The biocompatibility of dense and porous Nickel–Titanium produced by selective laser melting

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ARTICLE INFO

Article history:
Received 19 January 2012
Received in revised form 16 August 2012
Accepted 17 September 2012
Available online 23 September 2012

Keywords:
Nickel
Selective Laser Melting
hMSC
Nickel release
Biocompatibility

ABSTRACT

Nickel–Titanium shape memory alloys (NiTi-SMA) are of biomedical interest due to their unusual range of pure elastic deformability and their elastic modulus, which is closer to that of bone than any other metallic or ceramic material. Newly developed porous NiTi, produced by Selective Laser Melting (SLM), is currently under investigation as a potential carrier material for human mesenchymal stem cells (hMSC). SLM enables the production of highly complex and tailor-made implants for patients on the basis of CT data. Such implants could be used for the reconstruction of the skull, face, or pelvis. hMSC are a promising cell type for regenerative medicine and tissue engineering due to their ability to support the regeneration of critical size bone defects. Loading porous SLM-NiTi implants with autologous hMSC may enhance bone growth and healing for critical bone defects. The purpose of this study was to assess whether porous SLM-NiTi is a suitable carrier for hMSC. Specimens of varying porosity and surface structure were fabricated via SLM. hMSC were cultured for 8 days on NiTi specimens, and cell viability was analyzed using two-color fluorescence staining. Viable cells were detected on all specimens after 8 days of cell culture. Cell morphology and surface topography were analyzed by scanning electron microscopy (SEM). Cell morphology and surface topology were dependent on the orientation of the specimens during SLM production. The Nickel ion release can be reduced significantly by aligned laser processing conditions. The presented results clearly attest that both dense SLM-NiTi and porous SLM-NiTi are suitable carriers for hMSC. Nevertheless, before carrying out in vivo studies, some work on optimization of the manufacturing process and post-processing is required.

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1. Introduction

Nickel–Titanium shape memory alloys (NiTi-SMA) exhibit mechanical and chemical properties which make them attractive candidate materials for various types of biomedical applications (e.g., stents, shape memory staples for compression osteosynthesis, interbody fusion devices, hip endoprosthesis and acetabular cups with integrated self-expanding NiTi elements) [1]. These alloys demonstrate good deformability that is associated with their pseudoelastic behavior. A mechanically imposed strain as high as 8% can be reversibly recovered after unloading. This provides a damage tolerance which is not demonstrated by other metals or ceramics. Most importantly, porous NiTi-SMA exhibits an elastic modulus (28 GPa) closer to that of bone (0.3–20 GPa) than any other metallic or ceramic material [2]. Metals commonly used for orthopedic implants have a Young’s modulus in the range of 100 to 200 GPa (~110 GPa for Titanium, ~190 GPa for austenitic stainless steel, and ~193 GPa for Co-based alloys) [2,3]. This large difference in stiffness between implant material and the surrounding human bone leads to an inhomogeneous stress distribution on the bone. This stress-shielding effect may cause bone resorption and weakens bone locally which results in aseptic implant loosening [3]. Due to its elastic modulus, NiTi-SMA may reduce the stress-shielding effect when used as an orthopedic implant material. It has been reported that implantation of NiTi-SMA as a weight bearing bone graft substitute led to osseointegration and a good bone–implant contact [4]. Nevertheless, the production of highly complex NiTi parts, which could be used as bone substitutes or implants, is challenging due to poor machinability of the alloy [5]. Several powder metallurgical processing technologies are suitable for the production of both dense and porous NiTi parts [6–8], but they often have limitations with respect to complexity. Generally, the production of single parts, e.g., individual implants, is challenging. In recent years, additive manufacturing (AM) has established promising methods for medical applications [9,10]. These laser assisted freeform fabrication methods provide special opportunities due to their use of the additive operation principle.
By building up material by adding layers instead of removing material, they offer a nearly unlimited flexibility of the part’s geometry and complexity. AM, therefore, has a high potential for the production of scaffolds, individual bone substitutes and implants [9,10]. While NiTi-SMA are gaining interest for both engineering applications and for medical applications, AM of NiTi has received less attention so far. To date, there are almost a dozen international research papers or conference proceedings concerning the processing of NiTi via AM techniques [11–20]. This paper reports on the fitness and biocompatibility of additive manufactured NiTi parts as implant material.

