Machined surfaces as designed cell culture substrates

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ABSTRACT

Most living cells attach and proliferate on surfaces, but the reasons for attachment are not widely known when it comes to attachment on metal surfaces. Surface roughness is known to have an effect, and some materials are known to be toxic to cells. The aim of this study is to further investigate the effects of surface roughness on cell proliferation through the use of a model adherent cell line. To bridge the current gap in the scope of surface roughness investigations, and establish machined surfaces, i.e. through milling operations, as a valid surface for cell proliferation experiments. Thus, reducing both costs of materials, and the time for sample preparation. The highest cell proliferation was seen at an approximate surface roughness of 0.15 µm. All the samples (AZ31, Al 7075, and Ti6Al4V) except for the Cu-based (CW510L) performed better than the control group after undergoing milling operations. Therefore, establishing these metals and their surface roughness conditions as optimal for future testing and development to ultimately achieve new options to be used as implants in the human body.

1. INTRODUCTION

Certain living cells can attach in a plethora of different ways to surfaces depending on their geometry and surface chemistry. Understanding cell response and proliferation based on material surfaces is vital for various fields of research ranging from clean water supply to human implants [1]. In this area of research, a lot of experiments have been done both *in vitro* and *in vivo* to investigate the physical and chemical properties that affect cell proliferation [2-5]. Very little is known about cell growth and attachment onto machined surfaces. Majority of the researched cell attachment on metals is done through very precise sample preparation in the hopes of achieving specific surface roughness [1]. This study will explore a range of surface roughness values and material types to see how living cells react to the different conditions. One of the main benefits of this study is the juxtaposition of metal samples that are prepared through the traditional method, by grinding, and samples that undergo a milling operation, both yielding good surface conditions for cell proliferation. Combining machining and life sciences in this way allows for a collaboration that can result in quicker and more cost-effective solutions by performing direct experiments on metal surfaces. This study aims at investigating the surface roughness effects on cell proliferation, and determining the optimal surface roughness conditions for the different metals that would be viable options for use as implants in the future.

2. RELEVANT THEORY

There is a good understanding in this field of research that the physical and chemical properties of the material surface affect cell proliferation [2, 4, 6-8]. Huang *et al.* [7] have found that an arithmetical mean surface roughness value (Ra) of 0.15 µm is optimal to achieve the highest proliferation rate. They were able to determine a bell-shaped curve, which can be seen in **Error! Reference source not found.** labelled as areas I and II for cell proliferation within 0.05-1.20 µm range. Keller *et al.* concluded that a rougher surface (sandblasted) experienced an order of magnitude more percent cell attachment when compared to the smooth (1 µm diamond paste polished) surface [6]. This was combined with Huang *et al.*'s conclusion in **Error! Reference source**
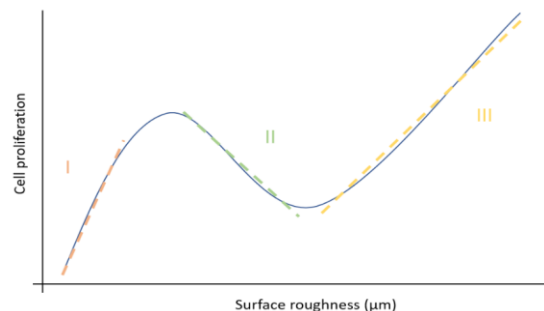
not found., where III shows Keller *et al.*'s experienced trend.

Figure 1: The three experienced surface roughness effects based on the investigated literature currently conducted in the area of cell proliferation

Chung *et al.* concluded that from a nano-scale perspective an increase in surface roughness lead to better cell attachment which is symbolized as I in **Error! Reference source not found.**. On the other hand, [2] has shown opposite findings, where a smoother surface has led to a higher initial adhesion as well as a more increased spreading on the surface. This poses the question, as to whether cell proliferation should only be counted in terms of cell density, or if types of attachment should also be investigated. Therefore, in this study the machined samples will be evaluated based on both cell density, and the type of attachment.

2.1 METAL SPECIFIC PROPERTIES

Magnesium (Mg) alloys are generally not used as biomaterials due to their poor corrosion properties [9]. However, they have many advantages such as; similar to bone density, they are readily available, have high specific strength even when compared with Ti6Al4V. Li *et al.* investigated the cytotoxicity of magnesium and concluded that there were no inhibitions in cell proliferation with regards to their exposure to magnesium alloys [9].

