Comparative study of cell growth and cellular adhesion on Ti-6Al-4V surfaces made by Selective Laser Melting followed by different surface post processing steps

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Abstract. Selective laser melting is generally considered as to improve the design of medical implants, thus supporting medical treatment and maintaining mobility of invalid and older people. In particular, medical grade titanium alloys are in favour for spinal implants, as being nowadays manufactured by, e.g., milling. Selective laser melting offers the advantage of an adapted elasticity as to avoid stress shielding within the backbone by including complex lattice structures inside the individualized implant. For the integration into the backbone, surface properties, particularly surface roughness, are crucial with respect to biocompatibility and cell growth. Opposite to conventional milling, selective laser melting, however, results in an inferior surface roughness, leading to the necessity of downstream process steps. We report on cell growth and cellular adhesion of human primary fibroblasts on medical grade Ti-6Al-4V fabricated by selective laser melting followed by combinations of milling, hot isostatic pressing, chemical surface treatment and steam sterilization to generate different surface conditions for cell growth. For example, cell growth is studied for varying milling path spacing on SLM parts exhibiting different surface roughness. Our results reveal good cell growth for milling path spacing lower than 0.18 mm as compared to higher milling path spacing and not milled surfaces. Cell fluorescence images and SEM images show that the cell growth is additionally hampered by the edges of the milling path. Conveniently, process failures such as pores originating from the selective laser melting process do not hamper the cell growth.

1. Introduction
Due to the worldwide increasing life expectancy, the number of elderly people rises strongly. This sociodemographic development effects numerous areas of life and results in both entrepreneurial and social challenges as well as high demands on medical technology. In addition to the treatment of degenerative diseases of the central nervous system, the main focus is on maintaining mobility. This increasing demand inevitably leads to growth in the medical technology sector. Selective laser melting (SLM) is generally considered as to improve the design of medical implants, thus supporting medical treatment and maintaining mobility of invalid and older people. In particular, medical grade titanium alloys are in favor for spinal implants, as being nowadays manufactured by, e.g., milling or casting. Despite numerous efforts, however, milled implants may still exhibit strong deficits, such as, e.g., stress shielding, which occurs due to the high stiffness of implants as compared to natural bone tissue. It leads
to a capacity underload of the protected natural bone, which in turn may result in a reduction in bone density and weakening of the healthy bone tissue. Stress shielding occurs when the insertion of the implant results in mechanical shielding of the remaining tissue, thus damaging the balance between formation and degradation in the bone tissue [1]. To avoid stress shielding in SLM produced implants, Fousová et al. produced complex lattice structures of 316L stainless steel to mimic mechanical properties (especially elasticity) similar to trabecular human bone [2]. With fully porous material produced by SLM, stress shielding was reduced by 75% compared to a fully solid implants [3]. With porous materials produced by SLM, the Young's modulus can be varied by one order of magnitude to adapt it to the application and to avoid stress shielding [4]. SLM even allows a mixture of powders like hydroxyapatite (a matrix for bones) and 316L stainless steel to produce a material with tensile strengths close to human bones [5].

Since literature reveals the possibility to deliver implants with the required elasticity [6], biocompatibility is a second important requirement to implants. While in the past, various materials were used for implants such as different high, medical-grade steels and cobalt-chrome based alloys [7], nowadays usually titanium alloys are preferred because of its good biocompatibility [8].

Next to the Ti-6Al-4V alloy also Ti-35Zr-28Nb [9], Ti-30Zr-7Mo [10] or Ti-10Ta-2Nb-2Zr [11] reveal high biocompatibility. To evaluate biocompatibility, mainly cell growth behavior tests with fibroblasts or osteoblast cells [12] are performed, the results of such studies, however, do not only depend on the material but also on the specific surface properties. The high surface roughness after the SLM process [13] is generally hampering the cell growth and results in a low biocompatibility [8], thus post processes are required. To improve the cell growth behavior after SLM, exhibiting pores with a size of up to 250 µm, Matena et al. functionalized the SLM surface with a polycaprolactone (PCL) coating [14]. In Ref. [15], chemical etching was found to be the most effective surface post-processing treatment (vs. sandblasting, polishing) to improve cellular affinity and simultaneously increase the fatigue performance.

In order to further improve the functional accuracy of the implant, i.e. to provide a reliable interface for the insertion tool or to remove unwanted support structures, generally, a milling process after SLM is compulsory. Therefore, the objective of this contribution is to evaluate the cell growth behavior of SLM build Ti-6Al-4V alloy which is post processed by milling compared to other surface treatments.