Only a few AM technologies are suitable for the production of load bearing metallic implants. One of those technologies is the Selective Laser Melting process (SLM). Fig. 1 shows several steps of the SLM-procedure. The starting point for this processing technique is a three-dimensional CAD model of the part (Fig. 1 A) which is sliced in horizontal layers of a defined thickness (Fig. 1 B). Each layer contains specific information regarding the geometry of the part. To fix the part on the substrate material and to enhance heat conduction during SLM processing, support structures are developed and added to the sliced CAD model (Fig. 1 B). These filigree support structures are built up from the same material and in the same SLM process as the desired part, but are removed mechanically afterwards. The SLM process is a series of repeats of the same procedure which applies powder layers and transfers the specific geometrical information of each layer into the material by melting the powder with a laser beam (Fig. 1 C). After solidification, a full metallurgical fusion between adjacent laser scan tracks (hatches) and layers yields the geometry of the part. Fig. 1 D exhibits a final NiTi structure which was realized by SLM. Further details on the SLM procedure in general and a detailed description of process parameters are reported elsewhere [18,21].

SLM enables the production of highly complex and tailor-made implants for patients on the basis of CT data. Such implants could be used for the reconstruction of the skull, face, pelvis, or vertebral body defects. For the reconstruction of critical size bone defects, precultivation of a scaffold with autologous mesenchymal stem cells can enhance osseointegration and lead to a stable bone implant contact [22]. Newly developed porous NiTi, produced by Selective Laser Melting (SLM-NiTi), is currently under investigation as a potential carrier material for human mesenchymal stem cells (hMSC).

hMSC can be differentiated in vitro into osteoblasts [23], chondrocytes [23], tenocytes [24] and adipocytes [23]. hMSC can be easily expanded in vitro and retain their developmental potential during extensive cultivation steps or cryopreservation [25]. It has been shown in animal models that the implantation of MSC supports the regeneration of critical sized bone defects [22]. For the treatment of local bone defects, expanded autologous hMSC may be applied when previously loaded on a porous SLM-NiTi carrier matrix. Osseointegration and wound healing may be improved. The scientific objective of the present work was to determine whether newly developed SLM-NiTi biomaterial is suitable for implants and a potential carrier for hMSC.

2. Materials and methods

2.1. Production of the specimens

The pre-alloyed powder used for these studies was initially produced by gas atomization (TLS Technik GmbH, Bitterfeld) of as-cast NiTi ingots (Fig. 2A) with the following chemical composition: 49.7 at.% Nickel and 50.3 at.% Titanium. Details are previously described [18]. Due to the moderate cooling rate during gas atomization, the powder particles feature a spherical shape (Fig. 2B). Particle size was ranged between $d_{10} = 45 \mu m$ and $d_{90} = 110 \mu m$ (Fig. 2C).

The SLM process was performed by using a commercial SLM system (Realizer SLM 100; MTT Technologies GmbH, Lübeck, Germany). This instrument is equipped with a continuous wave 100 W Ytterbium Technologies GmbH, now SLM Solutions GmbH, Lübeck, Germany). This instrument is equipped with a continuous wave 100 W Ytterbium fiber laser (wavelength 1070–1080 nm, TEM$_{00}$). Generally, high temperature processing of NiTi is highly complex. Powder or ingot metallurgical processing methods for NiTi are usually associated with a significant increase in impurity levels due to the high reactivity of the melt [26–28]. The functional properties of the material are very sensitive to the impurity content and can be decreased due to pick up of impurity elements [26,27]. Hence, the SLM process was carried out in argon atmosphere in order to minimize oxidation. In a recent study, we showed that successful SLM processing of NiTi is accompanied by only a slight pickup of oxygen (30 ppm) [18]. We also showed that using high quality ingot raw material resulted in an impurity level of both oxygen and carbon of SLM-NiTi below the limits for medical NiTi (oxygen: 0.05 wt.%, carbon: 0.05 wt.%) prescribed in ASTM 2063-05.

![Fig 1. The SLM-procedure: CAD model of the later part (A); CAD model prepared for SLM (B); laser–material interaction during SLM (C); finished NiTi-SLM structure (D; scale bar = 10 mm).](image-url)
The layer thickness was adjusted to 75 μm. Due to the particle size of the used powder material a further reduction of the layer thickness is impossible. The chosen layer thickness ensures an acceptable powder bed density and prevention of damage of both SLM part and the SLM system’s recoating mechanism.

