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CLINICAL APPLICATIONS OF BIOMATERIALS

Original Research

In vitro cytotoxicity and surface topography evaluation of additive manufacturing titanium implant materials

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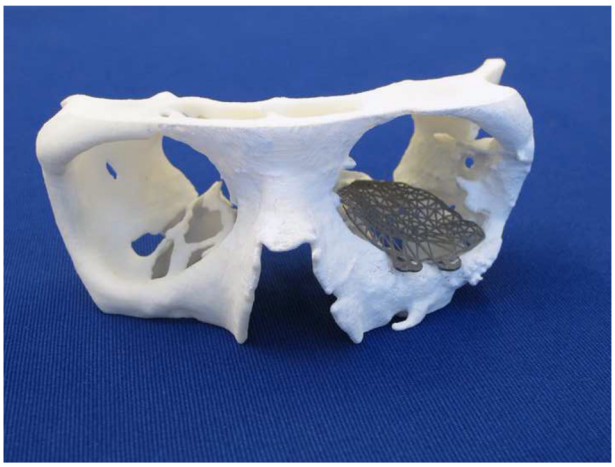
Abstract Custom-designed patient-specific implants and reconstruction plates are to date commonly manufactured using two different additive manufacturing (AM) technologies: direct metal laser sintering (DMLS) and electron beam melting (EBM). The purpose of this investigation was to characterize the surface structure and to assess the cytotoxicity of titanium alloys processed using DMLS and EBM technologies as the existing information on these issues is scarce. "Processed" and "polished" DMLS and EBM disks were assessed. Microscopic examination revealed titanium alloy particles and surface flaws on the processed materials. These surface flaws were subsequently removed by polishing. Surface roughness of EBM processed titanium was higher than that of DMLS processed. The cytotoxicity results of the DMLS and EBM discs were compared with a "gold standard" commercially available titanium mandible reconstruction plate. The mean cell viability for all discs was 82.6% (range, 77.4 to 89.7) and 83.3% for the control reconstruction plate. The DMLS and EBM manufactured titanium plates were non-cytotoxic both in "processed" and in "polished" forms.

Graphical Abstract



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1 Introduction

Large bone defects with variable shapes often pose a challenge in surgery. To date different materials are used to strengthen or replace different parts of the skeleton after trauma, resection of tumours or occasionally when treating congenital abnormalities. Currently most bony defects are managed using autologous bone grafts in combination with metallic and recently also with polymer/composite reconstruction plates. However, one major drawback of using

titanium plates is that during surgery such plates have to be manually manipulated, hence bent and cut to fit the defect site. This process of adapting stock titanium reconstruction plates to the bone defect can in some cases be cumbersome and time consuming, and more importantly, bending of the plate may predispose to fatigue fractures of the plate.

Recently there has been a rapid development in the area of customized implants for the treatment of aforementioned bone defects. Novel additive manufacturing (AM) technologies allow the pre-fabrication of patient specific, hence customized, reconstruction plates using computer tomography data of the patient. The major advantage of AM reconstruction plates is that they do not require any intraoperative bending and thereby offer a good passive fit to the defect site, better fatigue resistance and subsequently shorter operation time [1, 2].

In the literature, some studies [3] have reported that AM-processed material surfaces often result in a relative rough surface finish when compared with conventionally manufactured medical constructs. Therefore, in some applications like artificial joints the surface of the implant needs to be manually treated hence polished after the AM-process. Furthermore surface finishing is also required to eliminate surface flaws, which may predispose to fatigue failures. In this context, Sidambe et al. [3] reported that implant macrostructures and surface finishing have an affect on the overall clinical performance of AM implants.

To date a large group of patients have been successfully treated using customized metal implants that had been manufactured either using direct metal laser sintering (DMLS) or electron beam melting (EBM) [1, 4]. Currently, little is known about the surface structure and biocompatibility of the materials with respect to the AM technologies and surface treatment.

EBM technology utilizes a high power electron beam that generates energy to melt powder layer by layer. The electron beam is managed by electromagnetic coils, which provide an extremely fast and accurate beam. This allows several melt pools to be maintained simultaneously. DMLS technology uses lasers to sinter the metal powder layer by layer [5, 6]. Currently titanium and cobalt-chrome alloys are the most commonly used metals in medical AM.

