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:

a) *Biologically inactive (inert)*: alumina, zirconia, stainless steel, CoCrNi, CoCrMo, titanium, titanium alloys, carbon, latex, PE, PMMA;

b) *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.

c) *Bioresorbable*: tricalcium phosphate, calcium-aluminate, polylactic acid, poly-L-acetate.

Motivation of research

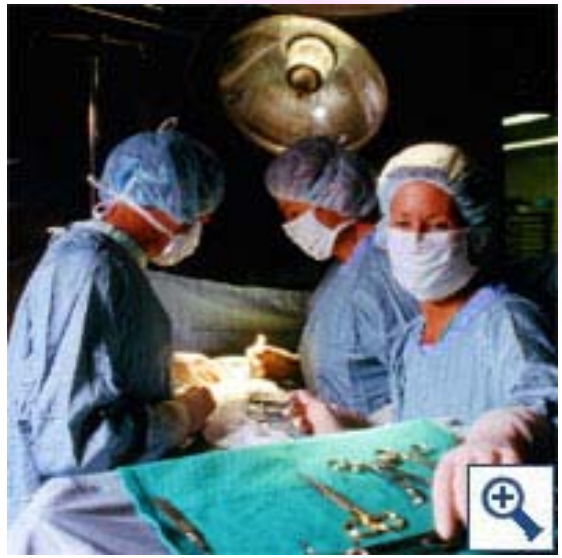
- **Major bone disease: Osteoporosis** - skeletal fragility; up to **1 in 2 women** and **1 in 3 men** will sustain an osteoporotic fracture during their lifetime;

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**

<u>Requirement:</u> better osteointegration resulting in improvement of fixation between hard tissues and implants

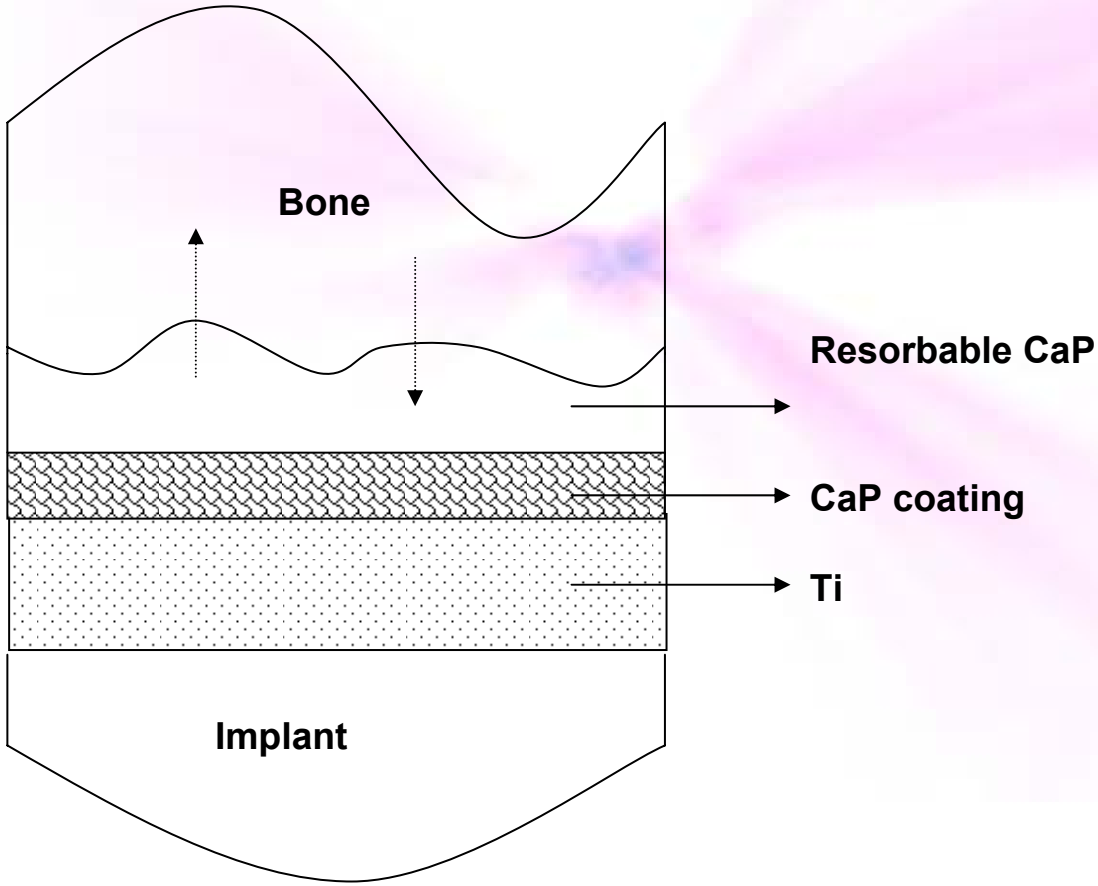
Our option: biocompatible, porous and bioactive CaPs

