Biocorrosion and cytocompatibility assessment of NiTi shape memory alloys

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Abstract

Ti-based alloys are characterised by an excellent corrosion resistance, high mechanical performances, an increased biocompatibility, and in particular by the deforming force of NiTi. Five alloys were examined by electrochemical assays and cell culture tests with different cell types. All tests show the high biological and electrochemical performances of Ti6Al4V and NiTi, and in particular a significant influence of living cells on corrosion.

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1. Introduction

Amongst the most interesting medical alloys developed in the last few years are the NiTi-alloys with nearly equiatomic nickel and titanium distribution. This provides them with several advantages. The most important are (i) their shape memory effect, (ii) their superelasticity and (iii) a good biocompatibility [1–4]. These alloys are successfully applied in orthopaedic surgery [5] where the superelastic property of NiTi produces a constant stress that accelerates wound healing. The shape memory effect is one of the greatest advantages for orthodontic wires and arches, which constitute the motor of dental shifting [6], and for self-expandable vascular stents [4,7]. The shape memory property in daily practice reduces significantly the time of treatment.

The aim of this work is to determine possible in vitro biological side effects of nickel–titanium alloys, with a high rate of nickel content: toxicity, allergenicity and carcinogenicity [8–12]. This work consists in assessing the NiTi-alloy electrochemical characteristics under biological conditions in relation to its components nickel and titanium. Therefore, corrosion assays were conducted with conventional artificial saliva [13] and especially with a culture medium with or without a human lymphoid cell line in suspension culture. The biological parameters were evaluated by means of the proliferation and cytotoxicity assessments with relevant human cell lines in direct contact with NiTi. They were completed with studies on cell adhesion and morphology and with a test of inflammatory effects [9]. The release of nickel and titanium was assessed in different cell culture systems.

2. Materials and methods

2.1. Samples and powders

NiTi-alloy (49% Ni, 51% Ti), highly pure nickel (hp-Ni), commercially pure titanium (cp-Ti), Ti6Al4V and stainless steel 316L were used for this work. The alloys were cast into cylinders of 12 and 15 mm in diameter using the standard laboratory techniques and were cut in disks of 1.2 mm thickness. Specimens were automatically polished at grade 1200 using carbide silicon paper. The surface cleanliness was assessed by SEM. Roughness was characterised by LASER confocal...
microscopy, the $R_p$ differences of all samples was between 1.7 and 2.1 $\mu$m. After polishing, the samples were cleaned in an ultrasonic ethanol bath, air dried, and sterilized. All metal powders were produced by the hydrogenation dehydrogenation process (HDH). Sieving and ethanol suspensions allowed to obtain particles with a size lower than 10 $\mu$m.

2.2. Electrochemical tests

The electrochemical device was a Bioreactor developed within an European Concerted Action BioMed [14] by our Laboratory and the Company Inceltech-SGI France (Toulouse). It consists of an electrochemical cell and a potentiokinetic setting. It is based on a classical fermentator arranged specifically for multiple investigations on the corrosion behaviour of metallic biomaterials, in particular to realize simultaneously bacterial or cell cultures and electrochemical tests.

A palladium foil is used as the counter electrode and potentials are controlled with respect to an Ag/AgCl electrode connected via a salt bridge and an adjustable Luggin capillary. The working electrode with a surface area of about 0.5 cm$^2$ is attached by means of an O-ring sliding assembly so that its surface pointed up parallel to the counter electrode (KMS1, Sensortek Minsberg Gmbh, Waldheim, Germany). The distance between both electrodes is about 1 cm. The bioreactor has a working volume of 650 ml.

The potentiodynamic control of the working electrode was provided by a potentiostat (PGP 201 Rodiometer-Tacussel, Copenhagen, DK). The overall system was controlled using a PC-compatible microcomputer with VoltaMaster 1 software from Rodiometer-Tacussel.

Electrochemical testing procedures: A first test series was conducted with the synthetic saliva according to the NF-standard S 91-141 as reference electrolyte [2]. Freshly prepared test samples (12 mm) were immediately introduced to the corrosion cell.