A recent animal study by Stübinger et al. reported no differences in grade of osseointegration between milled, grit blasted, etched and DMLS titanium implants [7]. Furthermore, EBM-made titanium alloys have been reported to be biocompatible [8]. However, there is no information on whether biocompatibility of DMLS and EBM processed titanium alloys differ from each other. It can be hypothesised that the finishing process to eliminate surface flaws can leave remnants of the finishing compounds to the grain boundaries of the metal, which may influence biocompatibility of the material.

Thus, the objective of this study was to use cytotoxicity tests to determine the biocompatibility of two different titanium alloys that were processed and finished in two ways and furthermore, to characterize the surface topography of the processed titanium discs by scanning electron microscopy.

2 Materials and methods

Disc shaped TiAL6V4 ELI alloy specimens were manufactured using an EBM device (equipment manufacturer Arcam AB, Mölndal, Sweden and service provider FIT Fruth Innovative Technologien GmbH, Lupburg, Germany). The aforementioned EBM alloy fulfils the ASTM F136 [9] specifications. Furthermore, disc specimens were manufactured using DMLS technology and a Ti64 ELI alloy (Electro Optical Systems GmbH, Krailling, Germany). According to the manufacturer's information, the Ti64 DMLS alloy fulfils the ASTM F136 [9] requirements. A forged stock titanium alloy mandible plate (TICP, Synthes GmbH, Zuchwil, Switzerland) was used as a reference sample.

Preliminary metallurgical grinding of the discs was done using silicon carbide abrasive paper with a FEPA grit size of P320 to P4000 (ISO 6344). Final grinding was performed using a 3 µm diamond suspension and a polishing cloth. Lastly, all discs were purified using ultrasonic cleaning (ethanol) for three minutes. The surfaces of the EBM and DMLS discs were subsequently examined using scanning electron microscopy (SEM) to assess differences in the surface microstructure and surface topography.

All of the discs and the control construct were subsequently sterilized using a dedicated autoclave in accordance to the ISO 17664 [10] specifications before cytotoxicity testing. The specimens are presented in Fig. 1. The number of test specimens was as follows: EBM as processed $n = 8$, EBM polished $n = 8$, DMLS as processed $n = 8$, DMLS polished $n = 6$, and the control material specimen. Nominal test specimen dimensions in the CAD model were: thickness $t = 2$ mm, diameter $d = 17$ mm and surface area 560 mm^2. Actual average test item dimensions were as follows. EBM as processed, $t = 2.5$ mm, $d = 17.2$ mm, 596 mm^2 (6% over nominal value); EBM polished, $t = 1.5$ mm, $d = 16.4$ mm, 503 mm^2 (10% under nominal value); DMLS as processed, $t = 2.1$ mm, $d = 17.3$ mm, 586 mm^2 (5% over nominal value); DMLS polished, $t = 1.8$ mm, $d = 16.7$ mm, 532 mm^2 (5% under nominal value).

The test procedure carried out in this study was performed according to the recommendations of the ISO standards and also the number of the used specimens and test repeats followed the instructions of these standards [11–13].