2. Experimental

2.1. Process Chain

The complete process chain, employed in this study, is shown in figure 1, beginning with the building the of the titanium samples using SLM. Subsequently, the samples are optionally milled, sandblasted or untreated handled to the following hot isostatic pressing step. After pressing, the samples are removed from the base plate by wire EDM. Before the cell growth tests, the surface is etched and cleaned to remove nitrogen residues from the hot isostatic pressing process.

![Figure 1: Process chain](image-url)
2.2. **Selective Laser Melting**
For the SLM process, DMG Mori Lasertec 30 machine is used to process Ti-6Al-4V (material number 3.7165). Samples are cylinders with a diameter of 15 mm and a height of 7 mm to fit into the cell growth measurement vessel. The samples are directly build on the base plate without support structures to increase the accuracy and stiffness for the milling step. The layer height during SLM is 50 µm which requires 140 passes to produce the cylinders. As laser process parameters, a laser power of 220 W and a scan velocity of 0.74 m/s are used in combination with a hatch distance of 0.10 mm. This parameter combination results in high density and comparatively low surface roughness of the samples in the range of 10 µm.

2.3. **Milling and other mechanical post processing**
For the milling process, which is depicted in figure 2a, a ball cutter with a diameter d of 2 mm is used. The most influential parameters on surface roughness after milling are the milling path distance a_s, the feed per tooth f_z and cut velocity v_c, respectively. While the feed per tooth and the cut velocity are limited by the manufacturer’s specification in order to reduce tool wear and are set to a cut velocity v_c of 190 m/min and a feed per tooth f_z to 0.03 mm, the milling path distance can be varied over a wide range. The required rotation speed n and feed rate v_f can be calculated with the equations 1 and 2.

\[
\begin{align*}
    n &= \frac{v_c}{d \pi} \quad (1) \\
    v_f &= z \cdot f_z \cdot n \quad (2)
\end{align*}
\]

The calculation results in a rotation speed of 30 240 1/min and a feed rate of 1 815 mm/min. According to figure 2b, the surface shows a defined waviness with a distance between the valleys and the depth of the valleys defined by the milling path distance. Alternative to the milling process and for comparative reasons, further samples are also sandblasted manually or are brought untreated to the following process step (as build).

![Milling paths](image)

**Figure 2:** Milling path on the specimen (a) and cross section of the milled surface (b)

2.4. **Hot isostatic pressing and chemical post treatment**
For medical implants, a high service life and thus the reduction of residual stresses in the material are required, which means in particular defect-free microstructures and high material strength. However, since additively manufactured components do not adequately meet these requirements, post-treatment of the specimen is inevitable. For this purpose, hot isostatic pressing (HIP) has been established, which allows for the reduction of inner defects such as pores and cavities and for an increase of density [16]. Furthermore, hot isostatic pressing can significantly reduce the residual stress in the component. This leads to an increase in the fracture toughness and thus to a significantly stronger plastic area. As a result,
the HIP process has also influence on the machinability [17] and therefore on the achieved roughness after milling.

The material Ti-6Al-4V used here is present as α-martensite after the SLM process, due to the high cooling rate. When the samples are hot isostatically pressed below the β-transus temperature (995 °C), it converts to a lamellar-shaped mixture of α and β fractions. In this state, fractions of pure α-martensite are still present. If, on the other hand, a temperature above the β-transus temperature is selected for the HIP cycle, large β-grains are formed, which convert to a lamellar α-β mixture during the cooling process [17, 18]. In detail for the hot isostatic pressing of our samples according to the norm ASTM F2924 – 14, a temperature of 925° C and a pressure of 100 MPa are used for a duration of 3 hours. This HIP process is necessary for the cell growth behaviour test because the ratio α and β phase influences the cell growth behaviour strongly [19] and so the parameters of the HIP process directly influence the biocompatibility.

To remove the samples from the base plate wire, EDM is used to ensure good height accuracy. Since the samples show undesired contamination of nitrogen at the surface after hot isostatic pressing, they are, additionally, chemically post treated. For this purpose, the samples are placed in boiling hydrogen peroxide for a total of 20 minutes and then cleaned with isopropanol and water.

