of the implants used. This problem raised the interest of researchers in increasing the quality of the prosthesis to reduce their physical wear and making them more biocompatible. One of the main cause that reduces the orthopedic implant longevity is the high wear rate of commonly used materials such as Cobalt-Chrome-Molybdenum (Co-28Cr-6Mo) alloy and Titanium–Aluminum–Vanadium (Ti-6Al-4V) alloy, (Grill, 2003; Khatir et al., 2015; Yingmin et al., 2016**).** The particles resulting from wear

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Received: 10 May 2016 / Accepted: 01 July 2016

of the prosthesis are harmful to the body and induce inflammation process (Grill, 2003; Hinüber et al., 2010). Many studies have shown that $Co^{-}$, $Cr^{-}$, and $Mo^{-}$ ions are considered toxic, carcinogenic and also able to promote inflammation (Andrews et al., 2011; Hinüber et al., 2010; Jakobsen et al., 2007). Aluminum is also considered to be a risk factor for the pathogenesis of Alzheimer's disease (McLachlan et al., 1990; Rusina et al., 2011). However, Ti-6Al-4V has an advantage over Co-28Cr-26Mo because of the presence of Vanadium. Vanadium may reduce memory deficits in individuals exposed continuously for a long time (Imtiaz et al., 2015). Due to these advantages, Ti-6Al-4V is considered better than Co-28Cr-6Mo in prosthesis production.

Different materials have been used as coatings on implants in order to improve cell adhesion and to prevent their wear, such as: ultra-fine hydroxyapatite powders (Kaya, 2008), carbon nanotubes (Kaya, 2008), Ag–ZrCN films (Ferreri et al., 2015) and DLC films (Casiraghi et al., 2005). All these coatings have shown good results, however only the DLC thin film has been considered able to promote a reduction on metal friction at the same time that increases the wear resistance, the hardness, and the cell adhesion rate (Grill et al., 1988; Grill, 2003; Ianno et al., 1995; Jelinek et al., 2016; Khatir et al., 2015; Marciano et al., 2009). In addition, DLC films may have antibactericidal effect (Marciano et al., 2009). All these properties make DLC suitable for a number of applications ranging from the coating of stents, heart valves, and prostheses in biomedical industry (Hauert and Müller, 2003). By other hand some researchers are also trying to improve the cell adhesion by only promoting changes in the material roughness, for example by surface etching or polishing, without the use of coatings on the prosthesis alloy. The results are so promising as are the works based on nanostructured coatings (Degasne et al., 1999; Musilkova et al., 2015; Safiullin et al., 2015; Varoni et al., 2015; Vrekhem et al., 2015).

Based on these, our study aimed to evaluate the effectiveness of macrophage cell adhesion on prosthesis samples based on Ti-6Al-4V alloy, correlating the results to the surface roughness of the sample and discriminating the effect of polishing and DLC coating.

## Methods

Ti-6Al-V alloy was taken as the experimental material from which 16 samples were prepared as discs of 1.7 cm diameter and 3 mm thick, all them with a standard surface roughness of 300 nm. These sixteen discs were grouped under four categories of samples, namely: rougher alloy (RA), polished alloy (PA), rougher alloy with DLC coating (RA-DLC) and polished alloy with DLC coating (PA-DLC), with four discs composing each group category, such that the statistical parameters of the measurements could be evaluated.

For the samples of groups PA and PA-DLC the titanium alloy was polished using commercially available sand paper with granulation size ranging from 180 to 2000, followed by felt polishing using diamond paste of two microns. Prior to DLC deposition and cell culture, the samples were immersed in a beaker with acetone and subjected to ultrasound for 10 min. Finally, they were placed inside the oven at 40 °C for the evaporation of acetone.