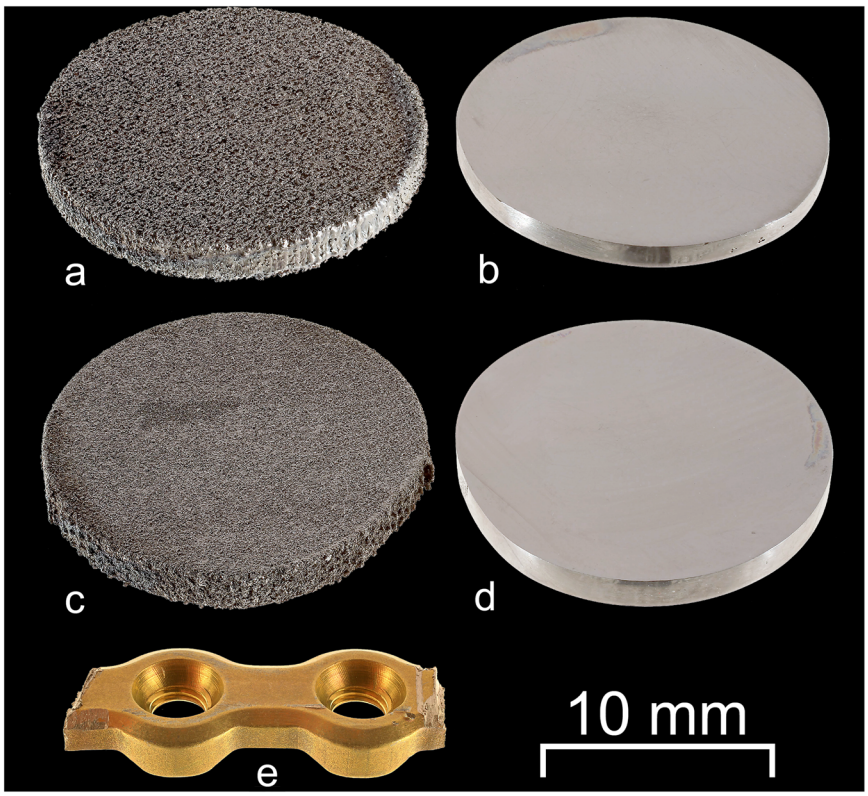

Fig. 1 **a** EBM test item as processed, **b** EBM test item polished, **c** DMLS test item as processed, **d** DMLS test item polished and **e** commercial mandible plate (control)

Surface topography of the specimens was analysed by measuring Ra values with the Innovatest TR-200 (Maastricht, The Netherlands) measuring equipment. The "EBM as processed"—specimens were out of the measuring range of the Innovatest TR-200 equipment and for these specimens the Taylor Hobson Form Talysurf Inductive 120 (Leicester, UK) equipment was used. The Ra values and standard deviations (SD) of three measurements were measured.

Positive Bioreaction (Lot: F0D014) (US Pharmacopeia, Rockville, MD, USA) was used as a positive control. The Positive Bioreaction test material contains an organo-tin compound, which, upon release in cell culture tests, causes cell damage. High-density polyethylene (Lot: H0F046) from US Pharmacopeia was used as a negative control. High-density polyethylene is considered to be non-toxic and non-hazardous. Cell culture medium was used as a blank control.

The extraction of the test items was done according to the ISO 10993 – 12 recommendations. The test items were submerged in Eagle's minimum essential medium with 10% FBS, at a ratio of 3 cm^2/ml medium, at $+37 \pm 1$ °C for 24 h. For test items 1, 3 and 4, four tests were performed. Each repeat consisted of two coin-like pieces. For test item 2, 3 repeats with two pieces were carried out. For test item 5 two repeats were carried out.

The total surface areas of the test specimens for each repeat and the medium volumes for extraction (3 cm^2/ml) were as following:

1. Test item 1 with 4 repeats ($T1_1$, $T1_2$, $T1_3$, $T1_4$), 11.2 cm^2, 3.73 ml
2. Test item 2 with 3 repeats ($T2_1$, $T2_2$, $T2_3$), 11.2 cm^2, 3.73 ml
3. Test item 3 with 4 repeats ($T3_1$, $T3_2$, $T3_3$, $T3_4$), 11.2 cm^2, 3.73 ml
4. Test item 4 with 4 repeats ($T4_1$, $T4_2$, $T4_3$, $T4_4$), 11.2 cm^2, 3.73 ml
5. Test item 5 with 2 repeats

$T5_1$, 6.9 cm^2, 2.3 ml
$T5_2$, 8.4 cm^2, 2.8 ml

The positive control (Positive Bioreaction) and the negative control (High-density polyethylene) were extracted in cell culture medium at a ratio of 3 cm^2 surface area/ml medium in same condition as those with the test items. Positive control: 2.6 ml (7.8 cm^2); negative control: 8 ml (24 cm^2).

The culture of L-929 cells was carried out based on instructions provided by ATCC. Cells were cultured for 2 passages in T75 flask in cell growth medium (Eagle's Minimum Essential Medium, 10% FBS, 1% antibiotic/antimycotic). Thereafter, 1×10^4 cells in a volume of 100 µl/per well were transferred to the 96 well microplates. Cells

were cultured in a cell culture incubator (Sanyo MCO 18) in humidified 5%CO_2 in air at +37 °C for 24 h, so that the cells formed half-confluent layers. Thereafter, cell culture medium was replaced by the test item extracts at concentrations of 100, 75, 50, 25 and 12.5% diluted in the normal culture medium as well as in the positive, the negative and the blank controls for a further 24 h. For the test specimens, the positive and negative controls, the extracts from each specimen will be applied to cells eight times (8 repeats = wells = 1 row). For the blank control (culture medium only), 16 repeats (2 rows) were used. Row 1 and Row 12 of each plate were not used.

