Modificarea suprafetelor implantabile, în vederea cresterii bioperformantelor. Anodizarea electrochimica, mijloc de modificarea suprafetelor metalice implantabile de la micro la nanostructuri.

I. Demetrescu
Motivatie si suport

- **Motivatie**: necesitatea de a imbunatati biomaterialele implantabile in contextul dezvoltarii acoperirilor de suprafața
- **Suport**: Proiecte si colaborari
  - C.N.C.S.I.S.TIPA. „Obtinerea si caracterizarea de noi micro si nanostructuri compozite cu utilizare in ingineria tisulara”
  - Elaborarea si testarea in vitro si in vivo a unor elemente de protezare pentru ortopedie, realizate din noi biomateriale romanesti
- **Bilaterala Franta Brincusi** «Couches minces d'oxyde d'aluminium et d'oxyde de titane pour différentes applications technologiques et biomédicales
- **Proiect CEEX** Micro si nanostructuri obtinute prin bioactivare chimica si electrochimica cu aplicatii in medicina regenerativa
- **Proiect PN2 IDEI** Studii exploratorii asupra mecanismului de formare si inducere de noi proprietati unor electrozi modificati cu forme structurale TiO₂ nanotuburi / nanoparticule si compozite polimerice
- **Proiect PN2 IDEI complexe PCCE** Noi concepte si strategii pentru dezvoltarea cunoasterii unor noi structuri biocompatibile in bioinginerie
The immunofluorescent signals revealed a better fibronectine expression and fine reticular organization on the treated substrate disc. This suggests a more tight cell adhesion on these supports than on substrate untreated disc; the cell density is higher on nanotubes indicating that it is preferred by the cells [1].

Generally a preference of HGF-1 cells for 120 nm-TiO2 nanotubes in comparison with native passive TiO2 surfaces in terms of cell adhesion was observed. Usually fibroblasts prefer smooth surfaces but recent papers sustained that microroughness on cellular response is inconclusive taking into account the etching. It is expected for nanotubes surface to appear slightly rougher than Ti, and our results are in agreement with this finding, the TiO2 nanotubes having a roughness (Sa 102 nm) comparing to passive TiO2 layer (Sa 26 nm)

Cell adhesion

- Immunofluorescence analysis of human osteoblasts with antibodies to fibronectin indicated a stronger deposition of fibronectin fibers on the cell surface of 15-nm nanotubes as compared to 100-nm nanotubes. (size dependent behavior of nanotubes was associated with the enhanced formation of focal contacts).

- Brammer et al. explained the highest adhesion potential of cell to the TiO2 nanotubes with an inner diameter smaller than 50 nm by the ECM (matrix extracellular) high concentration proteins adsorbed on the entire surface.

- On 100 nm TiO2 nanotubes the cell adhesion was shown to be impaired by a sparse deposition of proteins such as fibronectin. Brammer presents very different cell viability for nanotubes with various diameters, but almost no difference in roughness.

- Larger diameter (70-100 nm) nanotubes elicit a lower population of cells with extremely elongated cellular morphology and much higher alkaline phosphatase levels.

- Regarding wettability, a considerable number of studies have indicated that cells tend to attach better to hydrophilic surfaces than to hydrophobic surfaces. (our data as well)

- Contradictory reports argue that hydrophobicity enhances protein adsorption and promotes cell attachment and growth.

Osteoblasts adherence

- The number of adhered cells on the smallest 30 nm diameter nanotubes was notably higher than all the other sizes of nanotubes, but the cells started to be more elongated on nanotube diameters above 70 nm.
- Recently was found [1] that this phenomenon of adhesion vs. elongation is determined by the initial adsorption of proteins, namely fibronectin and albumin, from the serum in the culture medium.
- Osteoblasts cultured on the 30 nm TiO2 nanotubes easily adhered to the nanotube surface because of the large number and thorough distribution of protein nanoparticles already covering the whole surface of the nanotubes.

Park et al. [1] reported that the optimum length scale for cell vitality and differentiation is on smaller diameter (15 nm) TiO2 nanotubes and declined significantly with increasing pore size, with dramatically reduced cellular activity and a high extent of cell death in nanotubes of 100 nm diameter.

This difference in cell behavior might be caused by the substantially different nature of the TiO2 nanotubes (as-anodized, amorphous phase TiO2 nanotubes in Park et al.’s study vs. heat-treated and crystallized, anatase phase TiO2).

• Actin filaments are two-stranded helical polymers that maintain cell shape, form cytoplasmic protuberances and participate to cell–cell and cell-ECM junctions and to signal transduction.
• Actin bundles coupled with focal adhesion contacts can transmit forces to the substrate and help maintain cell shape and facilitate cell adhesion
• The function of focal adhesions is not only a structural one, linking the ECM to the actin cytoskeleton but also of sites for signal transduction, initiating signaling pathways in response to adhesion.
MTT and lactate dehydrogenase (LDH) assays

• MTT and LDH assays, were realized at 72 h of culture to provide information about cell proliferation and possible cytotoxic effects of the TiO2 (for natural passivated and nanotubes).

• MTT-assay allows evaluation of metabolically active cells by their activity of reduction of the yellow tetrazolium MTT to the corresponding blue formazan. The formazan crystals were solubilized with isopropanol and absorbance was measured at 550 nm. High absorbance values indicate an increase in cell activity.

