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Comparison of the cytotoxicity of molybdenum as powder and as alloying element in a niobium–molybdenum alloy

C. M. J. M. PYPEN*, K. DESSEIN, J. A. HELSEN
*Department of Metals and Material Engineering, Catholic University of Leuven, de Croylaan 2, 3001 Leuven, Belgium, * and also Department of Bone and Biomaterials Research, Histological-Embryological Institute, University of Vienna, Schwarzspanierstraße 17, and the Department of Oral Surgery, Dental University Clinic, University of Vienna, Währingerstraße 25a, 1090 Vienna, Austria*
E-mail: claire.pypen@univie.ac.at

M. GOMES, H. LEENDERS, J. D. DE BRUIJN
Biomaterials Research Group, University of Leiden, Professor Bronkhorstlaan 10, 3723 MB Bilthoven, The Netherlands

Commercially pure metal niobium (c.p. Nb) as well as niobium–molybdenum (Nb–Mo) alloys were produced following several powder metallurgical routes. In brief, niobium and molybdenum powders were blended and milled in order to form Nb–Mo alloys. The alloy powders and the c.p. Nb were then either pressed and sintered, or cold isostatically pressed followed by hot isostatically pressing. In order to assess the cytotoxicity of the c.p. Nb and c.p. Mo powders, a 72 h minimal essential medium-extraction test was performed according to ISO/EN 10993–5. The cytotoxicity of the c.p. Nb metal and the Nb–Mo alloys was tested in a 72 h direct contact test. Compared to a negative control (UHMWPE), c.p. Nb was non-toxic, but c.p. Mo was moderately toxic. None of the powder metallurgically produced materials were toxic. Neither differences in molybdenum concentration, nor in porosity of the samples, due to different production routes, had any influence on the toxicity of the materials. Rat bone marrow cultures showed that only on c.p. Nb was a mineralized extracellular matrix formed, while on the more porous Nb–Mo alloys, cell growth was observed, but no mineralization. In conclusion, c.p. Mo powder is moderately toxic, however, as an alloying element it is non-toxic. Material porosity seems to influence differentiation of bone tissue *in vitro*.

1. Introduction

In vivo studies have shown excellent bone apposition to commercially pure niobium (c.p. Nb) implants [1, 2]. However, the mechanical properties of unalloyed, not cold worked niobium are insufficient for load-bearing implants. Cold working of c.p. Nb by forging [1, 3], or alloying with, for instance, molybdenum (Mo) [4], both result in remarkable strengthening of this metal. When considering feasibility and costs, the powder metallurgical processing of near-net-shape implants seems to be a more efficient method for implant preparation compared to the conventional material production and implant machining.

Therefore, a Nb–Mo alloy has been produced using the powder metallurgical route with, as the final goal, the fabrication of net-shape dental implants with good mechanical properties. It has been shown that the alloy made out of 90 wt % Nb and 10 wt % Mo (Nb10Mo) seems to have satisfying mechanical properties [5].

In this paper, the biological evaluation of newly produced materials will be discussed and the toxicity of the starting powders in comparison to the final products evaluated.

2. Materials and methods

2.1. Materials

a) Pure ($> 99.1\%$) niobium and molybdenum (Fig. 1) with grain size 70 and 5 μm, respectively, were tested as-received (GfE, Nürnberg (D), Goodfellow, Cambridge (UK), respectively) (group A). These powders were also used in several powder metallurgical routes for the production of c.p. niobium and Nb–Mo alloys (Fig. 2).

b) Commercially pure niobium powders were uniaxially pressed at 900 MPa and these tablets subsequently sintered under vacuum ($< 10^{-3}$ Pa) in a home-built vacuum furnace at 2000 °C for 4 h (group B).