At the end of the experiment time point, culture medium was replaced by 50 µl MTT solution (1 mg/ml) and cells were further incubated for 2 h in an incubator. Then MTT solution was discarded and 100 µl of isopropanol was added to each well. Thereafter, the plates were shaken gently and subsequently transferred to a microplate reader (Hidex Chameleon V) with a 570 nm filter to read the absorbance (reference wavelength 690 nm).

To calculate the reduction of viability caused by the test item compared to the blank the following equation was used:

Viability% = 100 × OD_{570e}/OD_{570b} where

Table 1 Surface roughness (Ra) of materials processed with EBM and DMLS methods

Process	Ra	SD
EBM as processed	29.94	1.721
EBM polished	0.085	0.007
DMLS as processed	7.867	0.084
DMLS polished	0.028	0.003
Reference	0.491	0.016

Reference material: Forged stock titanium alloy mandible plate. *SD* standard deviation

OD_{570e} = mean value of the measured optical density of the 100% extracts of the test item and

OD_{570b} = mean value of the measured optical density of the blanks.

According to the ISO standard 10993, when cell viability is reduced to <70% of the blank, the test item is considered to have cytotoxic potential.

3 Results

Surface roughness (Ra) was higher for EBM processed than for DMLS processed (Ra 29.94 and 7.867). Polishing lowered the surface roughness (EBM: 0.085 and DMLS: 0.028) (Table 1). Figures 2–4 show magnified surface finishes of the processed and polished test specimens. Figure 2 shows EBM and DMLS discs that had been manufactured using different titanium particle sizes, and thus, resulted in different surface topographies. The DMLS manufacturing process used smaller titanium alloy beads than the EBM process. Figure 3 shows a larger magnification of the disc surfaces, compared with the control materials surface. Both AM processes resulted in surface flaws. Figure 4 shows that the polished EBM and DMLS have homogeneous microstructures with some small defect/flaw areas.

The viability of blank control (cells cultured with only normal medium) was set as 100%. The viability of cells treated with test item extract (100% without dilution) was then compared with viability of the blank.

Fifty percent extract of the same test specimen means 1 volume of test item extract plus 1 volume of normal culture medium. All the tested items were found to be non-cytotoxic. The cell viability of test item 1 (Ti64 ELI EOS DMLS as processed): 89.67%; the cell viability of test item 2 (Ti64 ELI EOS DMLS polished): 77.40%; the cell viability of test item 3 (TiAL6V4 ELI EBM as processed):

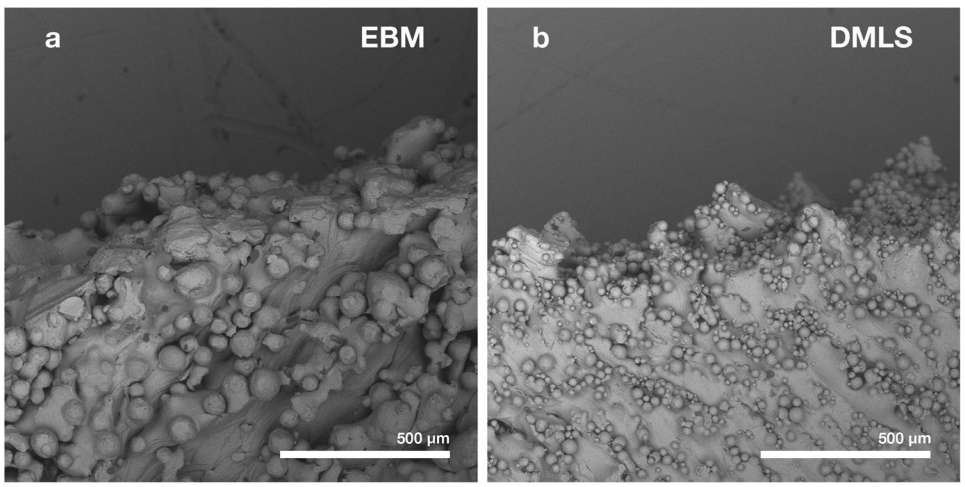

Fig. 2 SEM images of the surface finishes of as processed **a** EBM and **b** DMLS test specimens (original magnification: ×100)