• LDH leakage into the medium was evaluated by using a Sigma kit “In vitro toxicology assay kit lactate dehydrogenase based”. High OD490nm values are indicative of a reduction in the cell viability.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>native passive TiO2 surface</th>
<th>TiO2 nanotubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTT assay (OD550 nm)</td>
<td>0.526 (0.003)</td>
<td>0.543 (0.003)</td>
</tr>
<tr>
<td>LDH test (OD490 nm)</td>
<td>0.234 (0.003)</td>
<td>0.225 (0.001)</td>
</tr>
</tbody>
</table>
MTT test for nanoarchitectures elaborated in 2 kinds of electrolytes; inorganic and hybride electrolytes

Correlation between electrochemical stability and biocompatibility was established (better stability, smaller ion release for higher cell viability, as parts of bioperformance. As can be observed in our data [1] for some nanotubes dimensions the biocompatibility is decreasing for various periods of time. This is a more novel approach in evaluating nanomaterials performance admitting that there are merit and demerit aspects in biomaterials performance.


in domeniul nanomaterialelor
Fluorescence microscopic images of human osteoblasts or G292 grown on Ti6Al7Nb: (a) untreated, (b) coated with oxide nanotube layer obtained from electrolyte type 1 and (c) coated with oxide nanotube layer obtained from electrolyte hybrid.

The proportion of elongated cells characterized by strong adhesive strength was higher on the TiO2 nanotubes obtained in the hybrid electrolyte.

This cell elongation could be explained by a sparse adsorption of proteins from culture medium, at the top wall surface of large nanotube pore spaces.

Fluorescence microscopic images of human osteoblasts or G292 grown on Ti6Al7Nb: (a) untreated, (b) coated with oxide nanotube layer obtained from electrolyte type 1 and (c) coated with oxide nanotube layer obtained from electrolyte hybrid.
On titanium surfaces, self-assembled layers of vertically oriented TiO2 nanotubes with defined diameters between 15 and 100 nm show that adhesion, spreading, growth, and differentiation of mesenchymal stem cells are critically dependent on the tube diameter.

A spacing less than 30 nm with a maximum at 15 nm provided an effective length scale for accelerated integrin clustering/focal contact formation and strongly enhances cellular activities compared to smooth TiO2 surfaces.

Cell adhesion and spreading were severely impaired on nanotube layers with a tube diameter larger than 50 nm, resulting in dramatically reduced cellular activity and a high extent of programmed cell death.

Thus, on a TiO2 nanotube surface, a lateral spacing geometry with openings of 30–50 nm represents a critical borderline for cell fate.
GFP-labeled rat mesenchymal stem cells were plated on Ti coated with TiO2 nanotubes of six different diameters; polished TiO2- served as control (A)

cell adhesion, 1 h after cell plating, adherent cells were counted under the fluorescence microscope.

Cell proliferation rates were measured by counting adherent cells after 3 days (B) and by a colorimetric assay 6 days after cell plating (C).

(D) For measuring cell migration, cells were plated at a density of 50 000 cells/cm², and 3 h later, a track of 3.4 mm width was created in the confluent cell layer. Cell motility was evaluated by measuring the remaining width after 36 and 60 h.

(E) Apoptosis was analyzed by staining cells with Annexin V-FITC 2 (KIT) and 4 days after plating. Surface-labeled cells were counted using a fluorescence-activated cell sorter. (F) Osteogenic differentiation of stem cells after 2 weeks of cell culture in osteogenic differentiation medium was assessed by staining for calcium phosphate mineral deposition.
Focal contact formation, differentiation, and apoptosis of mesenchymal stem cells on 15 and 100 nm nanotubes. 
(A) On 1 and 3 days after plating, focal contact formation and stress fiber assembly was extensive on 15 nm nanotubes (a,c) but strongly reduced on 100 nm nanotubes (b,d). 
(B) Analysis of focal contacts by SEM using immuno gold staining with paxillin and α1-integrin antibodies revealed packing of paxillin in focal contacts on 15 nm tubes, while labeling was sparse on 100 nm tubes. 
(C) High extent of focal contact formation on nanotubes smaller than 30 nm as compared to 100 nm, and a flat TiO2 surface (control). 
(D) Osteogenic differentiation after 2 weeks in culture medium on 15 nm nanotubes as seen by osteocalcin staining (red, upper panels), but rarely detectable on 100 nm nanotubes. F-actin staining is in green. Bar, 20 μm. TUNEL staining (green, lower panels) showed extensive apoptosis on 100 nm tubes starting already. 
(E) Nanoscale spacing directs cell fate. Nanotubes larger than 70 nm diameter do not support focal contact formation and cell signaling, thus leading to apoptosis.
The effect of a titanium surface charge on fibroblast adhesion.

An electrical chamber was custom-made to generate negative and positive surface charges on commercially pure titanium cylinders with a potential difference of 4.5 V. Twenty-seven titanium cylinders were divided into three experimental groups.

- In each group, cell attachment to a positively charged titanium cylinder, a negatively charged titanium cylinder, and a titanium cylinder (control) was studied at three time intervals of 15, 30, and 60 minutes. NCTC clone 929 fibroblasts were used in these experiments. The effect of the potential difference in the pH of Dulbecco's Modified Eagle Medium (DMEM) was also evaluated using two new specimens at time intervals of 15, 30, 60, and 80 minutes.

**Results:** The fibroblast cell attachment was more statistically significant to the positively charged titanium cylinder than the negatively charged titanium cylinder and the control whereas the cell adhesion difference between the control and the negatively charged titanium cylinder was not statistically significant. The range of pH difference of the DMEM in the negative and positive parts of the electrical chamber was 0.46 and 0.30, respectively.