The electrolyte of the second test series was the culture medium RPMI (Gibco BRL) complemented with 10% foetal calf serum (FCS) (Eurobio). All assays were performed with each alloy or metal in the culture medium with and without CEM cells, a human lymphoid cell line (ATCC-TIB95). The applied cell concentration was 10,000 cells/ml.

The following sequence of measurements was used throughout the whole study [15]: (i) determination of the open-circuit potential over a 24 h period, the value obtained after stabilisation of the curve being called the rest potential; (ii) cathodic scouring of the working electrode at $-800$ mV/SCE for 10 min to reduce the surface films; (iii) assessment of the global polarisation curves between $-800$ and $+1000$ mV with a scanning rate of 60 mV/min.

2.3. Cell culture

Cell proliferation tests: Human epithelial embryonic cells (cell-line L132, ATCC CCL5), human embryonic palatal mesenchymal cells (cell-line HEPM, ATCC CRL 1486) and NIH3T3 mouse connective tissue fibroblasts (ATCC CRL 1658) were used for this test. The cells were incubated at 37 °C in a 5% CO$_2$ atmosphere with 100% relative humidity. The cell culture medium consisted of Earl’s minimum essential medium (MEM) containing L-glutamine (Gibco BRL), streptomycin (0.1 g/l) and penicillin (100 UI/ml), and supplemented with 5% foetal calf serum (Eurobio). Disks of each alloy or metal were placed in the bottom of a 15.5 mm multiwell plate (Costar). Cell suspensions (10$^5$ cells/ml for L132 and NIH3T3, 6 $\times$ 10$^4$ cells/ml for HEPM) were gently placed on each disk. Empty culture chambers filled only with cell suspension served as negative controls. Seventy-two hours later, cell counting was performed using a Coulter Z1 cell counter. The cell proliferation rate was calculated as the number of grown cells on the samples divided by the number of control cells. The data were expressed as the mean percentage ± SD with respect to the control cultures (100%). Triplicate assays were run in each test and the test was repeated five times.

Viability tests were performed by using the colony forming method on human epithelial cells (L132) in culture [10,16,17]. Cells were continuously exposed to increasing concentrations (0, 25, 50, 100, 200 and 400 $\mu$g/ml) of metal and alloy powders without renewal of the growth medium during the experiments. At least six repeated experiments were performed for each concentration.

The test of inflammatory reactions consisted of quantifying the multinucleated giant cells (MGC) in monolayer cell cultures of L132 in the presence of the metal or alloy powders [17]. This test reveals morphological modifications in a cell culture by the appearance of multinucleated giant cells, which are directly related to physiological, i.e. functional, alterations to the cells [18,19]. The concentration of Ni-powder used for this test was the 50% lethal concentration (LC$_{50}$) given by the colony forming method test. For cp-Ti, NiTi, Ti6Al4 and 316L we applied the strongest concentration, which was used for the viability test (400 $\mu$g/ml). The exposure time was 5 days. After staining and drying, the multinucleated giant cells were counted: at least 10 areas of 800–1000 cells. The final result was expressed as the mean percentage of MGC with respect to the total cell number [17].

Ion release was measured in the culture medium of L132 cells after the 3- and 6-days proliferation assays on the experimental alloys and metals. The media were analysed, after mineralisation, by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Ni and Ti were considered as elements to be analysed.
Cytoskeletal organisation: Actin filaments were labelled in HEPM cells with 1.2 μg/ml FITC-phalloidin (Sigma) [20]. After washes in buffer, the specimens are embedded in PBS-glycerol-DABCO (1/1) (Sigma) mounting medium and examined in a ZEISS epifluorescence microscopy or in a LEICA TCS NT LASER scanning confocal microscope.

