Nanostructured biomaterial thin films synthesized by pulsed laser technologies: new applications to implantology

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Why biomaterials?

**Critical use:**
- partial repair and reconstruct of parts of the musculo-skeletal system of vertebrates.

**Key asset:**
- Meet minimal biological requirements: biocompatibility combined with the absence of any adverse effect (non-toxic and non-allergic).

**Other requests:**
- resistance to physiological fluids;
- non-interference with the body’s natural immune system;
- lifelong resistance to mechanical stress;
- manufacturability in any desired shape.

**Tentative classification:**

- **Biologically inactive (inert):** alumina, zirconia, stainless steel, CoCrNi, CoCrMo, titanium, titanium alloys, carbon, latex, PE, PMMA;
- **Bioactive:** calcium phosphate ceramics, bioactive glasses (45S5 Bioglass®), bioactive glass-ceramics (Cerevital®, wollastonite A/W glass-ceramics, machinable glass-ceramics), bioactive composites (Palavital®, stainless steel-fiber reinforced Bioglass®, polyethylene-hydroxyapatite (PE-HA) mixtures), etc.
- **Bioresorbable:** tricalcium phosphate, calcium-aluminate, polylactic acid, poly-L-acetate.
Motivation of research

- **Major bone disease: Osteoporosis** - skeletal fragility; up to 1 in 2 women and 1 in 3 men will sustain an osteoporotic fracture during their lifetime;

Present day approach:

- **120,000 hip replacement** operations/year in USA; the total cost for treating all types of fractures in USA - $14 billions in 1999!

Limitations:

- **Inflammatory reaction (foreign body response):** fibroblast proliferation, collagen synthesis, blood vessel proliferation → encapsulation;

- **Mechanical wear (abrasion)** - 0.10–0.20 mm/year polyethylene abrasion; 0.002–0.006 mm/year cobalt–chromium–molybdenum alloy wear → Aseptic loosening

- **Ultimate solution:** 36,000 revision surgeries / year in USA

**Requirement:** better osteointegration resulting in improvement of fixation between hard tissues and implants
**Our option: biocompatible, porous and bioactive CaPs**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Chemical formula</th>
<th>Compound name</th>
<th>Ca/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Ca$_{10}$(PO$_4$)$_6$(OH)$_2$</td>
<td>Hydroxylapatite</td>
<td>1.67</td>
</tr>
<tr>
<td>FA</td>
<td>Ca$_{10}$(PO$_4$)$_6$F$_2$</td>
<td>Fluorapatite</td>
<td>1.67</td>
</tr>
<tr>
<td>CDHA</td>
<td>Ca$_{10-x}$(HPO$_4$)$_x$(PO$_4$)$_6$·(OH)$_2$·x (0&lt;x&lt;2)</td>
<td>Calcium-deficient Hydroxylapatite</td>
<td>1.33-1.67</td>
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<tr>
<td>BA</td>
<td>Ca$_{8.3}$(PO$_4$)$_4.3$(CO$<em>3$-HPO$<em>4$)$</em>{1.7}$(OH)$</em>{0.3}$</td>
<td>Biological apatite</td>
<td>1.38-1.93</td>
</tr>
<tr>
<td>Mn-CHA</td>
<td>HA with (0.4-2)% Mn$^{2+}$ and (2-6)% CO$_3^{2-}$</td>
<td>Mn$^{2+}$ doped carbonated hydroxylapatite</td>
<td>1.51-1.65</td>
</tr>
<tr>
<td>OHA</td>
<td>Ca$_{10}$(PO$_4$)$<em>6$(OH)$</em>{2-2x}$O$_x$</td>
<td>Oxyhydroxylapatite</td>
<td>1.67</td>
</tr>
<tr>
<td>OA</td>
<td>Ca$_{10}$O(PO$_4$)$_6$</td>
<td>Oxyapatite</td>
<td>1.67</td>
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<tr>
<td>MCPM</td>
<td>Ca(H$_2$PO$_4$)$_2$·H$_2$O</td>
<td>Monocalcium phosphate monohydrate</td>
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<tr>
<td>MCPA</td>
<td>Ca(H$_2$PO$_4$)$_2$</td>
<td>Monocalcium phosphate anhydrate</td>
<td>0.5</td>
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<tr>
<td>DCPD</td>
<td>CaHPO$_4$·2H$_2$O</td>
<td>Dicalcium phosphate dihydrate (Brushite)</td>
<td>1.0</td>
</tr>
<tr>
<td>DCPA</td>
<td>CaHPO$_4$</td>
<td>Dicalcium phosphate anhydrate (Monetite)</td>
<td>1.0</td>
</tr>
<tr>
<td>OCP</td>
<td>Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4$·5H$_2$O</td>
<td>Octacalcium phosphate</td>
<td>1.33</td>
</tr>
<tr>
<td>α-TCP</td>
<td>Ca$_3$(PO$_4$)$_2$ (monoclinic)</td>
<td>Tricalcium phosphate (phase α)</td>
<td>1.5</td>
</tr>
<tr>
<td>β-TCP</td>
<td>Ca$_3$(PO$_4$)$_2$ (rhombohedral)</td>
<td>Tricalcium phosphate (phase β, Whitlockite)</td>
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<tr>
<td>TTCP</td>
<td>Ca$_4$O(PO$_4$)$_2$</td>
<td>Tetracalcium phosphate</td>
<td>2.0</td>
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<tr>
<td>α-DCP</td>
<td>Ca$_2$P$_2$O$_7$ (orthorhombic)</td>
<td>Dicalcium phosphate (phase α)</td>
<td>1.0</td>
</tr>
<tr>
<td>β-DCP</td>
<td>Ca$_2$P$_2$O$_7$ (tetragonal)</td>
<td>Dicalcium phosphate (phase β)</td>
<td>1.0</td>
</tr>
<tr>
<td>ACP</td>
<td>Ca$_x$(PO$_4$)$_y$·nH$_2$O</td>
<td>(Amorphous Calcium pyrophosphate)</td>
<td>1.2-2.2</td>
</tr>
</tbody>
</table>