### *DLC coating process*

This section explains the DLC coating process. This process comprises three steps which includes (I) cleaning of the substrate by argon plasma, (II) deposition of silicon interlayer using hexa-methyl-disiloxane ($HDMSO_4$) diluted in argon plasma to improve DLC adhesion on Ti-6Al-4V (Grill et al., 1988; Ianno et al., 1995) and, (III) deposition of DLC coatings by methane plasma. Table 1 shows the experimental parameters maintained at each step of the DLC film deposition to produce a film of ~0.4 μm thickness. The coating were produced by plasma enhanced chemical vapor deposition (PECVD) in a commercial reactor

**Table 1.** Experimental parameters for the DLC coating.

| Parameters | Substrate cleaning | Interlayer deposition | DLC deposition |
|---|---|---|---|
| Time (min) | 10 | 30 | 120 |
| Power (W) | 200 | 200 | 200 |
| Pressure (Torr) | $3\times10^{-2}$ | $3\times10^{-2}$ | $3\times10^{-2}$ |
| Pulse (kHz) | 100 | 130 | 130 |
| Reverse pulse (μs) | 0.8 | 3.2 | 3.2 |
| Voltage (V) | 650 | 600 | 600 |
| Current (A) | 0.6 | 0.5 | 0.5 |
| Temperature range (°C) | 18 to 22 | 300 | 18 to 70 |
| Gas | Ar | Ar+ HMDSO | $CH_4$ |

manufactured by Nanomaster Inc. model NPE-4000 using a pulsed DC power supply.

### *Film characterization*

Different techniques were used for surface characterization of the samples. High-resolution images of the samples surface, coated and uncoated with DLC, were obtained by scanning electron microscopy (SEM, FEI - Inspect F50), operated in secondary electron mode. The film thickness as well as the surface roughness of the Ti-6Al-4V samples was obtained by optical profiler Veeco model Wiko - NT9000 with 20× magnification objective. The structure and degree of disorder of the DLC films were obtained by Raman spectroscopy (Renishaw 2000) with an Ar ion laser ($\lambda = 514$ nm), operated with backscattering geometry at room temperature. The diameter of the laser spot was maintained as 2.5 μm and the power at 0.6 mW on the surface of the film (Marciano et al., 2009).

### *Cells culture and cytotoxicity test*

J774 macrophages from murine cell line (ATCC) were grown in culture bottles, maintained in RPMI (Roswell Park Memorial Institute) medium plus 10% fetal bovine serum and antibiotics penicillin/streptomycin. The culture bottles were kept half closed at 37 °C in humidified atmosphere with 5% $CO_2$. The samples were placed in a Corning plate (24 wells) (NY- USA) and 5 ml of the medium with the cells was dripped.

The system remained untouched until the seventh day when it was found the formation of a monolayer of macrophages. At the end of the period, the medium was removed and images were taken by SEM.

To check the cytotoxicity of the coated and un-coated samples, J774 macrophages were used to make the neutral red dye uptake assay. The macrophages at concentration of $3.0 \times 10^5$ cells/ml were seeded in Petri dishes (15 × 60 mm) at a total volume of 5 ml of agar-overlay. They were incubated for 48 h at 37 °C in a humidified atmosphere with 5% $CO_2$. A sample of each experimental group was placed on the agar before their complete solidification. Two glass slides were taken as controls and under one of them 100 μl of phenol was dropped, to act as a positive control, the other acting as a negative control. The plates were then examined macroscopically and microscopically to check the cytotoxicity and cellular integrity around the sample (Allen et al., 1994; 2001).

### *Image processing and statistical analysis*

The amount of adhered cells was determined using ImageJ software and the mean and standard deviation (S.D.) values were calculated. Differences between the number of cells adhered on each group were individually analyzed by unpaired t-student tests in the Minitab 17.3 software. Significant interactions between the roughness and cells adhesion were also evaluated within a 95% confidence interval.

## Results

Figure 1 shows the Raman spectrum of the DLC films (blue line). The red and black spectra represent the deconvolution of the original spectrum. From this figure, it is observed that the D band and the G band were centered around 1350 $cm^{-1}$ and 1580 $cm^{-1}$ respectively. These bands are typical of DLC films. The ratio of the intensities of the D and G bands ($I_D/I_G$) was calculated to be 0.2, indicating that the film is a hydrogenated DLC.

Figure 2 depicts the surface images of the four groups of samples, obtained from profilometry analysis, from which the root-mean-square (RMS) surface roughness was inferred. It is quite remarkable that the four sample groups ended up with a large variation of roughness, ranging from 15 nm to 997 nm, depending on the raw material polishing and DLC coating. DLC alone provides a substantial increase, by a factor about 3.5, of the roughness.