Several different cylindrical SLM-NiTi samples (Fig. 2 D) were produced to analyze the influence of surface structures and porosity on biocompatibility. All specimens had the same geometrical dimensions (diameter: 9 mm, height 5 mm), but diverse strategies were used to adjust surface structures and differ amounts of porosity (Fig. 3). Due to the additive operation principle, the orientation of respective parts during SLM processing significantly influences the surface structure of the part. In the case of sample type I, the top face of the cylinder, which is formed by the upsides of the laser scanning tracks (hatches), is used for the analysis of biocompatibility. In contrast, the relevant face of sample type II is formed by the sidewalls of the laser scanning tracks due to the additive layer based process principle. Both sample types are produced with a hatch distance of 120 μm. This small distance assures a high density of each part. Sample type III represents a porous part which is produced in the same orientation as type I, but with a large hatch distance of 400 μm. The deficient connection of the scanning tracks results in a reduced relative density of 88.5%. The porosity of sample type IV was designed by a CAD model. The structure width as well as the width of the
quadratic pores was 500 μm, because this pore size seems to be optimal for bone ingrowth into porous NiTi implants [4]. All samples were manufactured by a bidirectional and xy-alternating hatchung scheme. The scanning direction is turned 180° for direct adjacent hatches and rotated 90° for the following slice. This scanning scheme ensures low porosities [21]. The density of each sample was determined by the Archimedes method.

The diameter of the laser beam at the position of the laser focus strongly influences the quality of SLM parts. Divergent laser focus positions can result in a rough surface with adherent powder particles. On the other hand distinct splatter of molten particles can follow from a sharp focused laser beam. Those particles can cause degradations of both surface quality and material density due to interruptions of the molten bath. To analyze the influence of the position of the laser focus on the particle release of the specimens, the four sample types were produced with two different diameters of the laser beam (61 μm and 128 μm) by shifting the focus position.

2.2. Cultivation of human mesenchymal stem cells

In order to analyze the biocompatibility of SLM-NiTi specimens, hMSC were cultured on different SLM-NiTi specimens. hMSC (3rd–6th passages) obtained from LONZA (Walkersville Inc., MD, USA) were grown in cell culture flasks (Falcon, Becton Dickinson GmbH, Heidelberg, Germany) using RPMI1640 medium (GIBCO, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Germany) using RPMI/FCS, and seeded on the specimens.

2.3. Reaction of cells

2.3.1. Viability

Live/dead staining in combination with fluorescence microscopy was used [29] to document cell viability. In live/dead staining, living cells give rise to a green fluorescence while dead cells appear red. After 8 days of cell culture, the cells were incubated with calcein AM (50 ng/ml, Calbiochem, Schwalbach, Germany) for 5 min at 37 °C using cell culture conditions. Subsequently, samples were washed with cell culture medium RPMI1640 followed by staining with propidium iodide (50 μg/ml, Sigma-Aldrich, Taufkirchen, Germany) for 15 min at room temperature while protected from light. After twofold washing with RPMI1640 the stained hMSC were analyzed using light and fluorescence microscopy (Olympus MVX10, Olympus; Hamburg, Germany).

2.3.2. Cytokine release

Interleukin-6 (IL-6), IL-8, and vascular endothelial growth factor (VEGF) released from hMSC were quantified in the cell culture supernatants using enzyme linked immunosorbent assays (ELISA). Antibodies, as well as recombinant human cytokine standards, were supplied by R&D Systems (Wiesbaden, Germany). Cytokines were quantified according to manufacturer’s instructions [30].

2.4. Nickel ion release

Nickel ion release was analyzed in the cell culture medium by atom absorption spectroscopy (AAS, SIMAA6000, PerkinElmer LAS GmbH, Rodgau, Germany). The detection limit for Nickel ions was 3.2 μg/l.

3. Results

The main objective of the experiments described here was to assess whether SLM is a suitable method to produce complex and highly porous hard tissue NiTi scaffolds. Therefore biocompatibility, including cell viability and cytokine release, was analyzed. Additionally, the nickel ion release during cell culture was quantified. Fig. 4 A–D shows a fluorescence micrographic overview of the cell-loaded NiTi specimens produced by different strategies. After 8 days of cell culture, all specimens were completely covered with a layer of viable (green fluorescence) cells (Fig. 4 A–D). Higher magnification images show more detailed information about cell–material interactions. A few dead cells (red fluorescence) could be detected at high magnification. The number of dead cells was comparable to the control (cells cultured without SLM-NiTi specimens in the same 24-well cell culture dish, not shown here). Inside the pores of the specimens, many viable cells were detected. Cell morphology was dependent on the specimen orientation during SLM processing (Fig. 4 E + F). The different processing strategies of the four specimens resulted in disparities in their surface topography. Those differences in surface topology are clearly visible in Fig. 5. The surface of sample type I (Fig. 5 A + E) is formed by the upsides of the hatchs. The low hatch distance produces a slight overlap of adjacent hatches and thus a closed surface structure. Sample type I produced with the smaller laser beam diameter (Fig. 5 E) exhibits an undulated surface structure whereas the greater beam diameter results in a relatively smooth surface (Fig. 5 A). In contrast to sample type I, the surfaces of sample type II exhibit considerably rougher structures (Fig. 5 B + F). This is due to the fact that the face of sample type II is formed by the sidewalls of the laser scanning tracks of the stacked layers. Remarkably, there are a high number of particles on the surfaces of the samples of type II. By downsizing the laser beam diameter the number of particles is reduced and the sidewalls of the hatches can be seen clearly (Fig. 5 F). In the case of sample type III, downsizing of the laser beam diameter results in an improved resolution of single hatchs (Fig. 5 C). The porosity produced by the large hatch distance appears well arranged by using the smaller beam diameter in contrast to the sample produced with a higher laser beam diameter (Fig. 5 C). A similar outcome can be obtained for sample type IV (Fig. 5 D + H). A reduction of the beam diameter results in an improved shape of the pores designed by the CAD model. In addition, we note that there are some spherical particles on the surface of each sample, which are significantly larger than the used powder fraction.