A lot of research on the chemical properties of metal samples has been conducted in the dental industry. One of the most prominent studies was by Grill *et al.* [5] where they found that Au is effective in maintaining cell viability [4] when combined with other alloying elements as long as the weight percentage (wt.%) is above 71 [10]. Through cell culture tests Craig *et al.* experimented on 29 alloys, and 6 pure metals. The pure metals are of interest to this study and those were: Au, Pd, Ti, Ag, Ni, and Cu. They concluded that Au, Pd, and Ti were least cytotoxic, whereas Cu carried the highest cytotoxicity when compared with the other pure metals [4].

3. EXPERIMENTAL METHODS

The metals used are magnesium alloys (AZ61a, AZ31), Cu-based alloys (CW510L), aluminum alloys (Al 7075), and titanium alloys (Ti Grade 2, Ti Grade 11, and Ti6Al4V). All samples went through two processes either grinding for specific surface roughness targeting, or milling with the use of the SECO tool. The samples were imbedded into a resin to allow for easier sample handling. This was done using the Struers CitoPress -5/-15/-30 machine with the MultiFast resin.

TARGETING SPECIFIC SURFACE ROUGHNESS

The grinding process used in this experimental process was surface grinding using the Struers Tegramin 30 grinding machine. The setup for grinding had to vary based on material hardness, to allow for proper surface roughness targeting. The hardness testing was performed using the Vickers hardness scale. The surface roughness values of Ra and Rt were recorded using the ALICONA IF-MeasureSuite.

MILLED SURFACES

One sample from each metal group underwent a milling process with the SECO tool (The tool holder: R217.69-2532.0-06-8AN, and tool insert: XOEX060204FR-E03 H15). The cutting data used was the same for all samples: 560RPM, 55 mm/min feed, and 0.05 mm depth of cut. The tool was used with only one insert to allow for a coherent surface finish.

CELL DEPOSITION

A549 cells were cultured in flasks until a specific cell density (cells/cm^2) was reached. The cells were lifted from the flask into the F-12K suspension using trypsin which is an enzyme that cleaves focal adhesion points thus removing surface adhesion [11]. PMMA tubes 10mm diameter, 1.5mm thickness, and 10mm height were attached to the areas of interest on the metallic samples using a double-sided tape. The samples, and tubes were all sterilized with 70% ethanol. The cells were then deposited into the tubes. Once deposited the cells were incubated for a period of 48 hours to allow for proliferation. The goals and cell amounts used in the respective trials can be seen in Table 1.

Experiment	Goal	Number of cells	Goal achieved
1	Determine effects of surface roughness on cell proliferation	17000	Y
2	Determine cell proliferation on machined surfaces	14000	Y

Table 1: Cell quantity and description of experiment for the 3 trials

QUANTIFICATION

Cells were rinsed with phosphate-buffered saline (PBS) solution twice to remove any dead cells from the surface. The cells were fixed during a 15min exposure to 4% paraformaldehyde. The cells underwent an ethanol dehydration series from 30% up to 95% ethanol to allow them to retain their cell morphology in the dried state necessary for secondary electron microscope (SEM) analysis. The surface of the samples was then sputter coated with more than 15nm of Au/Pd. The sputtering was done with chamber pressure $7x10^{-2}$ mbar, and a current of 15mA, for 250 seconds. This was done due to the non-conductive nature of cells which would result in them charging during SEM analysis, yielding poor image quality. Most of the SEM analysis was performed at 15kV, however for samples where an oxidized layer was present (AZ31, and AZ61a) the accelerating voltage was increased to 20kV. The characteristic sample field view was at 1 or 2mm which allowed for cell counting. The cell counting was performed using image analysis techniques, quantified through area fractions.

RESULTS & DISCUSSION

Ti Grade 2 and Ti Grade 11 were used with varying surface roughness values. Figure 2 shows the images that were taken for 4 different surface roughness values. Through x-ray energy dispersive spectroscopy (XEDS) analysis the cells, which are carbon-based, were determined to be the dark spots in the back-scatter electron (BSE) images.
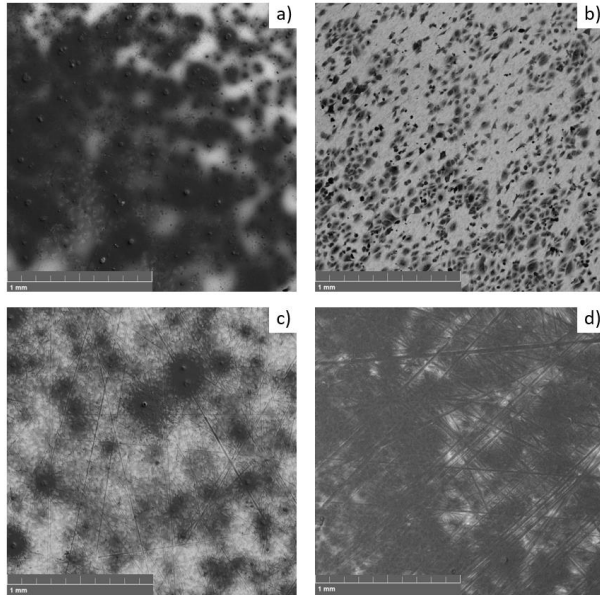