Acro-nym	Chemical formula	Compound name	Ca/P ratio
HA	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxylapatite	1.67
FA	$Ca_{10}(PO_4)_6F_2$	Fluorapatite	1.67
CDHA	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ (0<x<2)	Calcium-deficient Hydroxylapatite	1.33-1.67
BA	$Ca_{8.3}(PO_4)_{4.3}(CO_3\text{-}HPO_4)_{1.7}(OH)_{0.3}$ BA=carbonated CDHA (x=1.7)	Biological apatite	1.38-1.93
Mn-CHA	HA with (0.4-2)% Mn^{2+} and (2-6)% CO_3^{2-}	Mn^{2+} doped carbonated hydroxylapatite	1.51- 1,65
OHA	$Ca_{10}(PO_4)_6(OH)_{2-2x}O_x\square_x$ (0<x<1)	Oxyhydroxylapatite	1.67
OA	$Ca_{10}O(PO_4)_6$	Oxyapatite	1.67
MCPM	$Ca(H_2PO_4)_2{\cdot}H_2O$	Monocalcium phosphate monohydrate	0.5
MCPA	$Ca(H_2PO_4)_2$	Monocalcium phosphate anhydrate	0.5
DCPD	$CaHPO_4{\cdot}2H_2O$	Dicalcium phosphate dihydrate (Brushite)	1
DCPA	$CaHPO_4$	Dicalcium phosphate anhydrate (Monetite)	1
OCP	$Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$	Octacalcium phosphate	1.33
α-TCP	$Ca_3(PO_4)_2$ (monoclinic)	Tricalcium phosphate (phase α)	1.5
β-TCP	$Ca_3(PO_4)_2$ (rhombohedral)	Tricalcium phosphate (phase β, Whitlockite)	1.5
TTCP	$Ca_4O(PO_4)_2$	Tetracalcium phosphate	2
α-DCP	$Ca_2P_2O_7$ (orthorhombic)	Dicalcium phosphate (phase α)	1
β-DCP	$Ca_2P_2O_7$ (tetragonal)	Dicalcium phosphate (phase β)	1
ACP	$Ca_x(PO_4)_y\ nH_2O$	(Amorphous Calcium pyrophosphate)	1.2-2.2

The *Ca/P ratio* determines the solubility and activity of CaP compounds within the human body.