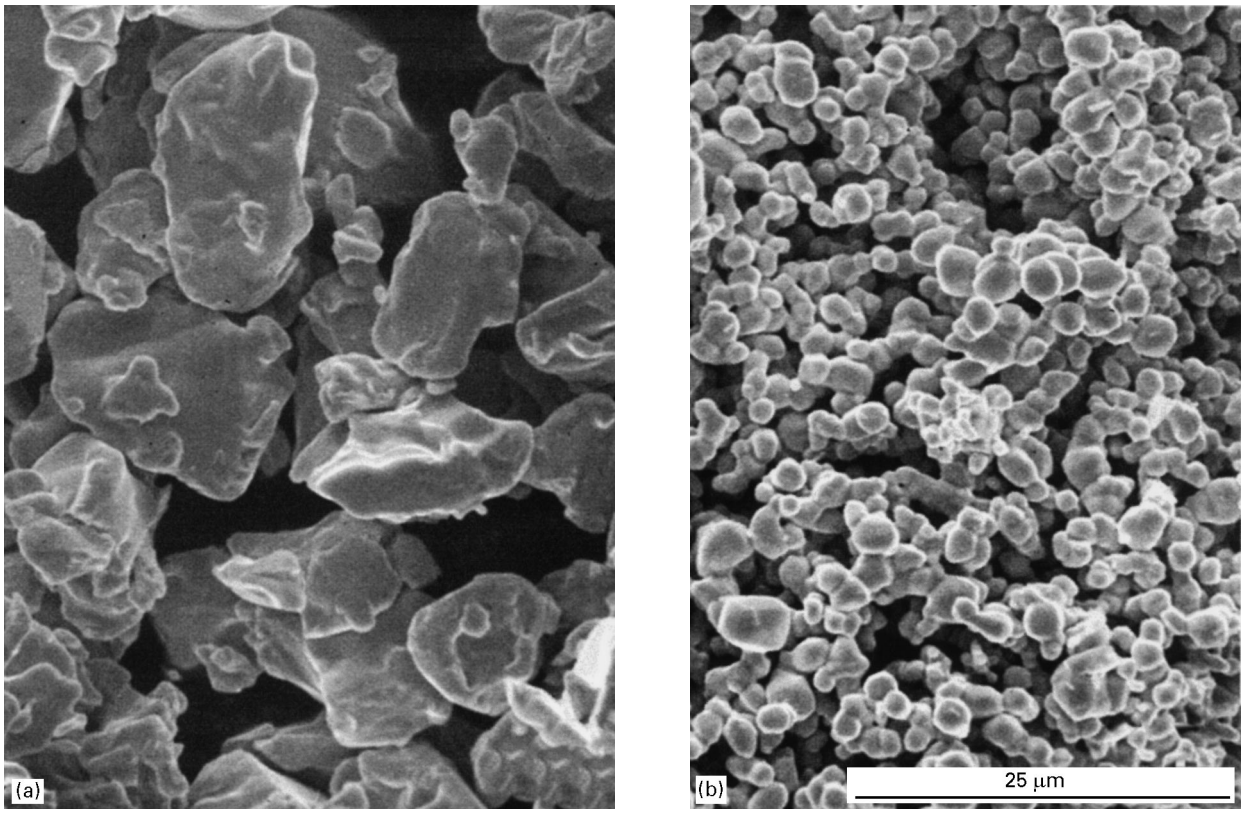

Figure 1 SEM of the powders (a) niobium and (b) molybdenum.

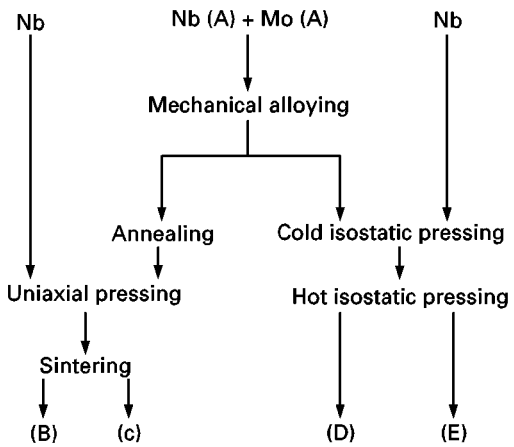

Figure 2 Production routes of the different testing samples.

c) Commercially pure niobium and molybdenum powders were blended into mixtures of Nb10Mo and Nb20Mo. These mixtures were mechanically alloyed in a planar Retsch RM4 ball mill under a protective argon atmosphere for several hours, ranging from 2–25 h. Niobium-coated chromium steel balls with a diameter of 6 mm were used for the milling in a weight of balls to powder ratio of 10 : 1.