The Ca/P ratio determines the solubility and activity of CaP compounds within the human body.
Main drawback of CaPs: brittle in bulk

Alternative solution: Biomimetic coatings for metallic implants
How difficult is to deposit CaPs? (1)

- very complex molecules

HA molecule: $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$

Projection in the (001) base plan of the hydroxyapatite unit cell (hexagonal structure)
How difficult is to deposit CaPs? (2)

Crystal structure of OCP projected down the c-axis

Octacalcium phosphate (Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4$·5H$_2$O)

Alternating apatite- and hydrated- layers, \( \parallel(100) \) planes.

The region with shaded atoms, the “apatitic layer”, is very similar to HA. The zone containing 10 water molecules is the “hydrated layer”.

H atoms are omitted for the sake of clarity.
## Deposition methods for CaP coatings

<table>
<thead>
<tr>
<th>Method</th>
<th>Working gas</th>
<th>Typical thickness</th>
<th>Surface morphology</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Vacuum and atmospheric plasma-spraying      | Ar, N₂, H₂           | 50-200 μm         | Very rough, irregular and porous    | Surface macro-porosity enhances bone ingrowth  
Large deposition area  
High deposition rate | Large amount of amorphousness  
Poor thickness control  
Undesired secondary phases  
Poor adhesion  
Mechanical failure |
| Magnetron sputtering                        | Ar                   | < 2 μm            | Smooth, uniform                     | High density  
Uniformity on large area  
High adherence  
Follows the substrate geometry | Amorphousness  
Presence of TTCP and CaO phases  
Low deposition rate  
Ca/P atomic ratio <1.67 |
| Ion/electron beam (assisted) deposition / ion beam sputtering | vacuum               | < 1 μm            | Smooth                             | High adherence                                | High vacuum needed  
Amorphousness  
Post-annealing at (400 – 600° C) in moisture media |
| Sol-gel                                    | -                    | ~ 1 μm            | Rough                               | Covering of various substrates shapes  
Medium temperature processing (300-500° C) | Precursors needed  
Poor integrity and microstructure |
<table>
<thead>
<tr>
<th>Method</th>
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<th>Typical thickness</th>
<th>Surface morphology</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic deposition</td>
<td>-</td>
<td>&lt; 20 μm</td>
<td>-</td>
<td>Covering of complex substrates</td>
<td>Poor bond strength</td>
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<td>Feeling of porous substrate cavities</td>
<td>Shrinkage and cracking</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Coated substrate sintered at (900 – 1000° C)</td>
</tr>
<tr>
<td>Laser cladding</td>
<td>Shielding Inert gas</td>
<td>300 – 400 μm</td>
<td>Smooth surface finish</td>
<td>Controlled clad shape</td>
<td>Undesired CaP, other than HA: TCP, CaP glass</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Localized processing heating</td>
<td>Formation of calcium titanates and titanium phosphates</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Controlled dilution levels</td>
<td></td>
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<tr>
<td>Pulsed laser deposition</td>
<td>Inert gas, O₂, H₂O or mixture of them</td>
<td>&lt; 1 μm</td>
<td>Smooth / rough, depending on target properties and deposition conditions</td>
<td>High density and crystallinity</td>
<td>Limited deposition on large areas</td>
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<td></td>
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<td></td>
<td>Proper stoichiometry</td>
<td>Limited thickness uniformity</td>
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<td>Controlled Ca/P ratio</td>
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<td>Good adherence</td>
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<td>Clean process</td>
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<td>Relatively low processing temperature (400 – 700° C)</td>
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</tbody>
</table>
HA, Mn doped - carbonated HA (Mn-CHA), Sr doped HA, octacalcium phosphate (OCP), Hydroxyapatite + maleic anhydride copolymer composite (HA + MP)