3. Results

3.1. Electrochemical tests

The potential vs. time curves obtained in the present study allow to determine the rest potential of the test samples; its value is obtained when the potential remains constant. The tests were carried out at 37 °C and with a pH stabilised at 7. Handling was repeated three times, the average results are represented in Table 1. The measured rest potentials of all alloys and metals are in accordance to those generally indicated in multiple investigations.

Ti6Al4V and cp-Ti, present logically the highest rest potentials, which significantly increase a first time in the organic electrolyte (RPMI) and further in the presence of CEM cells. A similar behaviour is observed for hp-Ni. The values of NiTi remain stable and those of 316L decrease having significantly more anodic rest potentials.

The global polarisation assessment (Figs. 1 and 2) allows the determination of the breakdown potential. Its value corresponds to the potential, from which the passivation film is altered or broken and the corrosion of the material subsequently accelerates. Although the density of current is relatively stable before the breakdown

Table 1
Electrochemical results after corrosion assays under standard and biological conditions

<table>
<thead>
<tr>
<th>Alloys and test conditions</th>
<th>$E_a$ (mV/ECS)</th>
<th>$E_c$ (mV/ECS)</th>
<th>$E_r$ (mV/ECS)</th>
<th>$I_p$ (μA at 400 mV/ECS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni (art. saliva)</td>
<td>−650</td>
<td>−500</td>
<td>−200</td>
<td>13,000</td>
</tr>
<tr>
<td>In RPMI</td>
<td>−412</td>
<td>−287</td>
<td>9</td>
<td>15,300</td>
</tr>
<tr>
<td>In RPMI + CEM</td>
<td>−347</td>
<td>−282</td>
<td>2</td>
<td>8000</td>
</tr>
<tr>
<td>Ti (art. saliva)</td>
<td>−350</td>
<td>&lt;800</td>
<td>&gt;1000</td>
<td>5</td>
</tr>
<tr>
<td>In RPMI</td>
<td>−145</td>
<td>−416</td>
<td>&gt;1000</td>
<td>2</td>
</tr>
<tr>
<td>In RPMI + CEM</td>
<td>−121</td>
<td>−287</td>
<td>&gt;1000</td>
<td>3</td>
</tr>
<tr>
<td>NiTi (art. saliva)</td>
<td>−322</td>
<td>−730</td>
<td>&gt;1000</td>
<td>19.4</td>
</tr>
<tr>
<td>In RPMI</td>
<td>−338</td>
<td>−425</td>
<td>600</td>
<td>44</td>
</tr>
<tr>
<td>In RPMI + CEM</td>
<td>−352</td>
<td>−615</td>
<td>530</td>
<td>56</td>
</tr>
<tr>
<td>Ti6Al4V (art. saliva)</td>
<td>−328</td>
<td>−600</td>
<td>&gt;1000</td>
<td>2</td>
</tr>
<tr>
<td>In RPMI</td>
<td>−274</td>
<td>−454</td>
<td>&gt;1000</td>
<td>2</td>
</tr>
<tr>
<td>In RPMI + CEM</td>
<td>−109</td>
<td>−454</td>
<td>&gt;1000</td>
<td>3</td>
</tr>
<tr>
<td>316L (art. saliva)</td>
<td>−290</td>
<td>−650</td>
<td>300</td>
<td>160</td>
</tr>
<tr>
<td>In RPMI</td>
<td>−341</td>
<td>−450</td>
<td>280</td>
<td>5300</td>
</tr>
<tr>
<td>In RPMI + CEM</td>
<td>−386</td>
<td>−400</td>
<td>150</td>
<td>42,600</td>
</tr>
</tbody>
</table>

RPMI: culture medium complemented with 10% foetal calf serum.
CEM: human lymphoid cell line.
$E_a$: rest potential, $E_c$: corrosion potential, $E_r$: breakdown potential, $I_p$: passive current.
potential, it increases abruptly after it. A remarkable influence of the organic electrolyte and living cells has been observed for hp-Ni and the stainless steel 316L, where the breakdown potential significantly increased. Although the breakdown potential of NiTi decreases, this alloy still remains passive at values higher than 500 mV/SCE.