Main drawback of CaPs: brittle in bulk

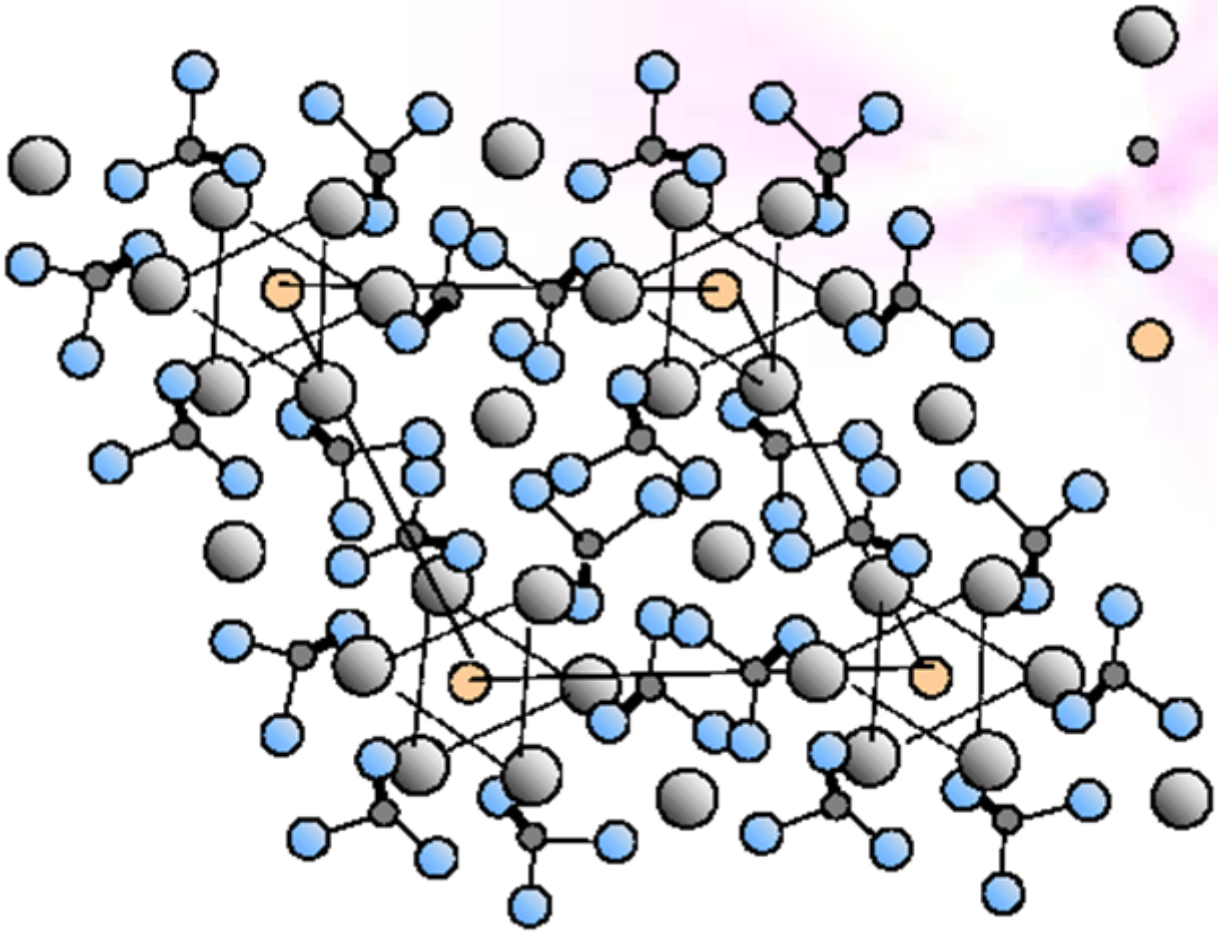
Alternative solution: Biomimetic coatings for metallic implants



How difficult is to deposit CaPs? (1)