2.5. Cell growth measurement
To make a statement about and rate the biocompatibility of SLM build parts, the cell growth behaviour of fibroblast cells is investigated which gives information of the cytotoxicity and adherence of the surface. For the cell growth test, the specimens are first steam sterilized at a temperature of 121° C for 20 minutes. The cells are seeded with a density of 5 000 cells/cm² together with a cell suspension on the specimen. After a period of 24 h, the nutrient solution is changed. After reaching confluency, the largely gapless coverage of the surface of a culture vessel with adherent cells, equilibration (reseeding of the cells) is performed in a period of 30 minutes. The seeding density corresponds to 10 000 cells/cm². After further 24 h in the incubator (37° C), another 300 μl cell suspension is given to the test specimens, so that uniform wetting can be ensured. The evaluation of the test results is performed after 72 h.

For optical examination and determination of fluorescence intensity, the agent Resazurin is used. This is a non-toxic, water-soluble, blue redox dye, which is converted by cell activity to pink, fluorescent Resorufin. Therefore with higher cell activity the amount of Resorufin increases, which the amount of Resorufin can be determined by fluorescence intensity measurement. Cell vitality can be determined first optically and in a further step via fluorescence intensity. Optically, the color purple represents a high cell presence and so the local distribution of the cell activity can be determined. The measurement of a high fluorescence intensity indicates that a lot of Resazurin has been converted and thus speaks for a high cell activity.

After optical examination of the specimens under the fluorescence microscope and measurement of the fluorescence intensity, the cells are fixed for further examination using the scanning electron microscope. The cell fixing is performed by subsequently dehydrating the cells through an ethanol series (10%, 30%, 50%, 70%, 85%, 95%, and 100% ethanol, 15 minutes each) in distilled water. Finally, the samples are dried. For this purpose, hexamethyldisilazane (HDMS) functions as a chemical drying agent. The samples are first placed in a mixture of 50% HDMS and distilled water for 60 minutes and then remain in 100% HDMS for 5 minutes.

3. Results

3.1. Surface roughness
To evaluate the different types of surface roughness directly after the SLM process, after milling and after sandblasting, SEM images of the surfaces are shown in figure 3. For the as build samples in figure 3a it can be seen that those samples have a large number of incompletely molten metal powder particles on the surface. Position and size do not follow any pattern. Furthermore, the melt paths and their
intersections can also clearly be identified. The samples are characterised by a high surface roughness of about 10 µm and a high standard deviation of the surface roughness for different sections of the surface. The sandblasted surfaces (figure 3b) are characterized by the highest roughness. The large sand particles result in large indentations on the surface with sharp edges. On the milled specimen (figure 3c), the valleys shaped by the path of the ball cutter can clearly be identified. Despite the milling process, individual metal powder particles can still be identified. The distribution of these particles are significantly responsible for the standard deviation of the roughness over the sample.

Figure 3: SEM Images of as build surface (a), sandblasted surface (b) and milled surface (c)

The surface roughness $R_a$ measured with a laser scanning microscope for the different post processing before HIP, after HIP and after the etching step is shown in figure 4. It is obvious to see that the roughness increases continuously with the milling path distance, which is caused by valley structures produced by the ball cutter (cf. figure 2b). With higher milling path distance, the valley gets deeper and therefore the roughness increases. For milling path distances lower than 0.18 mm, the roughness saturates in the range of 1.5 µm, i.e. lower distances only result in longer cycle times but not lower roughness. Generally, the roughness for the milled surfaces is lower as compared to the as build

Figure 4: Average roughness as function of the milling path distance
surfaces or sandblasted surfaces which have a similar $R_a$. The HIP process itself has only minor influence on the roughness but the following etching step reduces the roughness significantly, especially for the samples milled with high milling path distance and the sandblasted samples. The reason for this is that the ridge between the valleys is removed partly together with remaining melt particles.

![Figure 5: Surface topology with a milling path distance of 0.28 mm (a) and 0.14 mm (b)](image)

To evaluate the reason for the saturating roughness for milling path distances lower than 0.18 mm, scanning electron microscope images are taken to show the topology in figure 5. For a milling path distance of 0.28 mm (figure 5a), the valleys and the ridge between them can be seen clearly. Beside this valley structure, the surface is quite smooth. For the milling path distance of 0.14 mm (figure 5b), the ridge is ruptured, not continuous anymore and the roughness is caused by lined up peaks. These peaks are not removed by lower milling path distance because of the blade rotation so the roughness doesn’t decrease further.

3.2. Cell growth
In order to evaluate the cell growth behaviour, the described cell growth tests are performed with a series of samples with different milling path distances and the as build and sandblasted samples as control group. To handle the typical outliers during cell growth tests, three test specimens each are combined into a category and their characteristic values are averaged. A seventh category represents the comparison group of as build and sandblasted samples. The fluorescence intensity determined here is shown in figure 6. It can be seen that the fluorescence intensity and therefore the cell growth behaviour decreases with increasing milling path distance.