Figure 3 displays results of the cytotoxic studies to verify the cells biocompatibility with respect to RMS roughness of the Ti-6Al-4V samples covered by DLC. It is observed that the red-neutral dye was phagocytosed by the macrophages and remained inside the cells indicating that the DLC did not cause disruption of the J774 macrophages cell membrane. From the cytotoxicity studies using a biocompatible glass slides, it is observed that the positive control makes a roseate halo which indicates the cytotoxicity and the negative control did not make any cytotoxicity

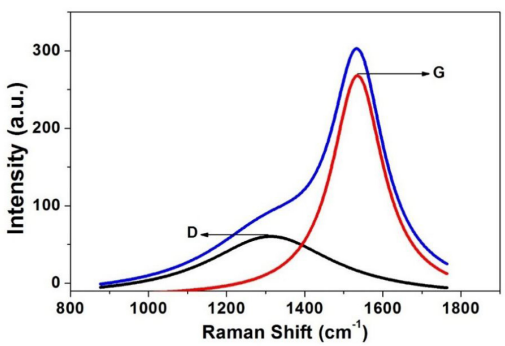


**Figure 1.** Raman spectrum of the DLC films (Blue line) from coated groups: Polished alloy covered by DLC (PA-DLC) and rougher alloy covered by DLC (RA-DLC). The black and red lines show the deconvoluted spectra of D (1350$cm^{-1}$) and G (1580$cm^{-1}$) bands.

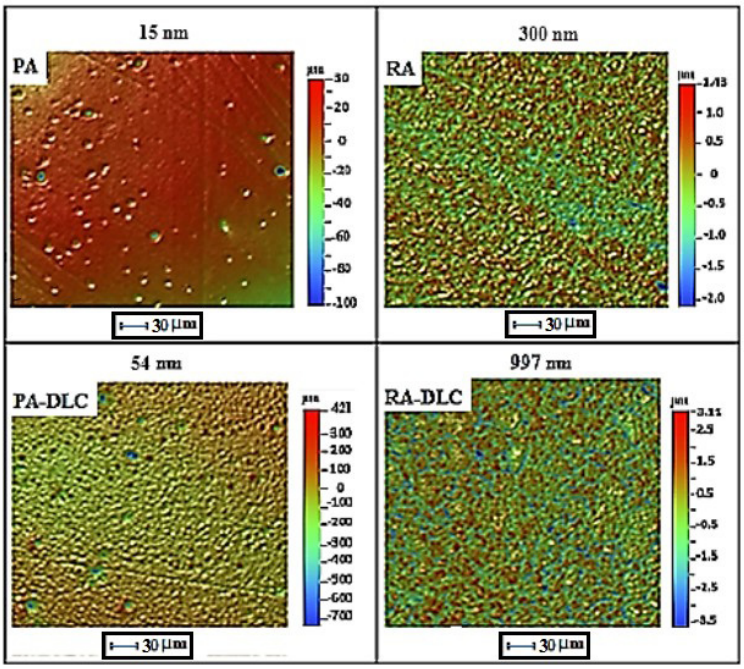


**Figure 2.** Sample groups images obtained on optical profiler with 20× magnification.

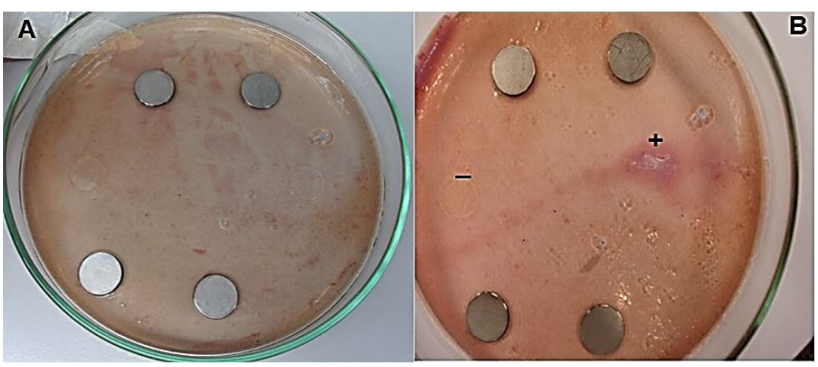


**Figure 3.** Result of cytotoxicity test. (A) Initial test image. (B) Image of the test after 24 hours. DLC on the samples are in contact with agar-overlay. The signs (+) and (–) mark the position of positive and negative controls, respectively.