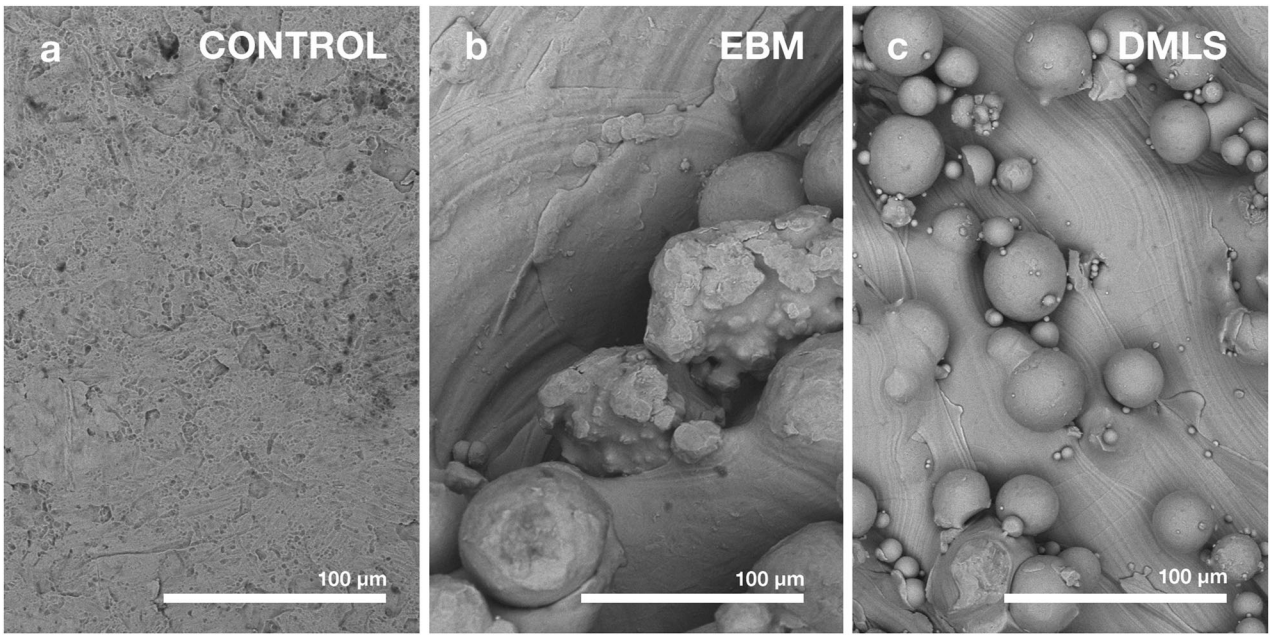

Fig. 3 SEM images of the surfaces of the as processed **a** control plate, and **b** EBM and **c** DMLS items (original magnification: ×500)

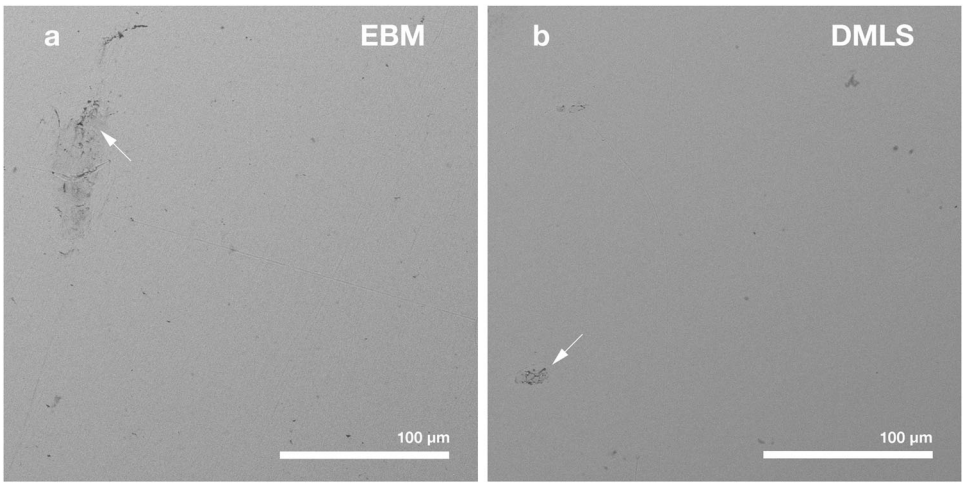

Fig. 4 SEM images of the surfaces of polished **a** EBM and **b** DMLS discs. Both samples contained defect areas of either not fully melted powder or gas pores in form of small fractures (*white arrows*), (original magnification: ×500)

80.74%; the cell viability of test item 4 (TiAL6V4 ELI EBM polished): 82.65%; the cell viability of test item 5 (Synthes lock mandible plate): 83.30%. Viability (%) of L929 cell 24 h after incubation with the extractions of the test items and the controls is summarized in Table 2.