In contrast, no increase of the current in the studied interval is observed for Ti6Al4V and cp-Ti. They remain passive up to 1000 mV/SCE, which indicates that both materials possess highly resistant passivation films. The passive current is an other important point. It is the density of current in its passivation domain of an alloy. hp-Ni has produces significant passive currents between 800 and 13,000 μA/cm². Similar high values are measured for 316L, in particular in the presence of living cells. The passive current of NiTi, cp-Ti and Ti6Al4V is very low. A significant influence of living cells is observed for NiTi.

3.2. Biological tests

The average results of cell proliferation tests are represented in Fig. 3. For all cell types lower proliferation rates were noted with respect to the control. NiTi and Ti6Al4V are the most cytocompatible materials, they induce proliferation rates close to those of cp-Ti. hp-Ni produces very low proliferation rates with HEPM cells (34%), NIH3T3 (16%) and L132 cells (11%). Thus its toxic potency seems to depend on the cell type, i.e. Ni is more toxic for L132 cells than for HEPM cells. The proliferation rates on 316L are situated between 24% and 35%.

A similar toxic effect of Ni (1 ± 0.2%) is revealed by the cell viability test performed with the various metallic powders. The survival rate in the presence of NiTi and cp-Ti is 79 ± 3.9% and 81 ± 4.1% respectively for the highest concentrations used (400 μg/ml). Ti6Al4V produces the best viability with a 96 ± 5.8% survival rate. 316L also induces only little cell death (76 ± 4.5%).

The appearance of MGC reveals an inflammatory response to a chemical substance (Fig. 4). Cells of characteristic areas are counted after colouration of the culture dishes. The total number of MGC is reported to the total number of normal cells. The frequency of the MGC appearance is approximately 1.3% for NiTi, 316L also induces only little cell death (76 ± 4.5%).
Ti6Al4V and cp-Ti. No significant differences could be stated with respect to the control cultures: 1.5%, whereas for 316L and in particular for hp-Ni, the frequency of MGC is significantly higher: 2.9% and 5.1% respectively.

Actin labelling in HEPM cells revealed a cytoskeleton composed of intracellular filaments (stress fibres) aligned to the longitudinal axis in control cells and in cells grown on cp-Ti and Ti6Al4V. NiTi and 316L induced spreading of cells with a strongly developed, i.e. nearly overloaded cytoskeleton (Fig. 5a). The actin fibres were strongly reduced in cells on hp-Ni (Fig. 5b), which mostly have retracted forms with a reduced cytoplasmic area.

The ion release has been determined for further explanation of any biological reaction. The Ni concentrations are significantly different to those of control cultures (Table 2). The nickel-content in the control medium does not exceed 0.01 ppm (normally contained in the foetal calf serum added to the culture medium), the Ni-release from the NiTi is very low (mean value 0.1 ppm), but rather high from pure nickel samples: 6.6 and 11.4 ppm after 3- and 6-days cultures respectively. Titanium is not detectable in the control medium. Its release is extremely low from the cp-Ti (detection limit) and is slightly increased from NiTi samples. No significant differences can be stated after 3- and 6-days cultures.

### 4. Discussion

Nickel and in particular nickel compounds are recognised to have more or less toxic and/or carcinogenic effects in animals and human beings [21,22]. These effects can be induced by occupational exposure and also—but on a limited scale—by long-lasting exposure to implants and prostheses [11]. Since nickel represents 49% of the composition of NiTi, it was interesting to assess whether this high amount has an unwanted biological side effect and to compare the results from electrochemical and biological assays with the alloy and its two pure constituents.