The alloyed powders were consolidated using one of the following techniques.

1. The mixed powders were first annealed at 1200 °C for 2 h in a vacuum Leybold furnace, uniaxially pressed at 800 MPa into rods, and finally sintered in the vacuum furnace for 4 h at 2000 °C (group C).

2. The powders were cold isostatically pressed (CIP) at 400 MPa into rods, encapsuled in titanium rods and then hot isostatically pressed (HIP) for 2 h at 1450 °C at a pressure of 200 MPa (group D). As a reference c.p. Nb powders were CIPed and HIPed (group E).

Platelets (6 mm × 6 mm × 2 mm) from groups B, C, D and E were ground with a final step on no. 4000 grit SiC and sterilized by dry-heat for biocompatibility testing.

2.2. Testing methods

2.2.1. In vitro cytotoxicity testing

To assess the cytotoxicity of the materials, 72 h minimal essential medium (MEM) extraction tests were performed on the powders (group A) and direct contact tests on the platelets (groups B, C, D, E). All tests were performed according to ISO/EN 10993-5 guidelines. In brief for the MEM extraction test, 4 g metallic powder was extracted in 20 ml minimal essential medium, supplemented with 10% foetal calf serum, 2 mM glutamine and antibiotics, for 24 h at 37 °C. The extract was filtered through a sterile 0.45 μm microfilter. For both tests, a monolayer of mouse lung fibroblasts (L929) was grown to 70%–80% confluency.

In the MEM extraction test, the extract was added to the cells, but in the direct contact test the sample was gently placed on the cell layer. After 24, 48 and 72 h the cell cultures were evaluated for both tests for confluency of the monolayer, degree of cellular lysis and change of cellular morphology and the results were related to a negative (UHMWPE) and a positive (latex rubber) cytotoxic control. After 72 h, a cell

count was performed to determine the percentage of growth inhibition in comparison to the negative control. For each material the test was evaluated in threefold.

2.2.2. Bone marrow cell cultures

Finally, tissue culture tests were performed on the samples of groups B and C as described before [6]. Bone marrow cells, collected from the femur from young male Wistar rats, were seeded on the samples at a density of 1×10^4 cells cm^{-2}. The cells were fed three times a week with fully supplemented α-MEM containing 15% foetal bovine serum, 50 μg ml^{-1} ascorbic acid, 10 mM β-glucerophosphate, 10^{-8} M dexamethasone and antibiotics. After 1 and 3 wk culture, the formation of a mineralized bone-like matrix was examined and analyzed with scanning electron microscopy (SEM) (Philips) in combination with energy dispersive X-ray microanalysis (EDX).

3. Results

3.1. Microscopical analysis of the test samples

Light microscopic analysis (Reichert-Jung, type Polyvar Met) of polished and etched surfaces of sintered samples (groups B, C) revealed that different milling times during the mechanical alloying resulted in different porosities. This is due to differences in grain hardness and grain size after milling and hence of the compressability of the powders. After 16 h milling, the lowest porosity of the samples was found and XRD showed that the molybdenum is fully substitutionally dissolved in the niobium lattice (Fig. 3). In addition, larger porosities were found on the Nb20Mo alloy samples than on the Nb10Mo alloy samples.

Light microscopical comparison of the pressed and sintered samples with the CIPed and HIPed samples shows that the CIPed and HIPed samples had a much denser microstructure (Fig. 4).