**Conclusion:** Within the limitations of this in vitro study, the positive surface charge of the titanium cylinder results in significantly favorable cell adhesion.
**TiO$_2$ nanotubes synthesis and annealing**

- Sample preparation: polishing, degreasing (acetone, ethanol)
- Electrochemical anodization of:
  - **Titanium substrate** (99.6% purity, 0.2mm thickness)
  - **Ti6Al7Nb substrate** (1.5mm thickness)
- Thermal treatment

**Surface analysis of TiO$_2$ nanotubes**

- Scanning electron microscopy (SEM) → nanotube morphology

**Phase composition of TiO$_2$ nanotubes**

- X-ray diffraction (XRD) → anatase and rutile %
Morphology controlled by the anodization conditions:

- electrolyte: CH$_3$COOH:H$_2$O (12.5:87.5 vol.%$)$ + 0.5 wt.% HF$^1,2$
- applied potential: 10V
- time: 1h
- temperature: room temperature


**Figure:** SEM images of: a) S1 – Ti substrate, b) S2 – Ti6Al7Nb substrate

$\beta$ phase (enriched in Nb)

$\alpha$ phase (enriched in Al)
Figure: XRD patterns of annealed S1 samples: a) 550°C, 2h, b) 10°C/min, 550°C, 2h; T-titanium, R-rutile, A-anatase

Chosen thermal treatment:

10°C/min, 550°C: 1h30min
Figure: SEM images of annealed samples: a) S1, b) S2

β phase (enriched in Nb)

α phase (enriched in Al)

Figure: XRD patterns of 10°C/min, 550°C, 1h30min annealed samples: a) S1, b) S2; T-titanium, R-rutile, A-anatase, * - Al₂O₃
Figure: Tafel plots for a) titanium samples, b) Ti6Al7Nb samples.
Nanotubes calcination
nanotubes elaborated in 1:7 volumetric ratio
CH₃COOH + 0.5% HF

Surface analysis of the obtained samples before and after annealing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nanotubes dim.</th>
<th>CA Value</th>
<th>Nanotubes dim.</th>
<th>CA Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d, nm</td>
<td>t.wall, nm</td>
<td></td>
<td>d, nm</td>
</tr>
<tr>
<td>Ti</td>
<td>—</td>
<td>—</td>
<td>S1</td>
<td>20-30</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>73.7</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>S2</td>
<td>25-40</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td>73.15</td>
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Modifications of nanotubes dimensions after annealing

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before annealing</th>
<th>After annealing</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d, nm</td>
<td>t.wall, nm</td>
<td>d, nm</td>
</tr>
<tr>
<td>S1</td>
<td>21.73</td>
<td>8.06</td>
<td>17.68</td>
</tr>
<tr>
<td>S2</td>
<td>34.10</td>
<td>9.51</td>
<td>22.36</td>
</tr>
</tbody>
</table>

Corrosion parameters for S1 annealed, S2 annealed and TiO₂ nanotubes – not treated sample and for Ti [17]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Eₜ₀, mV</th>
<th>iₜ₀, μA/cm²</th>
<th>iₚₜ₀, μA/cm²</th>
<th>vₜ₀, mm/Y ·10⁻²</th>
<th>Rₚ₀, kOhmcm²</th>
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<tbody>
<tr>
<td>cp-Ti</td>
<td>-606</td>
<td>2.32</td>
<td>8.5</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>TiO₂ nanotubes</td>
<td>-497.3</td>
<td>1.0160</td>
<td>0.0401</td>
<td>1.963</td>
<td>0.574</td>
</tr>
<tr>
<td>S1 annealed</td>
<td>666.2</td>
<td>0.6714</td>
<td>0.682</td>
<td>1.297</td>
<td>8.05</td>
</tr>
<tr>
<td>S2 annealed</td>
<td>429.5</td>
<td>1.8403</td>
<td>0.579</td>
<td>3.335</td>
<td>18.11</td>
</tr>
</tbody>
</table>

The annealing treatment was done at 500°C for 2 h.
Corrosion parameters for titanium samples

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ti</th>
<th>S1</th>
<th>S1 annealed</th>
</tr>
</thead>
<tbody>
<tr>
<td>$j_{corr}$ (A/cm²)</td>
<td>5.18 $\times$ 10⁻⁹</td>
<td>6.74 $\times$ 10⁻⁹</td>
<td>3.05 $\times$ 10⁻⁹</td>
</tr>
<tr>
<td>$E_{corr}$ vs. (Ag/AgCl) (V)</td>
<td>-0.09414</td>
<td>-0.1515</td>
<td>-0.09045</td>
</tr>
<tr>
<td>$R_p$ (Ω cm²)</td>
<td>3659100</td>
<td>2517600</td>
<td>7242800</td>
</tr>
<tr>
<td>$v_{corr}$ (mm/y)</td>
<td>4.51 $\times$ 10⁻⁵</td>
<td>5.85 $\times$ 10⁻⁵</td>
<td>2.64 $\times$ 10⁻⁵</td>
</tr>
</tbody>
</table>

Table 2: Corrosion parameters for Ti6Al7Nb samples

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ti6Al7Nb</th>
<th>S2</th>
<th>S2 annealed</th>
</tr>
</thead>
<tbody>
<tr>
<td>$j_{corr}$ (A/cm²)</td>
<td>4.93 $\times$ 10⁻⁸</td>
<td>1.60 $\times$ 10⁻⁹</td>
<td>1.72 $\times$ 10⁻⁸</td>
</tr>
<tr>
<td>$E_{corr}$ vs. (Ag/AgCl) (V)</td>
<td>-0.10196</td>
<td>-0.09358</td>
<td>-0.14126</td>
</tr>
<tr>
<td>$R_p$ (Ω cm²)</td>
<td>882880</td>
<td>183380</td>
<td>836130</td>
</tr>
<tr>
<td>$v_{corr}$ (mm/y)</td>
<td>13.46 $\times$ 10⁻⁴</td>
<td>41.82 $\times$ 10⁻⁴</td>
<td>4.5 $\times$ 10⁻⁴</td>
</tr>
</tbody>
</table>
Figure 6: *Electrochemical behaviour: EIS – Nyquist and Bode plots*

Equivalent circuits for Ti, S1 and S1 annealed, after fitting of EIS data

Table 3: Electrical parameters of equivalent circuits obtained after fitting results of EIS test for Ti, S1 and S1 annealed