- bioactive ceramic materials believed to enhance bioactivity and biocompatibility of the Ti-based bone prosthesis and tooth implants

**HA:**
- crystalline hydrate CaP; main constituent of bone (≈ 65% of volume); excellent biocompatibility and bioactivity;

**Mn-CHA:**
- \((\text{CO}_3)^{2-}\) ions, also present in biological apatite, generally enter the \((\text{PO}_4)^{3-}\) sites;
- \(\text{Mn}^{2+}\) ions activate integrins (*receptors mediating cellular interactions with extra-cellular matrix and cell surface ligands*) and should promote the interaction with the host bone tissue.

**Sr-HA:**
- benefic effect in osteoporosis (Sr ranelate based drugs)

**OCP:**
- the most likely precursor of biological apatites due to its structural resemblance to HA;
- prospective alternative to HA coatings for metal implants.

**HA+MP:**
- biopolymer capable of improving coating mechanical behaviour (adherence, elasticity);
- induces surface functionalization of the coating.
PULSED LASER DEPOSITION (PLD) METHOD

Laser parameters:
- Excimer KrF*
- $\lambda = 248$ nm
- $\tau_{FWHM} \sim 7$ or 25 ns
- rep. rate 2 - 50 Hz
- $F = (0.2 - 10) \text{ J/cm}^2$
Main differences between PLD and MAPLE: target preparation and mechanism of laser - material interaction

Target preparation:

- active material is dissolved in a solvent (matrix) forming a liquid composite;
- the liquid mixture is transformed in solid by freezing;
- target kept at low temperature with a cooler during deposition.
MAPLE

- Laser-Material Interaction:
  - Composite Target is Evaporated Using UV Laser Pulses;
  - Volatile Solvent:
    - Absorbs Most of Laser Pulse Energy
    - Does Not Form a Film
    - Is Pumped Away by the Vacuum System.

The volatile solvent absorbs most of the laser pulse. Upon heating, the solvent gently desorbs the biomaterial & organic molecule, forming a uniform thin film on the substrate surface.
CHARACTERIZATION

Physico-chemical analyses:
- GIXRD, SEM, TEM, HRTEM, SAED, XPS

Biological analyses:
In-vitro: - Biocompatibility tests:
  - Cell morphology;
  - Proliferation and viability (WST1 test);
  - Cytoskeleton labeling;
- Biodegradation tests
- Bioactivity tests:
  - Alkaline Phosphatase (ALP) activity
  - Collagen type 1 (CICP)
  - Transforming growth factor beta 1 (TGF β1)

In-vivo: - Pull out tests
ILUSTRATIVE RESULTS
HR ELECTRON MICROSCOPY OF OCP REVEALING NANO-CRYSTALLINE DOMAINS EMBEDDED IN AN AMORPHOUS MATRIX

a = 19.68(1) Å  
b = 9.50(1) Å  
c = 6.832(5) Å  
α = 90.21(8)°  
β = 92.52(9)°  
γ = 108.32(8)°

X-ray diffraction patterns are in agreement with an amorphous-poor crystalline structure. OCP presence is confirmed by the shoulder around 4.7° of 2θ, corresponding to the (100) reflection of OCP, and by the broad peak centered around 32-33° of 2θ.