4 Discussion

Recently there has been a rapid progress in the development of implant materials for the treatment of bony defects. The aim of this study was to investigate the in vitro cytotoxicity of titanium discs that were produced using DMLS and EBM technology. The acquired discs were subsequently compared to a control titanium reconstruction plate commonly used in maxillofacial surgery. In addition, the surface textures of the DMLS and EBM processed titanium alloys were characterized. Information of the surface texture of the AM-processed titanium helps our interpretation of the possible risks of fatigue failures which may initiate from the surface flaws of the material.

DMLS technology has been used to manufacture customized meshlike orbital implants [4] and mesh for bone augmentation [2]. Acetabular cup and femoral knee implants made by EBM are commercially available [14]. Also bone screws made using DMLS are in the market [15].

Table 2 Viability (%) of L929 cells 24 h after incubation with extractions of the test items and the controls

	100%	75%	50%	25%	12.50%
T1	89.67	89.53	90.47	91.45	91.53
T2	77.40	78.63	77.83	78.73	83.59
T3	80.74	79.78	80.15	81.15	81.15
T4	82.65	81.37	81.78	82.05	84.11
T5	83.30	82.54	82.07	85.49	87.89
PC	2.29				
NC	78.66				
Blanc	100				

Tn test item *n*, *PC* positive control, *NC* negative control, and *Blanc* blanc control

The Ti-6Al-4V brand used, EBM or DMLS design, polishing and sterilization all affect the implant macro-, micro- and nano-structure and chemical composition. Therefore, it was necessary to test the individualized implants for cytotoxicity using positive and negative controls and mass-produced clinical control implants. This was technically perfectly done using the current ISO standards.

Microscopical examination showed metal beads, which had not been melted in the DMLS or EBM process. Close to the titanium beads, some surface flaws could be detected. Surface flaws may behave as initiation areas for fatigue fractures if the implant is dynamically loaded like in mandible reconstruction. Polishing of the material surface seemed to remove majority of the surface flaw, although minor porosities could still be detected at the surface. Porosities could be either gas pores from trapped gas in the manufacturing process or unmelted powder beads.

All tested items were found to be non-cytotoxic after titanium had been subjected to the manufacturing, polishing and sterilization steps. It thus seems that DMLS and EBM produced individualized, custom-made implants do not cause any short-term adverse effects in form of cytotoxicity in peri-implant cells.

DMLS and EBM produced implants integrate to their surrounding tissues via their surface structures. Therefore, toxicity of the implants might impair both hard bone tissue (osseo)integration and soft tissue integration in short- and long-term [16]. Short-term cell and tissue damage might induce an acute inflammatory reaction, which might cause local site-specific damage and symptoms and contribute to the formation of an implant capsule and thus impair osteointegration, stability and the long-term outcome of the implant. Extraction method used according to the ISO standard 10993 – 1, 3, 5 gave assuring results considering the fact that the custom-made implant comes into contact with body fluids and cells upon implantation to the recipient. This interaction with the environment might initiate dissolution of harmful implant-derived products not necessarily derived from the parent biocompatible titanium compound [17] but from the substances and changes used and produced during additive manufacturing process. The EBM processed material used in this study showed a more porous structure than the DMLS surface and this might have an affect on the micromechanical locking and tissue-adhesion to the implant.

5 Conclusions

All the test pieces produced by Additive Manufacturing (AM) technologies and tested in the present study, seemed non-cytotoxic and would from this point of view be safe for clinical use as an alternative to the mass produced products which do not provide the patient- and site-specific adaptation and flexibility, which is characteristic to AM products. Surface of the titanium alloy showed some flaws, which were eliminated by polishing the surface. Surface finishing which in many clinical cases is required to finalize at least some surface areas of the AM implant does not influence to cytotoxicity of an implant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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