Before the cell culture experiments, specimens were sonicated (40 kHz, 30 min; room temperature) in ethanol twice in order to remove adherent particles. The size and shape of powder particles released during ultrasonic cleaning were visualized via SEM (Fig. 6). As shown in Fig. 6, both small and spherical particles matching the size of the starting powder, newly formed bigger spherical and non-spherical particles (Fig. 6 A–C, arrows), and non-spherical left-overs of the removed support structures (Fig. 6 D, arrowheads) were detected.

Fig. 7 A–C shows the results of the cytokine release from adherent hMSC including IL-6, IL-8 and VEGF. The co-cultivation of hMSC with the SLM-NiTi specimens did not lead to an increase in cytokine release compared to controls. IL-6 release is shown in Fig. 7 A. Cytokine concentrations in the cell culture media were 60% of control values. There was no difference in the cytokine release between the cells cultured onto different SLM-NiTi specimens. The release of IL-8 (Fig. 7 B) and VEGF (Fig. 7 C) was also lower than the control values. No significant influence of the focus diameter on cytokine release was detected. In general, the Nickel ion release of the dense samples (samples I and II) was lower than the release of the porous samples (samples III and IV, Fig. 7 D). Interestingly, the Nickel ion release is lower for sample type IV than for sample type III although sample type IV has a higher porosity. Nevertheless, the Nickel ion release of
all samples was lowered by reducing the diameter of the laser beam from 128 to 61 μm. For example, the ion release of sample IV decreased significantly from 258 ng/ml to 101 ng/ml.

4. Discussion

This study reports on the biocompatibility of NiTi biomaterial produced by Selective Laser Melting (SLM). The aim of this study was to assess whether SLM is a suitable method to produce complex and highly porous hard tissue NiTi scaffolds. In order to address this question, basic material properties, such as surface structure, ion release, and cellular responses, such as cell viability and cytokine release, were analyzed.

After 8 days of cell culture, all specimens were completely coated with a layer of viable cells. Inside the pores of the specimens, many viable cells were detected as well. These results are in agreement with our previous findings [31]. We previously showed promising results concerning the viability and adhesion of hMSC both on the surface and inside the pore structure of powder metallurgically produced NiTi samples produced by metal injection molding [31]. As shown in Fig. 4, cell morphology varies for all sample types. In general, cell morphology, orientation, and cytoskeletal organization are dependent on the stiffness and topography of the substrate [32,33]. As shown in Fig. 5, the topography of the samples strongly depends on CAD data and on SLM processing conditions (orientation, beam diameter, hatch distance). Therefore, in the future, surface topography and thus cell morphology, orientation, and cytoskeletal organization can be adjusted by varying the laser processing parameters.

Adherent particles were detected on the surface of all specimens. It is well known that in the human body particle release leads to adverse effects such as periprosthetic osteolysis and loosening of total joint arthroplasties [34]. Therefore, when applying a powder metallurgical approach, the analyses of loosely adherent particles and their release are of crucial importance when assessing the biocompatibility of the produced implant materials. In order to standardize the release of adherent particles, all specimens were sonicated in ethanol twice before conducting cell culture experiments. The powder volumes released during ultrasonic cleaning feature primary powder particles as well as
newly formed spherical and non-spherical particles. Some few larger particles (Fig. 6 A) were detected. That phenomenon is termed "balling" and can hardly be avoided during laser processing of powder materials. The reasons for the formation of these larger particles are not sufficiently understood. Kruth et al. trace this phenomenon back to melt pool instabilities and on the surface free energy of the melt. Gu and Shen identified two different causes for balling. The first cause is limited liquid formation during processing, and the second is laser-induced melt splashes caused by high capillary instabilities of the melt. Tolochko et al. described regrouping processes of powder particles under conditions of irregular laser heating as reasons for balling. This corresponds with the first cause of balling put forth by Gu and Shen. Additionally, some large and rough shaped particles in Fig. 6 D (arrowheads) represent leftovers of the supporting structure which must be used for the generation of the specimens via SLM (Fig. 3, red structures).