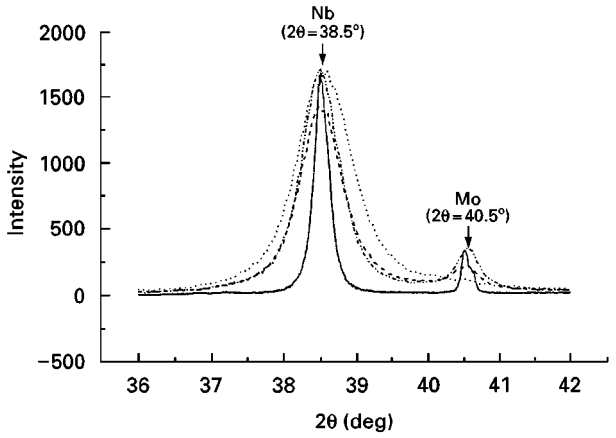

Figure 3 XRD of Nb10Mo for different milling times. After 16 h milling, the molybdenum peak has dissapeared, proving that the molybdenum is fully dissolved in the niobium lattice [5]. (———) 2 h, (– – –) 4 h, (· · ·) 16 h, (——) reference.

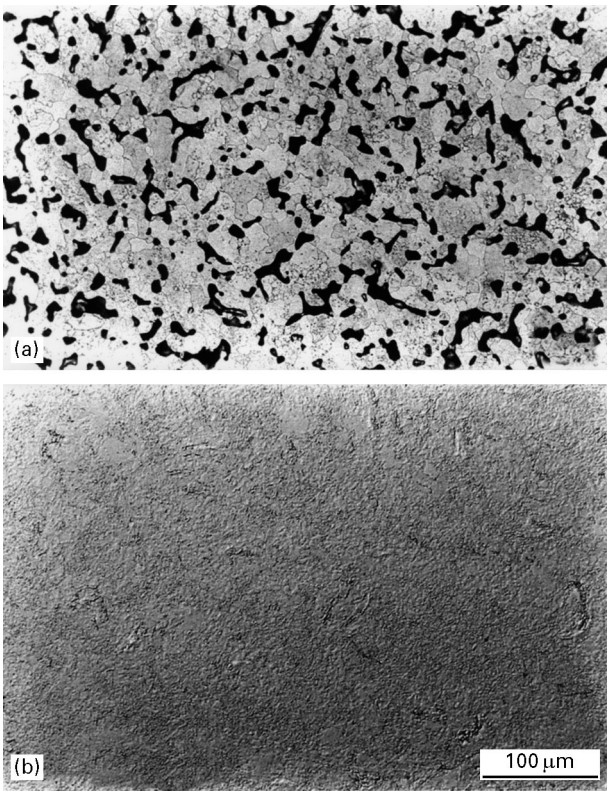

Figure 4 Microscopy of the samples Nb10Mo (a) before and (b) after HIPing.

3.2. *In vitro* cytotoxicity of the metallic powders

After 24 h culture, the cells in contact with the extract of the c.p. Nb powder showed a similar behavior to the cells in contact with the negative control. A normal cell growth was seen and no morphological changes could be observed. However, the cells in contact with the extract of the c.p. Mo powder were hardly attached to the substrate and were rounded.

After 72 h, the cells in contact with the c.p. Nb powder extract still did not show any change in cell morphology, a 100% confluent cell layer and hardly any cell lysis was seen, similar to the negative control (Fig. 5). The cell counting showed no inhibition of cell growth.

Although the cells in contact with the c.p. Mo powder extract showed 30%–60% confluency of the cell layer, major cellular lysis, and severe changes in cell morphology (Fig. 5), cell counting showed a 100% inhibition of cell growth, compared to the negative control. According to the ISO 10993-5, 72 h MEM extract test the c.p. Mo powder is only moderately toxic.