<table>
<thead>
<tr>
<th>Sample</th>
<th>Imposed potential [V]</th>
<th>EC model</th>
<th>$R_{\text{sol}}$ [Ω cm$^2$]</th>
<th>$R_{\text{pl}}$ [kΩ cm$^2$]</th>
<th>$Q_{\text{pl}}$ [μMho]</th>
<th>$n_{\text{pl}}$</th>
<th>$R_{\text{bl}}$ [MΩ cm$^2$]</th>
<th>$Q_{\text{bl}}$ [μMho]</th>
<th>$n_{\text{bl}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti</td>
<td>-0.05</td>
<td>$R_{\text{sol}} (R_{\text{bl}}Q_{\text{bl}})$</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>S1</td>
<td>-0.05</td>
<td>$R_{\text{sol}}(R_{\text{pl}}(Q_{\text{pl}}(R_{\text{bl}}Q_{\text{bl}})))$</td>
<td>128</td>
<td>4.98</td>
<td>5.25</td>
<td>0.908</td>
<td>99.6</td>
<td>7.43</td>
<td>0.758</td>
</tr>
<tr>
<td>S1 annealed</td>
<td>-0.05</td>
<td>$R_{\text{sol}}(R_{\text{pl}}(Q_{\text{pl}}(R_{\text{bl}}Q_{\text{bl}})))$</td>
<td>100</td>
<td>25</td>
<td>1.96</td>
<td>0.789</td>
<td>44.4</td>
<td>34.5</td>
<td>0.451</td>
</tr>
</tbody>
</table>
Figure 7: Equivalent circuits for Ti6Al7Nb, S2 and S2 annealed, after fitting of EIS data

Electrochemical behaviour: EIS – Nyquist and Bode plots

Table 4: Electrical parameters of equivalent circuits obtained after fitting results of EIS test for Ti6Al7Nb, S2 and S2 annealed

<table>
<thead>
<tr>
<th>Sample</th>
<th>Imposed potential [V]</th>
<th>EC model</th>
<th>$R_{sol}$ [Ω cm$^2$]</th>
<th>$R_{pl}$ [kΩ cm$^2$]</th>
<th>$Q_{pl}$ [μMho]</th>
<th>$n_{pl}$</th>
<th>$R_{bl}$ [MΩ cm$^2$]</th>
<th>$Q_{bl}$ [μMho]</th>
<th>$n_{bl}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti6Al7Nb</td>
<td>-0.08</td>
<td>$R_{sol}(R_{bl}Q_{bl})$</td>
<td>136</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>370</td>
<td>11.9</td>
<td>0.9</td>
</tr>
<tr>
<td>S2</td>
<td>-0.05</td>
<td>$R_{sol}(R_{pl}(Q_{pl}(R_{bl}Q_{bl})))$</td>
<td>141</td>
<td>22.9</td>
<td>8.31</td>
<td>0.904</td>
<td>227</td>
<td>24.2</td>
<td>0.466</td>
</tr>
<tr>
<td>S2 annealed</td>
<td>-0.10</td>
<td>$R_{sol}(R_{pl}(Q_{pl}(R_{bl}Q_{bl})))$</td>
<td>170</td>
<td>380</td>
<td>17.9</td>
<td>0.584</td>
<td>20</td>
<td>20.2</td>
<td>0.797</td>
</tr>
</tbody>
</table>
Figure 8: Fluorescent labeling of F-actin (green) and DNA (blue) of MG-63 cells. Images obtained by fluorescent microscopy, seven days after cell seeding. Scale bar - 25 μm.
• **Reverse transcription polymerase chain reaction (RT-PCR)**

is a variant of *polymerase chain reaction* (PCR), a *laboratory technique* commonly used in *molecular biology* to generate many copies of a *DNA* sequence, a process termed "amplification". In RT-PCR, however, an RNA strand is first *reverse transcribed* into its *DNA* complement (*complementary DNA*, or *cDNA*) using the enzyme *reverse transcriptase*, and the resulting cDNA is amplified using traditional *PCR* or *real-time PCR*. RT-PCR utilizes a pair of *primers*, which are complementary to a defined sequence on each of the two strands of the cDNA. These *primers* are then extended by a *DNA polymerase*. The analysis of the PCR products depends on the type of PCR applied. If a conventional *PCR* is used, the PCR product is detected using *agarose gel electrophoresis* and *ethidium bromide* (or other nucleic acid staining).
• **RT-PCR** includes three major steps. The first step is reverse transcription (RT), in which RNA is reverse transcribed to cDNA using reverse transcriptase. This step can act only on DNA templates.

• **The next step** involves the denaturation of the DNA at 95°C, so that the two strands separate and the primers can bind again at lower temperatures and begin a new chain reaction. Then, temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used.

• **The final step of PCR** amplification is DNA extension from the primers. This is done with thermostable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally.
Modulation of gene expression of bone specific markers in MG-63 osteosarcoma cells grown for 7 days.

a) mRNA levels of bone specific markers osteocalcin (a noncollagenic protein), osteonectin (a protein that binds selectively to both hydroxyapatite and collagen), BSP II (bonesyaloglicoprotein), B, osteocalcin, c) mRNA levels of osteonectin.

MG63 cells were grown on: 1- calcinated TiO2 nanotubes on Ti, 2 – uncalcinated TiO2 nanotubes on Ti, 3 – Ti (control), 4 - calcinated TiO2 nanotubes on TiAlNb, 5 – TiAlNb (control), 6 - uncalcinated TiO2 nanotubes on TiAlNb.

Total RNA was extracted and the mRNA levels of bone specific markers (osteocalcin, osteonectin and BSP II) were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the osteocalcin (panel B) and osteonectin (panel C) were quantified and are shown as a histogram.

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene
TiO$_2$ nanotubes obtained also on Ti6Al7Nb, in the same conditions as on Ti, but well-ordered TiO2 nanotubes were mainly on β phase.

Thermal treatment was optimized in order to obtain a higher rutile content without cracks on the surface or a tendency of nanotubes walls to coalesce.