- very complex molecules

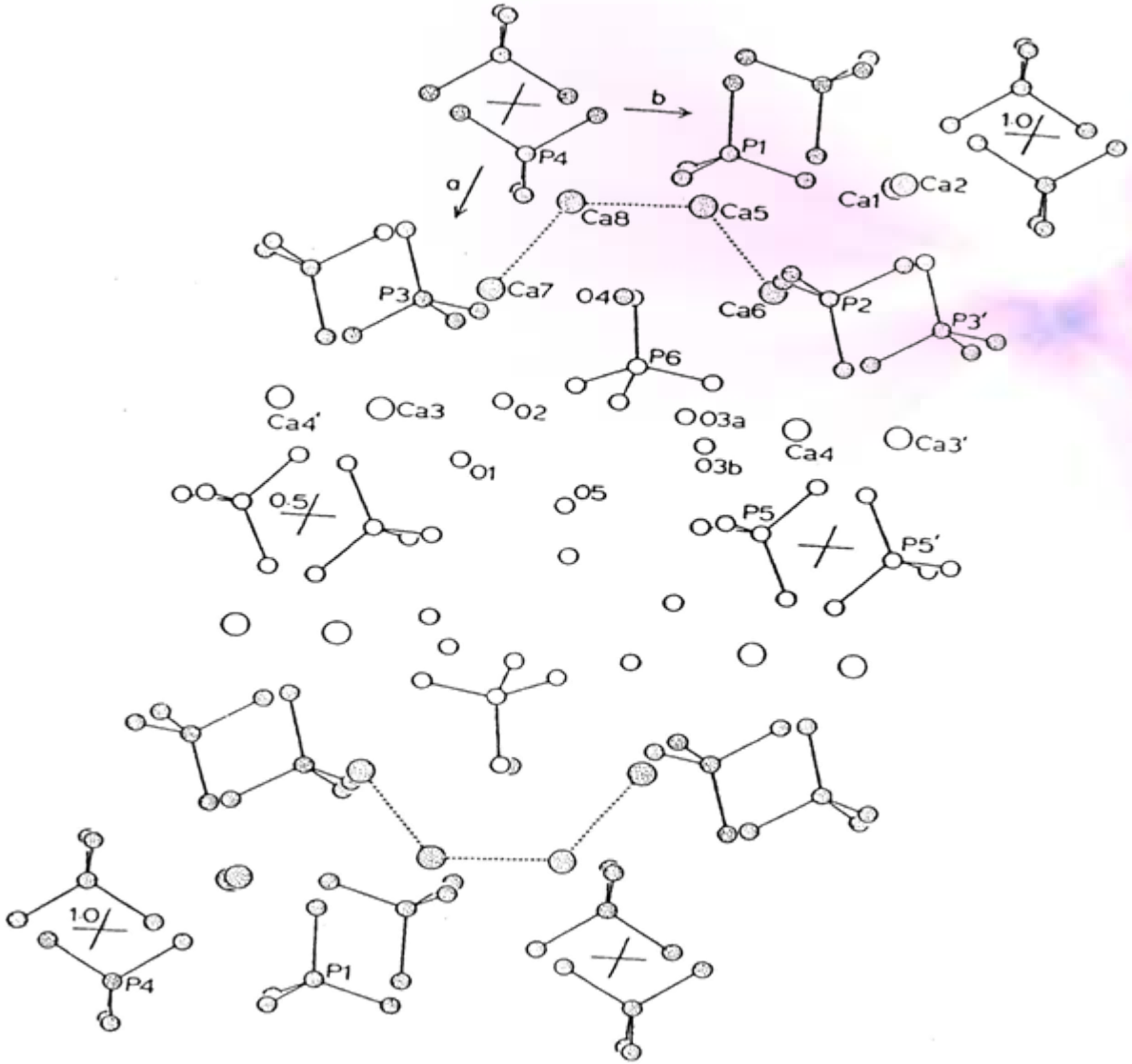
HA molecule : $Ca_{10}(PO_4)_6(OH)_2$



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 ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$)

Alternating apatite- and hydrated- layers, ‖(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

Method	Working gas	Typical thickness	Surface morphology	Advantages	Disadvantages
Vacuum and atmospheric plasma-spraying	Ar, N_2, H_2	50-200 μm	Very rough, irregular and porous	Surface macro-porosity enhances bone in-growth 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

Method	Working gas	Typical thickness	Surface morphology	Advantages	Disadvantages
Electrophoretic deposition	-	< 20 μm	-	Covering of complex substrates Feeling of porous substrate cavities	Poor bond strength Shrinkage and cracking Coated substrate sintered at (900 – 1000° C)
Laser cladding	Shielding Inert gas	300 – 400 μm	Smooth surface finish	Controlled clad shape Localized processing heating Controlled dilution levels	Undesired CaP, other than HA: TCP, CaP glass Formation of calcium titanates and titanium phosphates
Pulsed laser deposition	Inert gas, O_2, H_2O or mixture of them	< 1 μm	Smooth / rough, depending on target properties and deposition conditions	High density and crystallinity Proper stoichiometry Controlled Ca/P ratio Good adherence Clean process Relatively low processing temperature (400 – 700° C)	Limited deposition on large areas Limited thickness uniformity

BIOMATERIALS IN THIS LECTURE:

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:

➢ $(CO_3)^{2-}$ ions, also present in biological apatite, generally enter the $(PO_4)^{3-}$ sites;

➢ 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;