In our experiments, optimal cell growth conditions, indicating superior biocompatibility, are observed for smooth surfaces as obtained by milling path distances between 0.12mm and 0.18mm. For both, a high median and arithmetic mean fluorescence intensity are measured. In contrast, for milling path distances between 0.24 mm and 0.42 mm, being characterized by a higher surface roughness, the fluorescence intensity is significantly lower as being characterized by a low median. This, in turn, is associated to a lower biocompatibility. Beside the apparent influence of the surface roughness, we observe a pronounced impact of ridges between the milling paths, which are observed for path distances higher 0.18 mm (cf. figure 5 and 6). Though specimen groups with path distances of 0.24 mm and 0.42 mm exhibit an arithmetic mean of the fluorescence intensity of halve the intensity at 0.12 mm, this intensity can be attributed to one significant positive outlier in each group which increases the mean and the standard deviation. Please note, in contrast to the median, the arithmetic mean is no robust statistic because it can be greatly influenced by outliers. In the specimen groups with 0.3 mm and 0.36 mm path distance, these outliers are missing which results in both low median and arithmetic mean. The comparison group with the sandblasted and as build samples reveal a median intensity of less than one third of the best results with milling path distances below 0.18 mm. Also here a positive outlier increases the arithmetic mean significantly but still this is less than half of the intensity as compared to the intensity for milled specimen with less than 0.18 mm distance. In summary, milling path distances
of 0.18 mm and below without ridges reveal a good median cell growth behavior while higher milling path distances and the comparison group (as build and sandblasted samples) are characterized with a much lower median cell growth, which is in accordance to Ref. [8].

To perceive the different cell growth behavior for different milling paths distances, two exemplarily chosen fluorescence images are depicted in figure 7. Fluorescence microscopy reveals vital cells by their blue coloration, indicating vitality of the cells and delivering information in which areas cells grow best. In figure a, a sample with low cell growth is shown which is indicated by a small amount of slightly fluorescent dots which are distributed over the surface. In figure b, small lines without cell activities are visible. These dark lines are the ridges between the milling paths which are obviously not suitable for cell growth. On the other hand, the cells show a good growth in the valleys of the milling path. Apparently, the ridges hamper the cell growth and with milling path distances above 0.18 mm the height

Figure 6: Fluorescence intensity as a function of the milling path distance

Figure 7: Fluorescence images with low cell growth (a) and high cell growth (b), please note the different scales
and ridges of the ridges increase significantly which is the reason for the low cell growth at high milling path distances.

3.3. Influence of pores
Parts build with the SLM process do always have a certain porosity [20] which depends on the material and laser machine parameter combination. Typical porosities in Ti-6Al-4V with good process parameters are in the range of 0.1 % to 0.3 % [21]. Because open pores are usually not completely closed by the HIP process and while during the milling process, pores can laid open, the surface is characterized by holes. To determine the influence of the pores on the cell growth behavior, SEM images of pores at the surface are taken after the cell growth and fixing process. In figure 7 a pore with a size of about 40 µm and oval shape can be seen and a cell that grew over the hole. The cells are obviously able to grow as a bridge over the hole and can therefore also close holes. As a result it can be stated that the holes produces by the SLM process are no drawback for the cell growth.

4. Conclusion
In this contribution, we determine the cell growth behavior on SLM build Ti-6Al-4V with different mechanical post processing for spinal implants applications. The post processing is performed by milling with a ball cutter or sandblasting. For milling, different milling path distances are chosen to achieve a different surface roughness. Before the cell growth test, samples are hot isostatic pressed, removed from the baseplate by EDM, etched and steam sterilized. Roughness measurements show a low surface roughness of 1.5 µm for milling path distances below 0.18 mm and increasing roughness with increasing milling path distance. For the sandblasted and as build samples, the roughness is in the range between 6 µm and 12 µm, respectively. Biocompatibility tests based on fibroblast growth reveal a good cell growth behavior for milling path distances below 0.18 mm with an associated good surface roughness. Samples with higher milling path distances or sandblasted samples are characterized by low median cell growth, however but positive outliers in some groups can increase the arithmetic mean significantly. Fluorescence images show that ridges between the milling paths observed at high milling path distances above 0.18 mm hamper the cell growth and results in a low cell growth. Process failures like pores from the SLM process are yet not hampering the cell growth.

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