Corrosion parameters from Tafel plots showed a smaller corrosion rate for annealed nanotubes, both for Ti and Ti6Al7Nb.

EIS studies showed that TiO$_2$ nanotube surface is composed of bi-layered oxide consisting of an inner barrier layer (associated to high impedance and responsible for corrosion protection) and an outer porous layer (nanotubes of lower impedance).

Cell tests showed that annealed TiO$_2$ nanotubes are more suited for cell growth and increase osteogenic activity.

All these data suggest that annealed TiO$_2$ nanotubes represent a promising material for orthopedic cellular therapy.
• Monitoring fabrication and processing conditions of nanoarchitecture is a way to obtain a better interface biomaterial living-tissue and synergetic effects in enhancing bioperformance.

• Electrochemical behaviour is directly related to biocompatibility, being an important aspect in selecting materials with high performance and help in understanding the mechanism at the biointerface.

- Cell growth and proliferation should be improved manipulating structure, composition and surface of metallic biomaterials.

• TiO2 nanoarchitectures have a size dependence behaviour. The various diameter and hydrophilic character (a contact angle between 83 and 16°) of self-organized TiO2 nanotubes layers induced different characteristics which can be exploited in different bioapplications.
Is the nanostructure a way to improve TiO2 properties?

- Yes (1,2) it is possible to improved bone forming functionality or cell viability (3)
- NO (4,5) Nanotube Diameter Directs Cell Fate
  - Increasing nanotube diameters led to increased elongation/stretching of cell bodies, and greater bone-forming ability.

TiO2 nanotubes could be photocatalyst for cancer cell killing and in such way a potential treatment for cancer appears.

- Fabrication nanotubes with bioinspired structure and specific composition and **specific geometry**, is a way to obtain a better interface biomaterial living tissue[2] The immunofluorescent images of cytoskeletal actin seem to suggest that there is a trend of increasing cellular (osteoblast) elongation with increasing size of nanotube diameter.

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1. Das Kakoli; Bandyopadhyay, Amit Bose, Susmita Biocompatibility and In Situ Growth of TiO2 Nanotubes on Ti Using Different Electrolyte Chemistry J. American Ceramic Soc. Vol. 91, 9, 2008, pp. 2808
Alumina nanotubes

Previously, alumina nanotubes have been prepared; by wet-chemical etching of a porous alumina membrane, by anodizing of Al/Si, or by utilizing one-dimensional organic or inorganic nanowires as sacrificial templates in hydrothermal synthesis, a sol-gel technique, chemical vapor deposition (CVD), or atomic layer deposition (ALD). However, apart from processing time, these methods have inherent disadvantages in fabricating alumina nanotubes with uniform diameter and in tailoring the length of nanotubes simultaneously.

Now is available a novel continuous method for the preparation of structurally well-defined alumina nanotubes with controllable length. The approach is based on pulse anodization of aluminum in H$_2$SO$_4$ combining both mild (MA) and hard anodization (HA) conditions, where the pulse duration defines the length of nanotubes.

Woo Lee,*, Roland Scholz, and Ulrich Gö"{o}nza Continuous Process for Structurally Well-Defined Al$_2$O$_3$ Nanotubes Based on Pulse Anodization of Aluminum NANO LETTERS 2008 Vol. 8, No. 8 2155-2160
Cross-sectional SEM micrographs of AAOs prepared by pulse anodization, showing the effect of HA-pulse duration on the tube length; (a) $\tau_{HA} \,\, 2 \, \text{s}$ and (d) $\tau_{HA} \,\, 6 \, \text{s}$. Other pulse parameters were fixed at $j_{HA} \,\, 368.42 \, \text{mA cm}^{-2}$, $j_{MA} \,\, 3.16 \, \text{mA cm}^{-2}$, and $\tau_{MA} \,\, 5 \, \text{s}$. (b,e) SEM micrographs of alumina nanotubes obtained from the samples shown in panels a,d, respectively; (b) $\tau_{HA} \,\, 2 \, \text{s}$ and (e) $\tau_{HA} \,\, 6 \, \text{s}$. TEM micrographs of the corresponding samples are shown in panels c and f, respectively.
Schematics illustrating (a) cross-section of pulse anodized AAO, (b) two different fracture modes of AAOs (A-A’ cleavage plane for HA-AAO and B-B’ cleavage plane for MA-AAO). (c) A representative cross-sectional SEM micrograph of pulse anodized AAO ($j_{MA}$) 3.16 mA cm$^{-2}$ and $\tau_{MA}$) 10 s for MA-pulses, $j_{HA}$) 200 mA cm$^{-2}$ and $\tau_{HA}$) 2 s for HA-pulses).

Horizontal fragmentation of AAO at the interfaces of HA-AAO segments is marked by a white rectangle in (c). White arrows in (c) indicate voids around the interface of HA-AAO segments. (d) TEM micrograph of a single alumina nanotube, showing a modulated pore structure. (e) Planeview SEM micrograph of bottom surface of HA-AAO, manifesting crack propagation along cell boundaries (i.e., A-A’ cleavage).
Vanadium Oxide Nanotubes I

Synthesis

Vanadium oxide nanotubes (VO$_x$-NTs) are easily accessible as a pure product in gram quantities by a low-temperature, soft chemistry synthesis. Primary alkyl amines ($C_nH_{2n+1}NH_2$ with $n = 4 - 22$) or alkyl diamines ($H_2N[CH_2]_nNH_2$ with $n = 14 - 20$) are reacted with a vanadium(V) alkoxide, and, after aging and a hydrothermal treatment at 180°C for about 1 week, the nanotubes are obtained in very high yield.

Morphology.

The tubular structure can be already seen in high-resolution SEM images. The inner core appears to be empty while the tube walls show roll-up characteristics.
Vanadium oxide nanotube materials were prepared by hydrothermal method and the factors that affect their electrochemical discharge–charge performance have been investigated.