This comparison allows to confirm that hp-Ni is not very corrosion resistant. NiTi, Ti6Al4V and cp-Ti, however, are corrosion resistant, and their passivation film has a very good stability. By comparing the rest potentials of these materials with those of other examined alloys [15,23,24], we note, that all materials tested in the present study have frankly negative rest potentials. This indicates a high susceptibility to corrosion, which is checked, indeed, for hp-Ni and 316L by the global polarisation assessment. The NiTi, Ti6Al4V and cp-Ti have revealed in identical assays, however, a very good corrosion resistance with very low passive currents and very high breakdown potentials, hp-Ni presenting a 1000 times higher passive current. The low passive current of NiTi and cp-Ti indicate a slower corrosion speed and subsequently a lower ion release into electrolytes or biological fluids [25]. This is of great importance for the biocompatibility of any alloy [9,26].

Pure nickel has not only an unpropitious corrosion behaviour but also induces unfavourable biological responses as revealed by all applied cell culture tests. hp-Ni shows a weak proliferation rate with both cell types, but it seems to be less toxic for the HEPM cells than for the L132 cells. In contrast, NIH3T3 cells rather than HPEM cells have a better growth rate on cp-Ti, Ti6Al4V and NiTi, the latter inducing the best cell growth. It is important to take into consideration the differences of the cell types for future investigations in this field.

The cell proliferation test is a routine test method giving reproducible and quantitative results. In order to get more relevant results, other highly specific cytotocompatibility tests were performed. One is the quantitative determination of cytotoxicity by using the colony forming method [16,19], measuring only one criterion of cell reaction which is cell death or cell survival [10]. A second specific test is the quantitative assessment of inflammatory reactions by the appearance of MGC. These cells may appear not only in cellular or organotypic cultures [18], but also in vivo in patients with amalgam tattoos [26–28] or orthopaedic implants [29]. They are generally considered to be a specific inflammatory response [26–29]. Both tests consolidate the results obtained by the corrosion experiments and the cell proliferation.

The labelling of actin and the cytoskeleton are a qualitative assay. The results reflect in some way those obtained by the proliferation test. HPEM cells show similar cell morphology and actin filament distribution and intensity in control cells and in cells grown on cp-Ti and NiTi and Ti6Al4V. However, cells grown on hp-Ni only show some rare actin filament, and the green colour of the cell is probably due to the labelling of depolymerised actin in the so-called globular state. This

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Ni (ppm)</th>
<th>Ti (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
<td>6 days</td>
</tr>
<tr>
<td>Control medium (MEM + 10% SVF)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>hp-Ni</td>
<td>6.599 ± 0.037</td>
<td>11.364 ± 0.034</td>
</tr>
<tr>
<td>cp-Ti</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
<tr>
<td>NiTi</td>
<td>0.081 ± 0.006</td>
<td>0.176 ± 0.008</td>
</tr>
</tbody>
</table>

n.d.: not detected; n.m.: not measured.
depolymerising effect is less evident in cells grown on 316L, where there is still a certain number of cells exhibiting actin filaments.

The ultimate responsible for all these biological effects is the Ni-ion release from pure nickel by a low electrochemical corrosion resistance but also by a direct action of biological fluids and of living cells [30]. Nickel is nearly not released when it is allied in a high quality alloy with excellent physical characteristics [31].

5. Conclusion

New technology has been applied for the electrochemical and biological characterisation of the NiTi shape memory alloy compared to its pure components and two different currently used alloys. The electrochemical assays reveal a clear difference of corrosion behaviour in inorganic and organic (biological) electrolytes, and that living organisms such as cells have an additional effect on the corrosion resistance.

Routine cytotoxicity tests and in particular more realistic cell function assessments like the determination of inflammatory effects and the integrity of the cytoskeleton have confirmed that nickel is not only a corrosive but also a cytotoxic material. Its toxic potency depends on the cell type: it is more toxic for L132 cells than for HEPM or NIH3T3 cells. Its use in implants, prostheses and other medical devices should be regulated within the condition of lowest release.

cp-Ti and NiTi are biocompatible and particularly corrosion resistant. Evidence is shown here that the introduction of nickel into this alloy does not generate any cytotoxic reaction nor alters the physiological and functional behaviour of cells. In addition, it confers a very special property on this alloy which is shape memory and which provides this alloy with some important advantages in orthodontic and cardiovascular domains.

References