Figure 2: SEM images of the Ti Grade 2 sample using BSE imaging at the various surface roughness specifications; 0.158 µm (a), 0.212 µm (b), 0.263 µm (c), and 0.354 µm (d)

Error! Reference source not found. shows that the area fractions for the smoother and rougher surface roughness values are above the threshold. In this case the threshold value is based on the cell proliferation on glass slides which were used as a reference. As previously mentioned by Huang *et al.* the most cell proliferation was seen around a surface roughness of 0.15 µm [7]. Additionally, the rougher end of the spectrum shows an increase in cell proliferation. A further investigation with a larger range of surface roughness values would be beneficial to determine whether 0.15 µm is a local maximum as suggested previously. Chung *et al.* concluded that on a nano-scale an increase in surface roughness leads to more cell proliferation and better cell adhesion [3]. This would suggest that inversely from the 0.15 µm surface roughness the cell proliferation and adhesion should decrease. Or if this increase in cell proliferation continues increasing with reduced surface roughness as suggested by [2]. Since the literature related trends were observed in this method testing experiment, it is valid to assume method correctness.
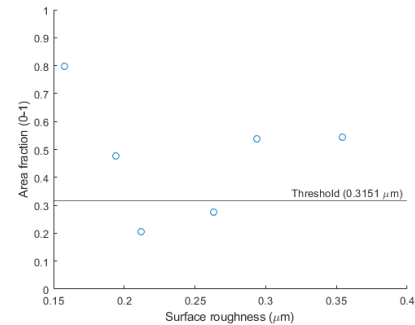

Figure 3: Area fraction graphical representation for the Ti Grade 2 and Ti Grade 11 samples, with the 'Threshold' value from the Glass (reference)

MACHINED SURFACE ANALYSIS

The goal of classifying the cells into specific groups is to identify which samples performed best in terms of cell density, and whether cells attached. The first is easy since it is a direct result of the area fractions which can be seen in Figure 4. To determine whether the cells attached and were thriving in the metal conditions the shape and surrounding areas around the cells were analyzed. Firstly, if the cells were adhered and thriving the areas around the cells would be a lighter shade of grey with carbon deposits which would be the result of the cells spreading and adhering on a larger area. Secondly, the actual shape of the cells can be used to determine if the cells deemed the surface as toxic, or uninhabitable. If the cells were more circular in shape, they were in an environment that is not as prosperous when compared to cells that were of a more elongated/amorphous shape.

Error! Reference source not found. (b) shows the CW510L sample which consists of copper-rich environments which are known to be toxic to cells, as a result the cells remained in circular shapes and did not spread out. This is consistent with previous research [4, 5, 10], however a preferred attachment site can be seen. An XEDS spot analysis would be beneficial in order to determine if the less copper-rich surface was the one with higher cell attachment.
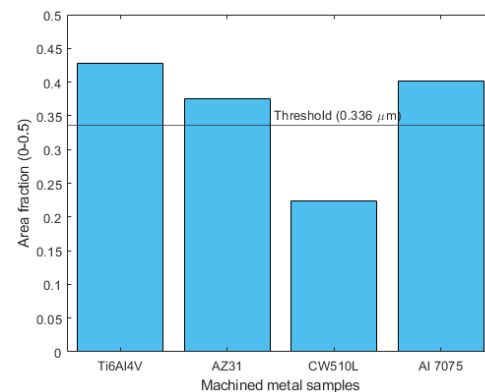

Figure 4: Area fraction values for cell proliferation for the milled samples compared to the threshold value of 0.336 µm
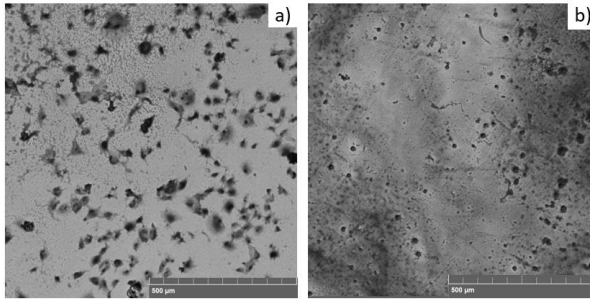
Figure 5: BSE images of different cell attachment types; (a) amorphous cell spreading, (b) circular cell spreading

Error! Reference source not found. (a) shows the Ti6Al4V sample and the cells in their amorphous shape **Error! Reference source not found.**shows the ranking of the different samples based on their attachment.