The morphology, structure and electrochemical properties of the VO\textsubscript{x}-NT materials depend on the post-treatment temperature and heat treatment media. The controlled post-treatment (200 °C for 1 h)

Hai Xia Lia, Li Fang Jiao, Hua Tang Yuan, Ming Zhang, Jian Guo, Li Qin Wang, Ming Zhao and Yong Mei Wang

Factors affecting the electrochemical performance of vanadium oxide nanotube cathode materials Electrochemistry Communications volume 8, Issue 11, November 2006, Pages 1693-1698

A new kind of cathode materials for rechargeable lithium-ion batteries, lithium vanadium oxide nanotubes synthesized by a combined sol–gel reaction and hydrothermal treatment procedure is reported.
SEM, TEM, XRD and XPS techniques were performed to investigate the morphology and structure of the resulting materials.

The results confirmed that the synthetic materials are composed of uniformly open-ended multiwalled nanotubes with a length from 1 to 3 μm. The inner and the outer diameters of the obtained nanotubes vary from 30 to 50 nm and 50 to 120 nm, respectively.

The electrochemical performance as a cathode material was examined and evaluated by cyclic voltammetry, galvanostatic charge–discharge cycling and AC impedance spectroscopy techniques. The results indicated that the resultant lithium vanadium oxide nanotubes have a high initial discharge capacity of 457 mAh g⁻¹ in the potential range of 1.0–4.0 V (vs. Li/Li⁺) and good cycling performance. The improved electrochemical performance of the products should be due to its special one-dimensional multiwalled tubular structure and the contribution of lithium-ions.
• V oxide nanotubes were prepared in a modified sol-gel reaction of V oxide triisopropoxide in the presence of hexadecylamine and followed by hydrothermal treatment.
• The tubes consist of concentric shells of highly crystalline Voxide separated by alternating organic amine layers.
• The template molecules were removed without structural breakdown of the nanotubes by **combined ion-exchange reaction and extraction process**. The V oxide nanotubes are redox-active and can electrochemically insert Li reversibly.
• A specific charge was measured for Li$^+$ insertion into porous electrodes containing template-free nanotubes. The specific charge decreased during cycling, indicating a loss of electroactivity.

TEM image of a template-containing vanadium oxide nanotube showing the typical open end.

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SEM image of the template-containing vanadium oxide illustrating its nanotubular morphology with open ends.

\[ x\text{Li}^+ + x\text{e}^- + \text{VO}_{2.45}(\text{TEMP})_{0.34} \rightarrow \text{Li}_x\text{VO}_{2.45}(\text{TEMP})_{0.34} \]

electrochemical insertion of lithium into the nanostructured material

\( \text{VO}_{2.45}(\text{TEMP})_{0.34} \) (\( \text{TEMP} = \text{C}_{16}\text{H}_{33}\text{NH}_2 \)), indicating that a relatively large amount of organic template was built into the oxide structure during synthesis.
Lithium vanadium oxide nanotubes (Li$_y$VO$_x$-NTs) are composed of uniformly tubular-like morphology with a length from 1 to 3 $\mu$m, which are mostly grown-together in the form of bundles. As can be seen from the TEM image (Fig. b), the obtained nanotubes exhibit a well-defined multilayered structure, which have an open-ended tube and consisted of several concentric closed cylinders. The morphological characteristic indicated that the resulting materials are almost made up of multiwalled nanotubes with an inner diameter from 30 to 50 nm and an outer diameter from 50 to 120 nm.
(PPy/VOx-NTs) as a new high-performance cathode material for rechargeable lithium-ion batteries are synthesized by a combination of hydrothermal treatment and cationic exchange technique.

SEM and TEM images of VOx-NTs (a and c) and PPy/VOx-NTs (b and d); both samples present a uniform tubular-like morphology with a length of 1–3 μm, which are isolated or grown-together in the form of bundles. Both samples exhibit the open-ended cylindrical shape of the tubes and the dark lines correspond to vanadium oxide layers. It is clear that VOx-NTs (Fig. c) exhibit a well-ordered multilayered structure. For PPy/VOx-NTs (Fig. d), however, the interlayer walls are out of order after exchanging templates with PPy. The substitution of hexadecylamine by polypyrrole does almost nothing affect the shape of the nanotubes, with the exception of a slight difference in the interlayer distance.
• The miniature weight loss for the both samples from 50 to 200 °C is assigned to the removal of adsorbed water molecules;

• 49% weight loss for VOx-NTs from 200 to 380 °C is due to the adsorption and decomposition of residual organic template molecules.

• 35% weight loss for PPy/VOx-NTs from 200 to 400 °C corresponds to the combustion of the organic polymer PPy.

• Two broad exothermic peaks of PPy/VOx-NTs could be associated to the decomposition of the Ppy and the crystallization of V2O5, respectively.

• These results indicate that PPy/VOx-NTs have better thermal stability compared to VOx-NTs, and the substitution of the templates by the polypyrrole is also proved indirectly.

Three sharp exothermic peaks of VOx-NTs may be attributed to the decomposition of the organic template molecules at the surface and interlayer and the crystallization of the yellow V2O5, respectively.
• The results indicate that the organic templates are mainly substituted by the PPY without destroying the previous nanotube structure.

• Their electrochemical properties are evaluated via galvanostatic charge/discharge cycling, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). It is found that PPy/VOx-NTs exhibit high discharge capacity and excellent cycling performance at different current densities compared to vanadium oxide nanotubes (VOx-NTs).

• After 20 cycles, the reversible capacity of PPy/VOx-NTs (159.5 mAh g⁻¹) at the current density of 80 mA g⁻¹ is about four times of magnitude higher than that of VOx-NTs (37.5 mAh g⁻¹).