effect (Xuan et al., 1990) (Figure 3B). Comparing Figure 3A, B, even after 24 hours of contact with films, it is clear that the dye was not released, thereby indicating no cytotoxicity with regard to all samples. In addition to the cytotoxic study, the shape of cell grown under the sample discs was observed by optical microscopy (Figure 4A), and by SEM (Figure 4B). By this exam it was observed that the cells did not undergo any alteration or structural changes that could affect their activity.

Figure 5 displays the amount of adherent cells in the four groups of samples, as measured by Image J software. It can be inferred from the figure that cell adhesion is higher in the rougher surface (RA) than on polished one (PA) and DLC coating further enhances the cell adhesion by more than 75%.

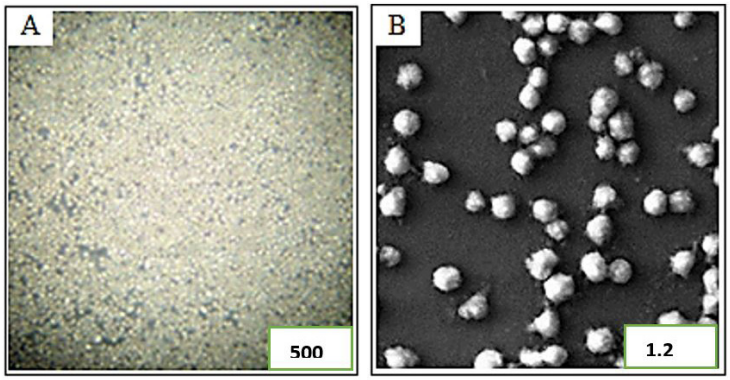


**Figure 4.** Optical microscopy image (A) and the SEM image (B) of cells that were under the Ti-6Al-4V discs.

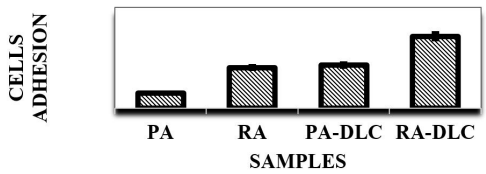


**Figure 5.** Amount of cells adhered on polished alloy (PA), rougher alloy (RA), polished alloy covered by DLC (PA-DLC), and rougher alloy covered by DLC (RA-DLC).

## Discussion

The improvements on mechanical, chemical and biocompatibility characteristics of prosthesis are currently a focus of great interest in the biomedical sector, attracting growing number of researchers to work in this area of science and technology. Good results have been reported by the use of coating with nanostructured thin films such as DLC and by techniques that promote changes on the surface morphology and topography of raw materials used in prosthesis. In this context, the present study deals with a careful evaluation of cell adhesion and biocompatibility respective to culture of macrophage cells on prosthesis based on Ti-6Al-4V alloy, considering the effect of surface polishing and DLC coating of the alloy sample.

Figure 1 shows the Raman spectrum of DLC film coated on Ti-6Al-4V samples. The two observed peaks, D and G at ~1380 $cm^{-1}$ and ~1560 $cm^{-1}$, respectively, confirm the deposition of a-C:H DLC film (Casiraghi et al., 2005). The D and G peaks are both sensitive to the $sp^2$ content and the D peak is due to the bonds of carbon in rings while the G peak is due to the bonds of carbons in both rings chains (Ferrari and Robertson, 2000; 2004).The ratio of the intensity at peaks D and G, $I_D/I_G$, was used to determine the disorder degree and it was found here to be approximately 0.2. According to Casiraghi et al. (2005), a DLC film with this $I_D/I_G$ ratio presents a content of ~35% of hydrogen and ~20% of $sp^3$ bonds between the C atoms.