Table 2: Cell attachment type

Sample	Cell attachment type
7075 Al	Amorphous
AZ31	Slightly amorphous
CW510L	Circular
Ti6Al4V	Amorphous

CONCLUSION

The highest cell proliferation was seen around 0.15 µm. The two lowest cell proliferation results were seen in the middle range of surface roughness values at 0.212, and 0.263 µm. Both of these results were also below the control sample. All the samples (AZ31, Al 7075, and Ti6Al4V) except for CW510L were above the threshold value of 0.3364 µm. Ti6Al4V and Al 7075 showed clear amorphous shapes meaning the cell thrived on those surfaces. AZ31 was considered 'slightly amorphous' which meant that some of the cells looked circular, while most exhibited an amorphous shape. CW510L was seen to only contain circular shaped cells. Finally, AZ61a did not yield any conclusive results due to a heavily oxidized layer which prevented cell counting. The thesis findings can be used as a suitable workflow for future evaluation of cell proliferation on metal surfaces. It can be further utilized as a screening process for future implants based on the proliferation requirements.

ACKNOWLEDGEMENTS

REFERENCES

[1] B. Boyan, "Role of material surfaces in regulating bone and cartilage cell response," Biomaterials, vol. 17, no. 2, pp. 137-146, 1996-01-01 1996, doi: 10.1016/0142-9612(96)85758-9.

[2] K. Anselme et al., "The relative influence of the topography and chemistry of TiAl6V4 surfaces on osteoblastic cell behaviour," Biomaterials, vol. 21, no. 15, pp. 1567-1577, 2000-08-01 2000, doi: 10.1016/s0142-9612(00)00042-9.

[3] T.-W. Chung, D.-Z. Liu, S.-Y. Wang, and S.-S. Wang, "Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale," Biomaterials, vol. 24, no. 25, pp. 4655-4661, 2003-11-01 2003, doi: 10.1016/s0142-9612(03)00361-2.

[4] R. G. Craig and C. T. Hanks, "Cytotoxicity of Experimental Casting Alloys Evaluated by Cell Culture Tests," Journal of Dental Research, vol. 69, no. 8, pp. 1539-1542, 1990-08-01 1990, doi: 10.1177/00220345900690081801.

[5] V. Grill et al., "The influence of dental metal alloys on cell proliferation and fibronectin arrangement in human fibroblast cultures," Archives of Oral Biology, vol. 42, no. 9, pp. 641-647, 1997-09-01 1997, doi: 10.1016/s0003-9969(97)00055-1.

[6] J. C. Keller, "Tissue Compatibility to Different Surfaces of Dental Implants: In Vitro Studies," Implant Dentistry, vol. 7, no. 4, pp. 331-337, 1998. [Online]. Available: https://journals.lww.com/implantdent/Fulltext/1998/07040/Tissue_Compatibility_to_Different_Surfaces_of.12.aspx.

[7] H.-H. Huang, C.-T. Ho, T.-H. Lee, T.-L. Lee, K.-K. Liao, and F.-L. Chen, "Effect of surface roughness of ground titanium on initial cell adhesion," Biomolecular Engineering, vol. 21, no. 3-5, pp. 93-97, 2004-11-01 2004, doi: 10.1016/j.bioeng.2004.05.001.

[8] R. Lange, F. Lüthen, U. Beck, J. Rychly, A. Baumann, and B. Nebe, "Cell-extracellular matrix interaction and physico-chemical characteristics of titanium surfaces depend on the roughness of the material," Biomolecular Engineering, vol. 19, no. 2-6, pp. 255-261, 2002-08-01 2002, doi: 10.1016/s1389-0344(02)00047-3.

[9] L. Li, J. Gao, and Y. Wang, "Evaluation of cyto-toxicity and corrosion behavior of alkali-heat-treated magnesium in simulated body fluid," Surface and Coatings Technology, vol. 185, no. 1, pp. 92-98, 2004-07-01 2004, doi: 10.1016/j.surfcoat.2004.01.004.

[10] D. Brune, "Metal release from dental biomaterials," Biomaterials, vol. 7, no. 3, pp. 163-175, 1986-05-01 1986, doi: 10.1016/0142-9612(86)90097-9.

[11] M. A. H.R.H Patel, I.S. Shergill, Basic science techniques in clinical practice, British Library: Springer-Verlag London Limited, 2007.

[12] K. Sharma, "Machined surfaces as designed cell culture substrates", Master's thesis, Department of Mechanical Engineering Sciences, Lund University, 2021.