• The improved electrochemical performance could be attributed to the enhanced electronic conductivity and the improved structural flexibility resulted from the incorporation of the PPY.
zirconia nanotubes

zirconia nanotubes formation by electrochemical anodization of zirconium in a 1 M \((\text{NH}_4\text{)}_2\text{SO}_4\) electrolyte containing 0.5 wt% \(\text{NH}_4\text{F}\). Highly self-organized zirconia nanotubes can be formed with a diameter of \(\approx 50\) nm and a length of \(\approx 17\) \(\mu\text{m}\), i.e. with an aspect ratio of more than 300.

The nanotubes show a distinct smooth and straight morphology. XRD investigation reveals that the nanotubes have a cubic crystalline structure directly after anodization, that is, without any further annealing.
• compact oxide layers with cubic structures are formed in a wide variety of electrolytes.

• The observed crystalline structure is particularly noteworthy as porous layers formed on other valve metals such as Ti, W, Nb and Ta typically have an amorphous structure. Therefore – if a crystalline structure is desired – the layer on such valve metals must be transformed to a specific crystalline structure by annealing.

• zirconia nanotubes have a cubic crystalline structure directly after anodizing without annealing and this is of a particular advantage in applications that are sensitive to thermal annealing.

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• nanotube layer has a crystalline structure, having peaks corresponding to a cubic zirconia.

• Additional XPS investigation also showed that the structure essentially consists of a ZrO2 though only traces of F could be detected in the nanotube layer.
The zirconia nanotube arrays with diameter of about 130 nm, a length of up to 190 μm were prepared by anodizing a zirconium foil in a mixture of formamide and glycerol (volume ratio = 1:1) containing 1 wt.% NH4F and 3 wt.% H2O. The as-prepared nanotube arrays were amorphous zirconia.

Monoclinic and tetragonal zirconia coexisted when annealed at 400 °C and 600 °C, while monoclinic zirconia was obtained at 800 °C. The ZrO2 nanotubes retained their shape after heating up to 800 °C.

The lower dissolving rate of zirconia in organic electrolytes might be the main reason for fabrication of zirconia nanotube arrays with high aspect ratio.
Effect of ageing in the electrolyte and water on porous anodic films on zirconium

- It is a significant influence of ageing in the formation electrolyte on the morphology and composition of anodic films grown on zirconium in 0.35 M ammonium fluoride in glycerol.
- Ageing after anodizing, by immersion in the electrolyte for 1 h, is shown to promote a transition from a porous to a nanotubular morphology, due to the dissolution of the fluoride-rich intratubular material in which the nanotubes are embedded. The morphological change is accompanied by a significant loss of zirconium and fluorine from the film. In contrast, ageing in deionized water has little influence on the films.

SEM of an anodic film grown on Zr to 780 mC cm$^{-2}$ at 40 V in 0.35 M NH4F in glycerol, in the absence of added water: (a) before ageing; after ageing for 3 h in (b) the formation electrolyte and (c) deionized water.
TEM of non-aged nanotubes grown in the electrolyte with no added water revealed a rough appearance, due partly to remnants of the fluoride-rich intratubular material, in which the nanotubes are embedded, that remained attached to the tube walls following scraping of the nanotubes from the anodized surface. (Fig. a).

Ageing in the formation electrolyte for differing times resulted in nanotubes with a smoother morphology, since the intratubular material had been dissolved (Fig. b); the particular nanotubes shown have a base thickness of 9–13 nm and internal and external diameters ($D_{\text{int}}$ and $D_{\text{ext}}$) of 50 and 70 nm, respectively.

The featureless appearance of the nanotube material is indicative of an amorphous structure. When cleaned of the intratubular material by the ageing treatment, the nanotubes revealed an intrinsic roughness, which appeared to be due to the tendency of the pores to form incipient branches, most of which do not develop beyond the stage of a short protuberance (Fig. b).

TEM micrographs of nanotube bases of a film grown on Zr to 780 mC cm$^{-2}$ at 40 V in 0.35 M NH$_4$F in glycerol, in the absence of added water, (a) before and (b) after ageing for 6 h in the formation electrolyte.
• Ageing affected the films grown with 5 vol.% added water in a similar manner to those formed with no added water. Fig. a shows a SEM of a film immersed for 24 h in the formation electrolyte; nanotubes can be distinguished clearly, with a length of 730 nm.

• The nanotubes are attached to a thin surface film, and the external walls of each nanotube appear to be free of intratubular material and smooth (contrasting with the nanotubes formed without added water) and are evidently well-separated from the walls of the adjacent nanotubes. No major differences were observed in the morphologies of nanotubes aged in the formation electrolyte for other times. Unlike the influence of the formation electrolyte, the nanotubes remained mainly embedded in the intratubular material following ageing in deionized water for times from 1 to 24 h, as shown in SEM of Fig. b. which discloses a thin, relatively flat fluoride layer, and a small region where the layer has detached from the nanotubes, revealing approximately hemispherical nanotube bases; the detachment may be related to a local difference in the composition and morphology of the etched Zr substrate.
Fig. presents TEM of an as-formed nanotube (in the electrolyte with 5 vol.% added water) (Fig. a) and a nanotube aged in the formation electrolyte for 1 h (Fig. b). The appearance of the nanotubes indicates an amorphous structure, similar to the observation for the nanotube formed without addition of water to the electrolyte.

The thicknesses of the barrier layer of the non-aged and aged nanotubes were 25 and 20 nm, respectively. The bases of the nanotubes were significantly thicker than those of the nanotubes formed with no added water, which were in the range from 9 to 13 nm. The average external diameters of the non-aged and aged nanotubes were 75 and 55 nm, respectively, and the average internal diameters were 35 and 30 nm, respectively.
• Fig a and b shows SEM of films formed with 0 and 5 vol.% added water, aged in water for 3 h; the areas selected for observation show locations where the films have detached from the Zr substrate.