From the surface topography analysis (Figure 2), DLC deposition makes the difference between the heights of peaks and valleys to increase substantially, i.e, approximately 111% increase between RA and RA-DLC and approximately 762% between PA and PA-DLC. Concomitantly, the surface roughness also increases, with coated groups representing a higher RMS roughness when compared to the groups that are not coated. DLC deposition makes the roughness to increase by 232% and 260% for the rougher and polished groups respectively. This analysis suggest that one effect of DLC coating by PECVD technique is to promote, approximately, a 3.5 fold amplification of the sample roughness.

There are results reported by Prado et al. (2012), Hinüber et al. (2010), and Corbella et al. (2005) who also observed that the DLC coating increased the RMS surface roughness on different prosthesis based on polymeric as metallic materials. Some hypotheses were proposed to explain this effect, for example, as due to the changes in the plasma density during the HMDSO injection which favors DLC adhesion (Bonetti et al., 2006). Corbella et al. (2005) proposed that the DLC roughness is directly related to the bias voltage causing the effect of "etching" on surface of polymeric substrate by fluorine species in the plasma during DLC growth (Prado et al., 2012).There are also claims that roughness increases when the kinetic energy of the hydrogen ions from the plasma is insufficient to remove certain roughness of the material surface (Corbella et al., 2005). Overall there are only unconfirmed suggestions for the mechanisms related to the increase of surface roughness by DLC coating. Although the above hypotheses should not be discarded, the most probable explanations for

this effect, observed in the present work, are: as the metallic substrate is exposed to the plasma in the PECVD reactor, its surface becomes surrounded by a plasma sheath where and averaged electrostatic field is produced, high enough to accelerate plasma ions towards the sample surface so enhancing the deposition rate of the film material. Moreover, the electric field is stronger on sharper edges (a classical problem of electrostatic), so the plasma sheath electric field is stronger on the edges of hills than on bottom of the valleys. The dimensions of hills and valleys are the topographic features that better define the dimensions of the micro and nano-craters on the sample surface, consequently defining the surface roughness. Accordingly, the film grows faster on the top (hills) than on the bottom (valleys) because ion flux follows direction of electric field; moreover the electric field promotes ionization, enhancing the plasma density where the field is stronger. This explain the higher deposition rate on top than on bottom of the micro-craters of the sample, increasing their depth, consequently the surface roughness, as evidenced by the topography analysis of the samples.

A comparison of the amount of cells adhered on the samples reveals that the four groups can be ordered as displayed in Figure 5, so as to make evident the effect of polishing of the substrate raw material and of the DLC coating on the number of adhered cells. Polishing of the Ti-6Al-4V is detrimental while DLC coating is favorable to cell adhesion. The DLC makes the adhesion of cells to increase by 184% and 76%, on polished group and on rougher rough group, respectively. In Figure 6 a plot of the number of cells versus roughness is presented, distinguishing the behavior of cell adhesion on coated and uncoated groups. It can be inferred from the figure that higher the surface roughness of the group sample, higher is the cell adhesion rate, moreover, as discussed before, the DLC substantially increases the roughness of the substrate surface, by a factor of ~3.5.