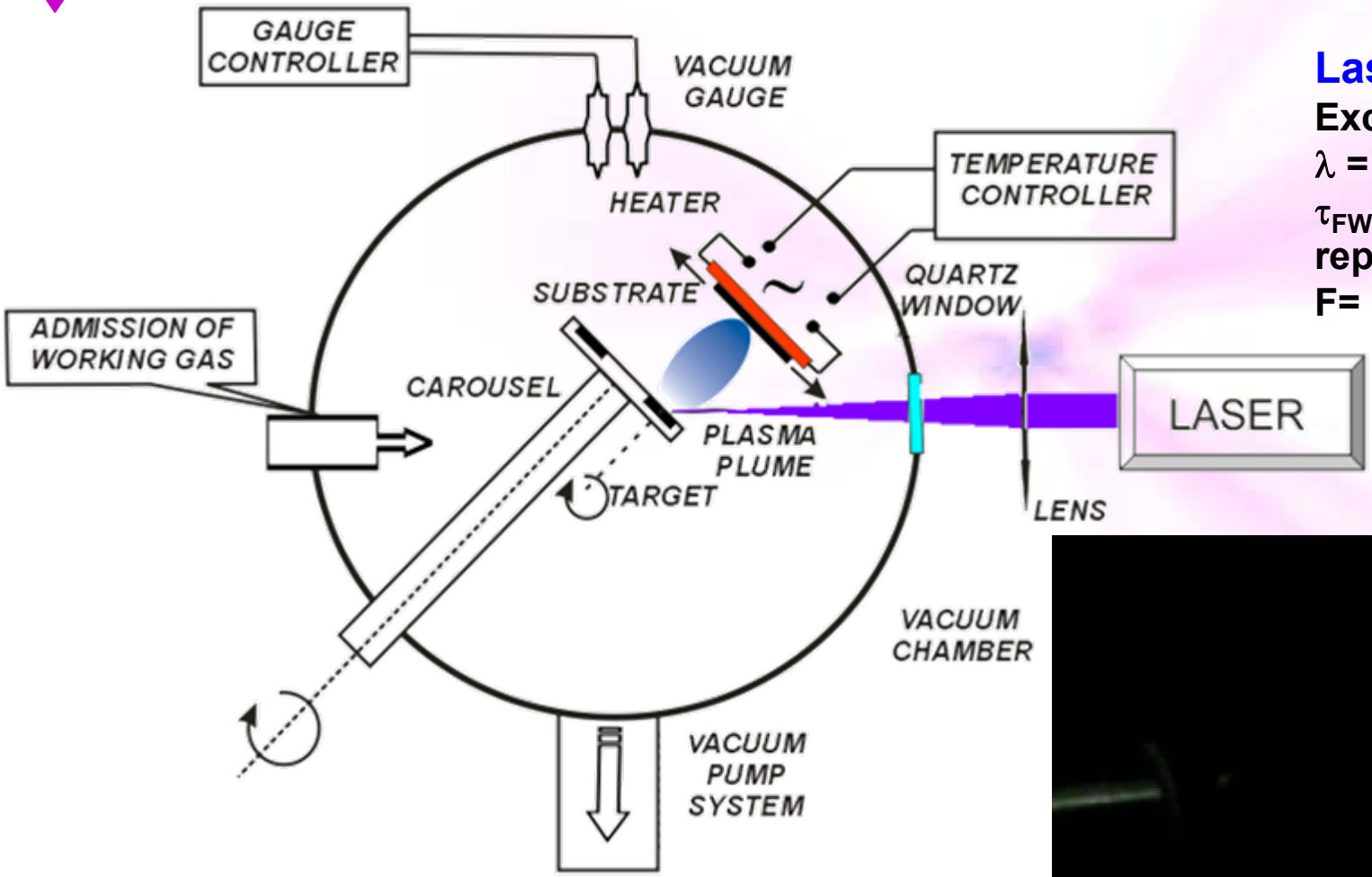
➢ prospective alternative to HA coatings for metal implants.

HA+MP:

➢ biopolymer capable of improving coating mechanical behaviour (adherence, elasticity);

➢ induces surface functionalization of the coating.

PULSED LASER DEPOSITION (PLD) METHOD



Laser parameters:

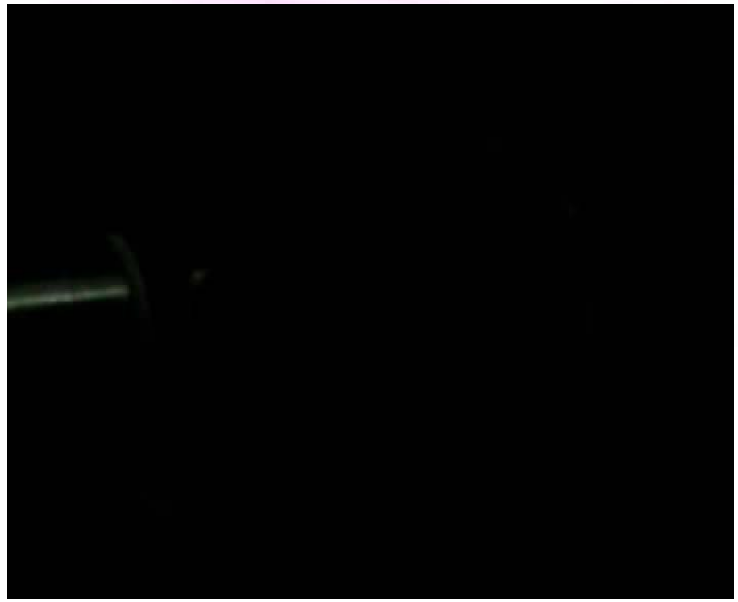
Excimer KrF*

λ = 248 nm

τ_{FWHM} ~ 7 or 25 ns

rep. rate 2 - 50 Hz

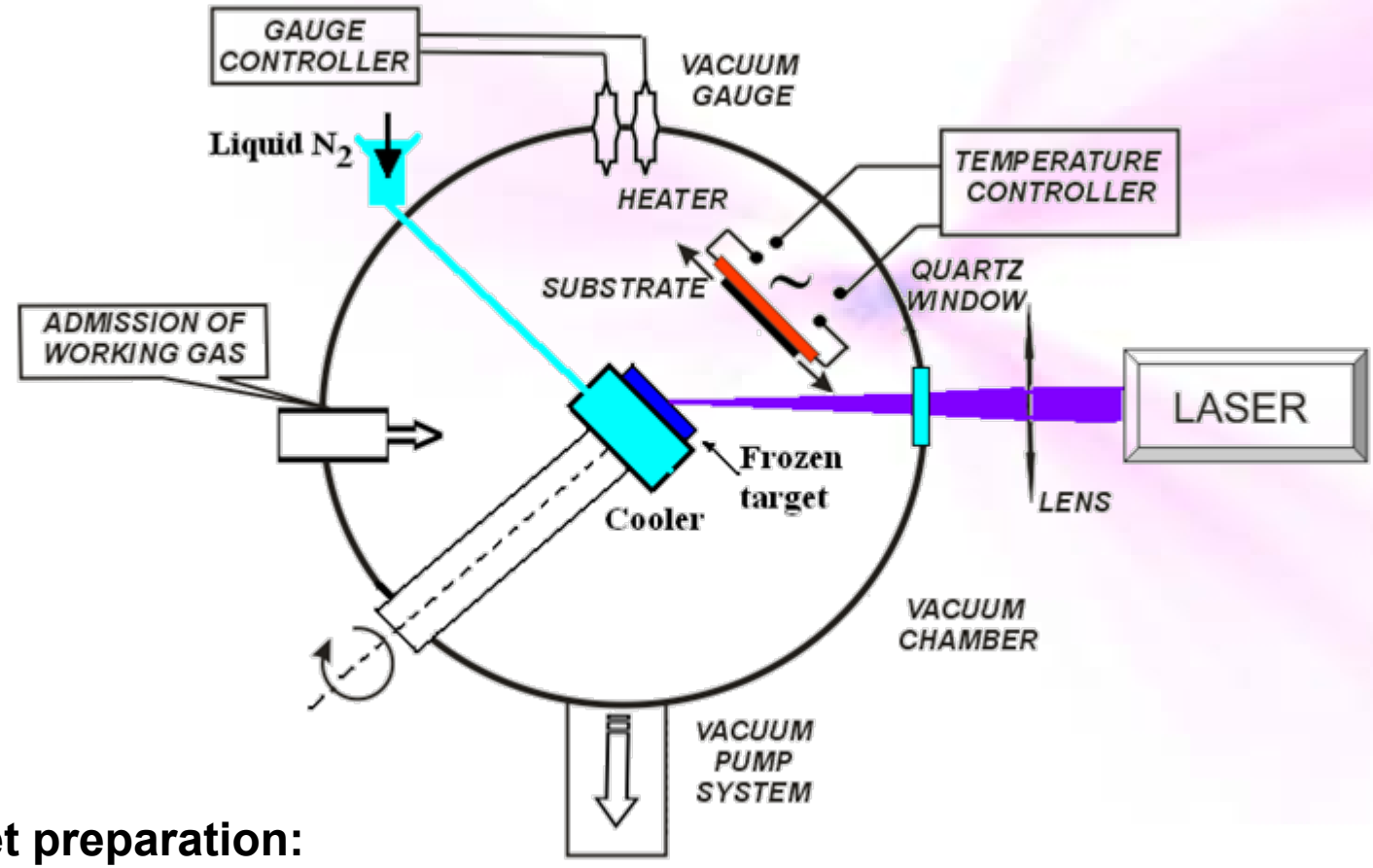
F= (0,2 – 10) J/cm^2




LAMBDA PHYSIK
COMPex Pro

MATRIX ASSISTED PULSED LASER EVAPORATION (MAPLE)