**OCP AND Mn-CHA STRUCTURES EXHIBIT DIFFERENT MORPHOLOGIES**

- **OCP**: porous, tree-like morphology

- **Mn-CHA**: granular, more compact morphology
Sr-HA STRUCTURES EXHIBIT RATHER POROUS MORPHOLOGIES

SEM micrographs of thin films deposited from (a) Sr0; (b) Sr10 samples.
Scale bars = 1 μm

Larger Sr doping induces an increase of porosity
Sr-DISTRIBUTION IN HA COATINGS

EDS maps recorded from the coatings: (a) TiSr1; (b) TiSr5; and (c) TiSr10

Increase of Sr doping confirmed by EDS

Sr-red, HA blue.
Biocompatibility Tests - Cell Cultures
(Proliferation and Viability)

Human primary osteoblasts (hOB) were cultured on OCP-coated Ti, Mn-CHA-coated Ti, HA-coated Ti, bare Ti, control (polystyrene)

hOB response: SEM micrographs

- on bare Ti: (a) after 7 days, (b) after 21 days

Elongated, rod-like morphology
Over time, the cells spread and expand with flattened, polyhedral-morphology.

Numerous cytoplasmatic extensions $\rightarrow$ firm attachment
- **Mn-CHA coatings**: (e) after 7 days, (f) after 21 days

- The cells spread and expand overtime, showing a flattened, polyhedral-morphology;
- Fewer cytoplasmatic extensions
**Sr-HA coatings:** SEM micrographs of osteoblasts after 21 days of culture on: (a) TiHA; (b) TiSr5; and (c) TiSr10. Scale bars = 50 μm.

→ **Ti/HA**: hOB were flattened, with polygonal configuration and dorsal ruffles; well attached to the substrate by cellular extensions.

→ **Ti/Sr doped HA**: hOB appear much more flattened and better spread across the surface.
Florescence microscopy images of hOB on Sr-HA coatings

Percentage of osteoblast adhesion 1 hour after seeding on (a) TiHA, (42±4%); (b) TiSr1, (48±8%); (c) TiSr5, (58±5%); and (d) TiSr10, (71±13%*). Bar: 50 μm.
Proliferation of osteoclast (hOC) culture on Sr-HA coatings: 21 days

(a) TiHA (3.285±0.021); (b) TiSr1 (3.252±0.047); (c) TiSr5 (3.211±0.008*); and (d) TiSr10 (3.193±0.019*). Bar: 50 μm.

hOC percentage decreases while cells pill
**HEK293 on HA+MP MAPLE coatings by cytoskeleton labelling**

Fluorescence microscopy images

Human embryonic kidney (HEK293) cells

- cell morphology: polyhedral
- good spreading, establish cell-cell contacts, tend to occupy the culture surface

The actin filament pattern of **cytoskeleton** of cells on HA+MP → indicative of **biocompatibility**

- Prominent focal adhesions: firmly anchorate cells to the substrates → good adhesion;

A - Hek293 cells grown on HA - maleic anhidride copolymer; B - Hek293 cells grown on standard glass material;
HEK293 on HA+MP MAPLE coatings by cytoskeleton labelling

Fluorescence microscopy images

A - Hek293 cells grown on HA - maleic anhidride copolymer; B - Hek293 cells grown on HA

1. Differences in cell actin staining may work as a sensor of the biomaterial surface coating quality.
2. Polymer enhances adhesion/proliferation qualities of the biomaterial coating surface
DEGRADATION TESTS

SBF composition

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>142</td>
</tr>
<tr>
<td>K⁺</td>
<td>5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.5</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>103</td>
</tr>
<tr>
<td>HPO₄²⁻</td>
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<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>27</td>
</tr>
</tbody>
</table>

OCP coatings dissolve and disappear almost totally after 7 days of immersion in SBF.