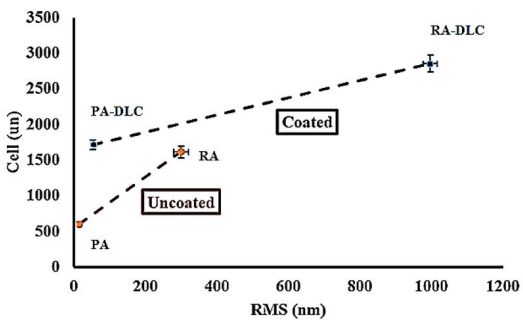


**Figure 6.** Relation between cells adherence and surface RMS roughness of the sample groups.

Although the findings of this study show a good statistical significance at a confidence interval of 95%, they do not necessarily cover the other types of cells, such as osteoblasts, monocytes, thrombocytes and other implantable materials such as polymeric materials, nickel and chromium alloys. The works of Hinüber et al. (2010) and Kornu et al. (1996), used very similar methodologies and concluded that DLC films do not cause changes in osteoblast adhesion rate to Ti-6Al-4V and Co-28Cr-6Mo substrates. Linder et al. (2002) demonstrated that the adhesion of monocytes on DLC deposited on glass slide, a material with low surface roughness, was slightly higher than on the control. In the case of thrombocytes, Tran et al. (1999), Scheerder et al. (2000) and Alanazi et al. (2000) reported that the adhesion of these cells to polytetrafluoroethylene is reduced by DLC coating, however Khatir et al. (2015) showed that the DLC grown in an atmosphere of 30% to 40% nitrogen may improve the adhesion of platelets to polytetrafluoroethylene. The later work is especially interesting because, beyond demonstrating that DLC influences cell adhesion, they also showed that this coating is able to both increase or decrease this effect.

Overall, the results of the present studies indicate that the surface roughness is a prominent factor for correlating properties of the Ti-6Al-4V sample groups with their respective macrophage adhesion rates. Figure 7 illustrates this correlation and highlights that, as far as the adhesion rate is concerned, the best substrate is that with rougher raw material surface and coated with DLC.

The benefits that DLC and high roughness offer to macrophages J774 adhesion on Ti-6Al-4V sample cannot be assigned to others material, and cell types, without further studies. Nevertheless, the present results corroborate with the sum of findings by Bendavid et al. (2007) and Lowenberg et al. (1991) who used silicon and titanium substrates and showed that DLC films increases the roughness of the substrate and favors the cell adhesion.

Figure 3 shows the cytotoxicity analysis of the four samples used in this study. The positive control indicated by (+) symbol, shows the behavior of a toxic sample that cause change in the environment color of the agar medium and the negative control (–) shows the behavior of non toxic components that causes no alteration in the culture medium. The same observations were made from our samples and shows that the DLC coated and uncoated Ti-6Al-4V samples exhibit no toxic or inflammatory response to J774 macrophages. This is in good agreement with previously published results by Hinüber et al. (2010), Linder et al. (2002), Wachesk et al. (2013)

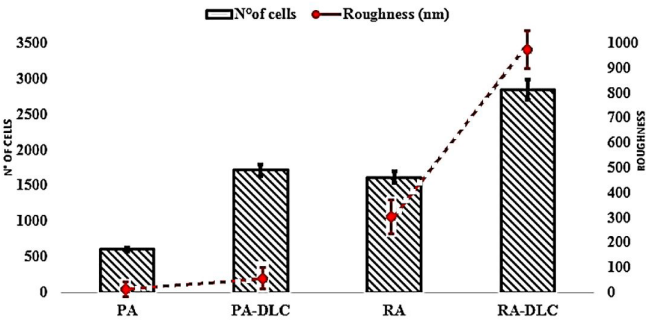


**Figure 7.** RMS Value versus number of cells adhered on samples group.

and Kornu et al. (1996). The analysis of the culture plates under inverted microscope shows no changes in the cell morphology and are in good agreement with the cytotoxicity results (Figure 4). The SEM analysis of the macrophages grown in area under the samples showed some clusters of macrophages and this effect assures the occurrence of mitosis. Because of this, it is possible to observe the long and slender cytoplasmic extensions of the cells in multiple directions, which indicates an excellent macrophages adhesion on the samples (Figure 4B). This shows that the Ti-6Al-4V alloy, covered or not covered by DLC, do not have cytotoxic effects in relation to J774 macrophages.

Finally, from the observed results it is evidenced that micro and nanostructures of the Ti-4Al-6V surface play a key role for the cell (macrophage) adhesion rate, the surface RMS roughness being a prominent characteristic of the surface topography to be considered in a search for correlation with the cell adhesion rate. This roughness can be tailored by the polishing of the raw material followed by deposition of DLC thin film. Beyond increasing the surface roughness, the DLC coating is favorable to the bio-integration of the prosthesis, also protects the implant against wear. A new explanation for the mechanism of roughness increase by DLC coating is presented. The numerical results, obtained by quantification of the effects of polishing and DLC coating on cell adhesion rate, are relevant for the search of optimal condition to prepare biomedical implants based on Ti-4Al-6V.

## Acknowledgements

We would like to thank the São Paulo Research Foundation (FAPESP) for funding the research carried out under the project PRONEX processes number 2011/50773-0 and 2013/05269-8.

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