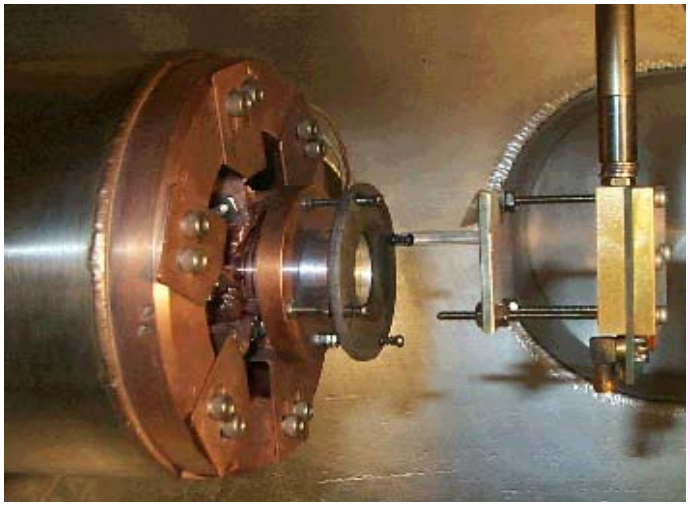
Main differences between PLD and MAPLE: target preparation and mechanism of laser - material interaction



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

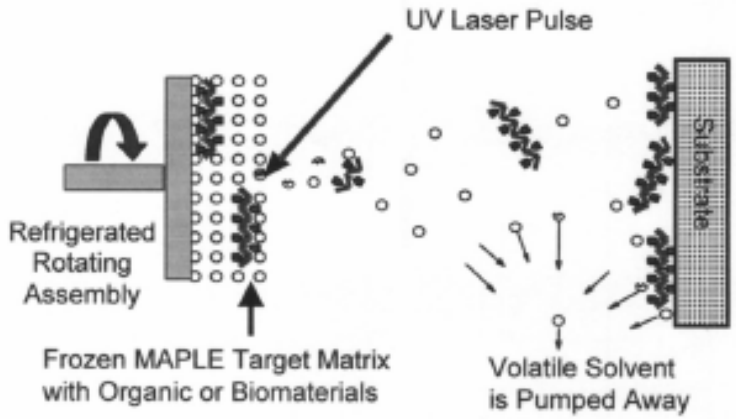


- **Laser-Material Interaction**:

➢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.



Simplified schematic of the MAPLE desorption process.

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:**

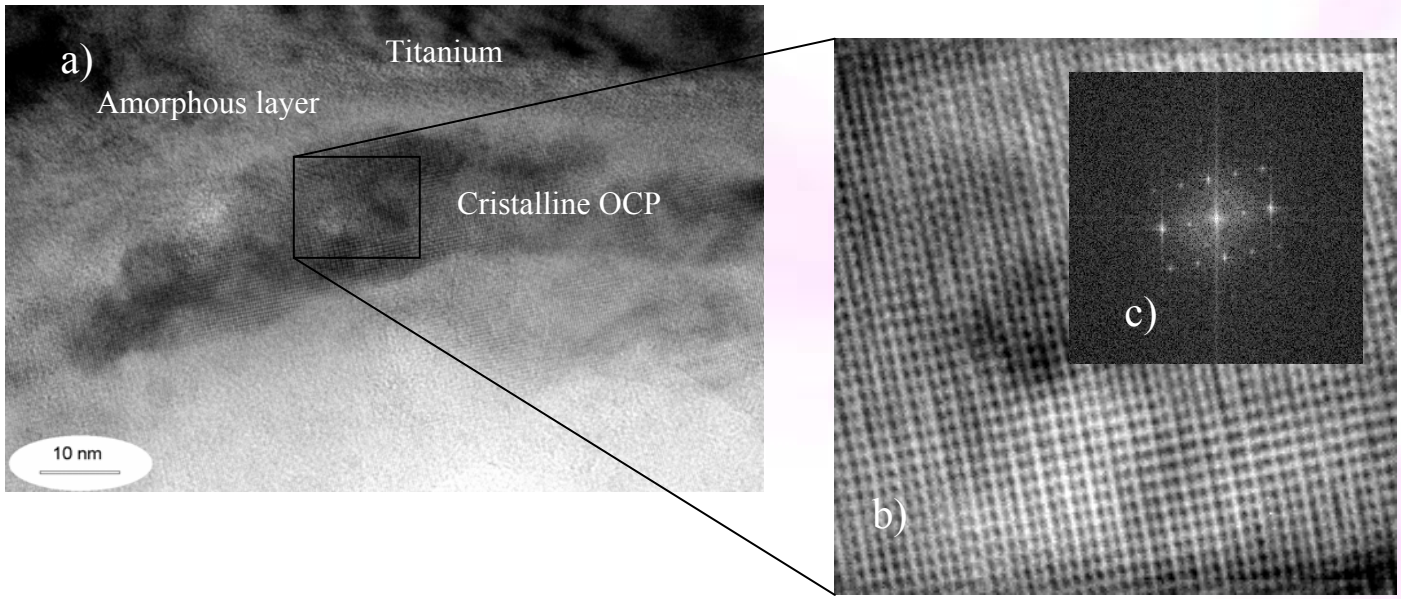
- *Cell morphology;*
- *Proliferation and viability (WST1 test);*
- *Cytoskeleton labeling;*