• The films had detached near to regions from which nanotubes had been removed from the Zr by scraping the anodized surface; the detachment was probably initiated by the stress induced in the film near to the scraped zone by the scraping procedure. In Fig. a, a piece of detached film formed with no added water is evident adjacent to a region where the film has been lost entirely from the Zr surface. The relatively thick, F- rich region is readily seen at the base of the film indicating that the detachment occurred either at or very close to the interface between the F- layer and the substrate. The exposed Zr reveals grain boundaries and facet-like features on the grain surfaces that differ in appearance between the grains. The crystallographic features suggest an influence of the grain orientation on the film growth. Fig. b shows a dimpled surface at the region of detachment of the film formed with 5 vol.% added water.
Superhydrophilic ZrO2 nanotube layer

• prepared by anodizing pure Zr in aqueous solutions 1 M (NH₄)₂SO₄ and 0.15 M NH₄F. The effect of annealing and (UV) irradiation on the microstructure, water contact angle and bioactivity of the ZrO₂ nanotube layer.

• The as-anodized nanotube layer consists of cubic and amorphous ZrO₂, no apatite crystals are deposited on its surface even after immersion in simulated body fluids (SBF) for 30 days, exhibiting weak apatite-inducing ability.

• After annealing at 450 °C for 3 h, the nanotube layer is composed of cubic and monoclinic ZrO₂, and its apatite-forming ability is enhanced because of its lattice structure matching that of apatite, apatite can be induced after immersion in SBF for 15 days. UV irradiation of the ZrO₂ nanotube layers does not alter their surface morphologies and phase components, however, can improve the bioactivity only when the ZrO₂ nanotube layer is well crystallized.

• The enhanced bioactivity by UV irradiation is thought to result from the abundant basic ZrOH groups on the crystallized ZrO₂ nanotube layer. Annealing and UV irradiation treatment do not alter the superhydrophilic nature of the ZrO₂ nanotubes.

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• Area of visible pores increases with increasing anodizing time but the precipitate layer always remains present to a certain extent. The slow potential sweep 20 mV s−1 leads to relatively etched structures. There are islands and/or clusters, but highly ordered porous structures are observed between islands.

• The top part of the porous layers consists of many protruded regions ("hillocks"). These hillocks consist of numerous nano-tubes. The tubes are concentrated at the tips of the hillocks and widen into a regular porous structure underneath the surface. (hillocks can be identified as some islands and/or clusters from the top Fig. (c) and the open porous structures are visible between the hillocks.

• With potential sweep rate (1 V s−1) the structure is as in Fig. (b) highly ordered porous zirconium oxide layer consisting of pores with an average diameter of approx. 50 nm.

**FIG** shows plan-view images of the porous zirconium oxide layer observed after anodizing using the fast potential sweep such as 20 V s−1. This type of anodizing seems to result in inhomogeneous structures, far from being organized and the surface is partially covered by precipitates or intermediate reaction products (presumably Zr-hydroxide).
HA is performed at relatively low temperatures and high current density (typically, \( j > 50 \text{ mA cm}^{-2} \)) by using H2SO4 is convenient for industrial applications due to the high-speed growth (50 - 100 \( \mu \text{m h}^{-1} \)) of anodic films with high technical quality. However, pores of the resulting anodic films are less ordered than those produced by MA processes, and thus HA processes have been out of focus in academic, Pulse associated with HA of aluminum and its alloys and thus to produce anodic films that are superior in hardness, corrosion resistance, and thickness uniformity at an efficient rate of production in comparison with the conventional HA methods.

However, the process has not been employed in current nanotechnology due to the nonuniform and disordered pore structure of the resulting anodic alumina, although have recently demonstrated that pulse anodization can successfully be implemented to develop novel three-dimensional (3D) porous architectures.
SEM of anodic porous zirconia layer. (a) Top-view and (b) cross-sectional of the porous layer formed on Zr in 1 M H2SO4 + 0.2 wt% NaF electrolyte at 20 V. The pores that were formed showed a wavy and somewhat irregular wall morphology with a diameter of \( \approx 50 \text{ nm} \) and a length of \( \approx 17 \text{ \textmu m} \). The pore walls are completely smooth and straight.
Compositions of aged nanotubes were investigated qualitatively by high resolution TEM/EDX analyses. TEM are shown in Fig. a and c. The presence of Zr, F and O was detected in the nanotubes, as shown in the EDX spectra of Fig. b and d that were recorded at the locations shown in Fig. 7 and c. EDX spectra that were recorded at other locations, including both the barrier layer and the tube walls, were very similar to those shown in Fig. b and d, suggesting that there was little variation of the composition of the nanotube material. Fig. a clearly shows that the roughness of the tubes formed in the absence of added water is due to a tendency to develop branches, although most appear to terminate at an embryonic stage.

Dark field TEM of nanotubes grown on Zr to 780 mC cm$^{-2}$ at 40 V in 0.35 M NH4F in glycerol, in the absence of added water, and aged for 24 h in the formation electrolyte.

(b) High resolution EDX spectrum related to the location indicated in Fig. a. (c and d) Results of TEM and EDX analysis for nanotubes formed and aged in electrolyte containing 5 vol.% water.
HfO$_2$ films and ultrathin-wall nanotubes

Many metal oxides exhibit size-dependent phase transitions among multiple polymorphs. In this work, the microstructure and crystallinity of ultrathin HfO$_2$ films and ultrathin-wall nanotubes were investigated by high-resolution electron microscopy and electron diffraction after high-temperature annealing. Nanotubes were formed by atomic layer deposition of HfO$_2$ on epitaxial Ge $<111>$ nanowire arrays on Si (111) substrates followed by selective etching of the Ge wires.

A size-dependent phase transition sequence from amorphous (a-HfO$_2$) to tetragonal (t-HfO$_2$) and from tetragonal to monoclinic (m-HfO$_2$) phase was observed with increasing film and nanotube wall thickness. These results are analyzed in light of recent predictions of surface energy-driven phase transitions in nanoscale fluorite-structure oxides.