3.3. *In vitro* cytotoxicity on the metallic samples (groups B, C, D, E)

In the direct contact tests on the c.p. Nb and Nb–Mo alloys platelets, a 100% confluent cell monolayer was observed. Minor (0%–5%) cellular lysis was detected and no or only slight changes in cell morphology were seen. Cell counts showed no cell growth inhibition compared to the negative control. According to the

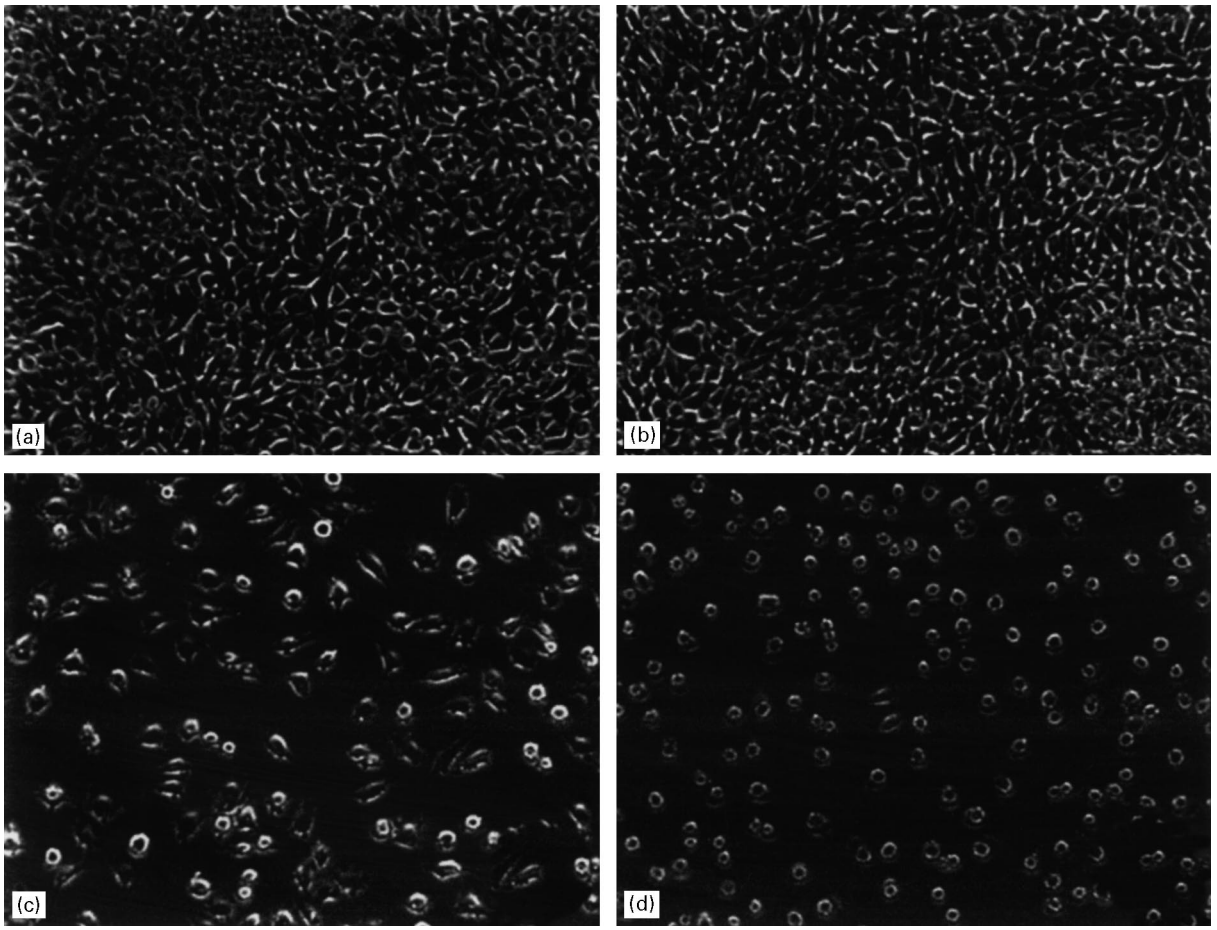

Figure 5 Cellular responses to various extracts: (a) negative control, (b) positive control, (c) c.p. Nb and (d) c.p. Mo.

ISO 10993-5, 72 h direct contact test none of the metallic materials that were produced via the powder metallurgic route were cytotoxic.