Mn-CHA coatings remain almost intact after 7 days of SBF immersion.

XPS spectrum of OCP before (OCP3) and after (OCP2) degradation tests

XPS spectrum of Mn-CHA before (HA) and after (HA1) degradation tests
BIOACTIVITY TESTS
ALKALINE PHOSPHATASE ACTIVITY (ALP)

ALP level is an early index of cell activation and differentiation. The mineralization stage correlates with a reduced ALP activity.

- Increase, days 3 to 14 ⇒ a shift to a more differentiated state;
- Slight decrease, days 14 to 21 ⇒ the mineralization matrix is formed;
- Higher values for CaP coatings ⇒ coatings are capable of improving tissue integration
Osteoblast proliferation and activity after 7, 14, and 21 days of culture on Sr:HA, ALP test

7 days: n.s.;
14 days: **TiSr5 versus TiHA; **TiSr10 versus TiSr1, ***TiSr10 versus TiHA;
21 days: *TiSr5 versus TiHA, **TiSr10 versus TiHA, TiSr1.

-Similar time evolutions - mineralization stage correlates with a reduced ALP activity;
- higher values after doping with Sr – further improvement of tissue integration
**COLLAGEN TYPE I (CICP):**

Collagen type I is synthesized by osteoblasts as the major organic macromolecule in the extracellular bone matrix.

- the values for polystyrene (control) and Ti were highest on day 3; they gradually decreased during days 7 to 21;
- on OCP and Mn-CHA coatings, an increase from days 3 to 7 was followed by a decrease after day 14
TRANSFORMING GROWTH FACTOR BETA 1 (TGFβ1):

TGF-β1 protein, synthesized by osteoblasts, modulates cell proliferation and differentiation and enhances the deposition of extracellular matrix during developmental processes.

- Values for Control (polystyrene) and Ti peaked after 7 days and then constantly decreased;
- TGF-β1 of coated materials increased from day 7 to day 21, indicating bone growth 3 weeks after implantation.
**IN VIVO - PULL OUT TESTS**

- Pull out test discriminates between different implant attachment mechanisms. The model involves the use of a flat coin shaped implant placed on the cortical bone of rabbit tibia.

- New Zealand White adult female rabbits, 8 months, 3000-3500 g weight

- Moderate Ti substrate roughness was chosen:
  - High enough to stimulate bone repair and growth; but
  - low enough to allow separation of biological effects;
  - threatened on reverse side
PULL OUT PROCEDURE (1)

Surgical procedures (a - g)
Tensile test procedures (h - k)

• Pullout test conducted after 8-week healing time;
• Cross head speed was set to 1,0 mm/min
All CaP PLD-coated Ti implants reveal enhanced bone healing/repairing (pull out force), about two times better than in the case of control machined-Ti implants.

New CaPs (OCP and Mn-CHA) lead to significant increases in osteointegration efficiency significantly higher pullout forces (up to ¼ of maximum value).
**CONCLUSIONS**

- New “intelligent” CaPs (OCP and Mn-CHA) nanostructured coatings have been successfully produced by PLD.
- The presence of OCP nano-crystalline domains inside an amorphous matrix was evidenced in a close similitude with the actual human bone structure.
- Mn-CHA films have been found to display a good crystallinity and granular surface morphology.
- Degradation in SBF has suggested that behaviour of OCP and Mn-CHA coatings varies in terms of their stoichiometry and degree of crystallinity, stable or resorbable CaP interlayers can therefore be designed.
- *In-vitro* tests have proved that human osteoblasts proliferate, reach a normal morphology and remain viable when cultured on CaP coatings.
- Biochemical studies showed that the presence of Sr in the CaP coatings enhances osteoblasts activity and differentiation, while it inhibits osteoclasts production and proliferation. This effect increases with Sr concentration.
- Cells grown on HA+polymer coatings grown by MAPLE show excellent biocompatibility: normal morphology, good adhesion and spreading to the substrate.
- *In-vivo* pull out tests on OCP, Mn-CHA and HA-coated implants clearly show that CaP coatings activate and enhance bone repair. New CaPs (in particular Mn-CHA) lead to a 20% supplementary improvement of the implant bioactivity/adherence as compared to pure HA.