However, any release of loose particles is not tolerable for implants, but cannot be avoided completely by powder metallurgical processing technologies. Especially SLM parts tend to be susceptible. Nevertheless, we showed that both orientation and focus adjustment strongly influence the number of loosely sintered particles (Fig. 5 A–H). SEM micrographs of sample types I and II (Fig. 5 A–F) illustrate that a variable adjustment of the focus can improve the surface quality. Horizontal faces (sample type I) should be produced with a less sharp focused laser beam because of a large but plain laser intensity distribution within the laser–material interaction zone. Perpendicular walls (sample type II) should be produced with a focused laser beam. This can be ascribed to the sharp bordered melt region where less sintering of ambient powder particles on the surface occurs. Further reduction of particle release could be achieved by decreasing the layer thickness during SLM processing. However, this requires the application of small-sized primary powder fractions. Admittedly, impurity contents of the primary powder increase with decreasing powder size and, furthermore, the flowability of smaller powder fractions is limited because of powder agglomeration. However, an entire prevention of particle release of SLM parts seems to be impossible. Hence post-processing procedures like long-time ultrasonic cleaning or additional sintering processes to fix loose powder particles must be adjusted or developed. Further work has to be done.

hMSC are known to produce a typical set of cytokines such as IL-6, IL-8, and VEGF. In addition to the biological effects of the released factors, the release of these cytokines is an indicator of cell activation. In this study, the cytokine release profile of the hMSC cultured on the NiTi specimens showed no sign of cell activation. The mean cytokine release was lower than the control at all times (Fig. 7 A–C). The Nickel ion release strongly depends on specimens' characteristics (Fig. 7 D). In general, the Nickel ion release of the dense samples was lower than the release of the porous samples. However, it seems that density is not accountable for the Nickel ion release completely. Highest Nickel ion releases were obtained for sample type III, although sample type IV has a higher porosity. Taking a look at the SEM micrographs presented in Fig. 5, it is obvious that sample type IV is characterized by large pores. In contrast, sample type III shows very small pores but a higher number. We assume that sample type III has a higher surface area than sample type IV and thus a higher Nickel ion release. Nevertheless, the Nickel ion release was significantly below cytotoxic concentrations for all specimens and a reduction of the laser beam diameter from 128 μm to 64 μm leads to a significantly decreased Nickel ion release (Fig. 7 D). We have reported previously that NiCl₂ concentrations below 25 mg/l are well tolerated by hMSC cultured for 8 days. A significant decrease in cell viability occurred at threshold values of 200 (24 h) and 25 mg/l (8 days). It was also shown that hMSC were activated in response to high, but non-toxic, NiCl₂ concentrations (25 mg/l).
The cells responded with a highly significant increase in IL-8 release under these conditions [30]. These Nickel concentrations were not reached in the present study.

5. Summary and conclusions

In the present work we investigated the reaction of mesenchymal stem cells cultured on NiTi implant materials produced by Selective Laser Melting. NiTi specimens were incubated for 8 days. Cell viability was analyzed via fluorescence microscopy after live/dead staining of the cells. Cytokine release from cells was quantified via ELISA. The Nickel ion release from the specimens was determined via AAS. From the results obtained in the present work the following conclusions can be drawn:

- Dense and porous SLM-NiTi samples are suitable carriers for hMSC.
- By variation of the diameter of the laser beam the surface structure and volume of loose powder particles can be influenced significantly.
- By reducing the diameter of the laser beam, the mean Nickel ion release decreases significantly.
- Although the Nickel ion release of porous specimens was higher than the ion release of dense specimens, for all specimens the ion release was significantly below cytotoxic concentrations.

- The cytokine release profile of the hMSC cultivated on the NiTi specimens showed no sign of cell activation.
- Surface topography and cell morphology can be adjusted by varying the processing conditions.

We showed that SLM can provide an attractive technology to produce biocompatible NiTi implants. Nevertheless, before carrying out in vivo studies, the manufacturing process including post-procedures must be optimized in order to reduce the particle release.

Acknowledgments

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 459: Shape Memory Technique).

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