Comparison of the cell counts for the different materials under investigation, c.p. Nb, Nb10Mo and Nb20Mo, revealed that neither differences in concentration of molybdenum in the alloy, nor differences in porosity resulted in significant differences in cell proliferation or in the amount of cell death. No significant differences in toxicity between the sintered (groups B, C) and the HIPed (groups D, E) samples was found.

3.4. Bone marrow cell cultures on the metallic samples (groups B, C)

Only on the c.p. Nb samples did SEM in combination with EDX reveal a confluent cell layer with a mineralized extracellular matrix, consisting of mineral globules and calcified collagen fibrils. On all the Nb–Mo alloys only cell growth was found, but no mineralization. The best results, consisting of several confluent cell layers and non-mineralized collagen fibrils, were seen on samples made from Nb–Mo alloy powders, mechanically alloyed for 16 h.

4. Discussion

It was the aim of this study to evaluate first, if the constituents in powder form or the powder metallurgically produced materials are cytotoxic, and secondly to test the reaction of bone marrow derived cells on these materials.

Although the molybdenum powder was shown to be moderately cytotoxic, no cytotoxicity of the Nb–Mo alloys was measured. No differences in cytotoxicity between the Nb10Mo and the Nb20Mo alloys were found. Comparison of sintered and HIPed samples showed that variations in porosity apparently had no influence on the toxicity of the Nb–Mo alloys. This direct contact test showed that the Nb–Mo alloys were as biocompatible as the c.p. Nb metal.

Although there was little found in the literature on the toxicity of molybdenum some reasons could be given.

1. It was found that small particles are much more toxic than larger particles [7, 8]. This seems normal because smaller particles have a larger specific surface and a larger surface imperfection for leaching toxic ions (MoO_4^{2-}, $Mo_7O_{24}^{6-}$) into the solution. Further, the amount of molybdenum available in the alloy and the oxides are beyond the detection limit by X-ray diffraction, and hence less molybdenum is available for leaching out of the alloy, compared to the molybdenum powder. The difference in grain size between the c.p. Mo powder and the c.p. Nb powder (5^{10}–70 μm) could also be an explanation for the higher toxicity of the c.p. Mo powder compared to the c.p. Nb powder.

2. The dissolution rate of molybdenum, which is fully dissolved in the niobium lattice (Fig. 3), from the

alloy could not be sufficient for producing a toxic level in the medium.

3. The molybdenum has a lower chemical activity in the Nb–Mo alloy than in the powder. This lower activity of molybdenum in an alloy was also found in the CoCrMo alloy where the cytotoxicity of the alloy is due to leaching out of cobalt and not of molybdenum [9].

Bone marrow tissue cultures showed that only on the dense c.p. Nb material was a mineralized matrix found. On the Nb–Mo alloys, several cell layers and not mineralized collagen fibrils were seen, and the thickest layer was found on the Nb–Mo alloy produced out of the 16 h milling powder. This alloy showed the lowest porosity for the Nb–Mo alloys. This means that the porosity of the test sample seems to have an influence on the cell proliferation and mainly on the differentiation of bone tissue *in vitro*. Abiko *et al.* [10] also found reduced osteoblastic activity on porous alloys when compared to denser alloys of the same material.

5. Conclusions

1. The c.p. Mo powder induces a 100% inhibition of cell growth.

2. The c.p. Nb powder is biocompatible.

3. None of the metallic materials (c.p. Nb, Nb10Mo, Nb20Mo) are cytotoxic.

4. For the Nb–Mo alloys, no influence of the molybdenum concentration nor of the porosity on the toxicity of the alloy was found.

5. Only on the dense c.p. Nb metal was a mineralized matrix found.

To confirm our supposition that the porosity of the Nb–Mo alloys has an influence on the differentiation of bone tissue *in vitro*, the bone marrow culture test will be repeated on the much denser HIPed Nb–Mo alloys and the results compared to those on the sintered Nb–Mo alloys.

Acknowledgment

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