- **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)°

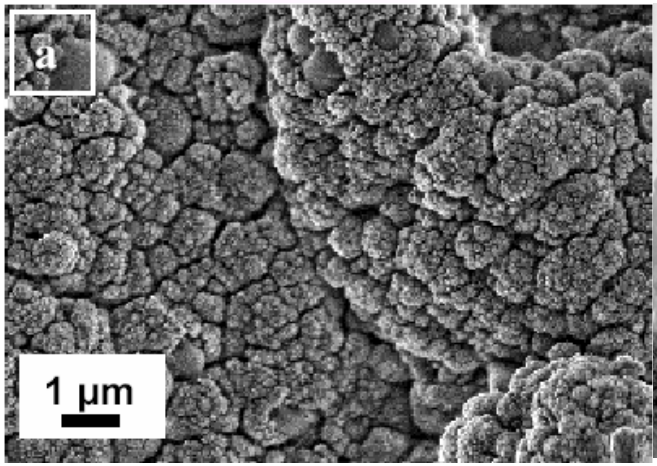
XHRTEM image at the interface a); b) enlargement and filtered image of the domain in a); c) FFT of the "core" domain in a)

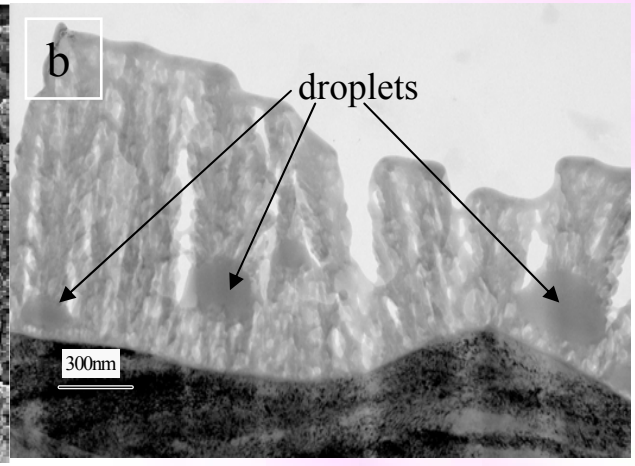
X-ray diffraction patterns are in agreement with an amorphous-poor crystalline structure. OCP presence is confirmed by the shoulder around 4.7° of 2θ, corresponding to the (100) reflection of OCP, and by the broad peak centered around 32-33° of 2θ.

(Lo et al., J. Biomed. Mater. Res., 2000: CaPs films in an amorphous or nanocrystalline matrix exhibit ideal dissolution and cell compatibility properties)

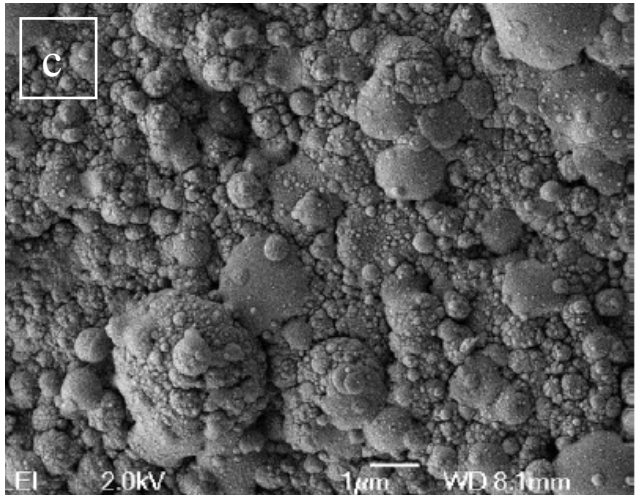
OCP AND Mn-CHA STRUCTURES EXHIBIT DIFFERENT MORPHOLOGIES

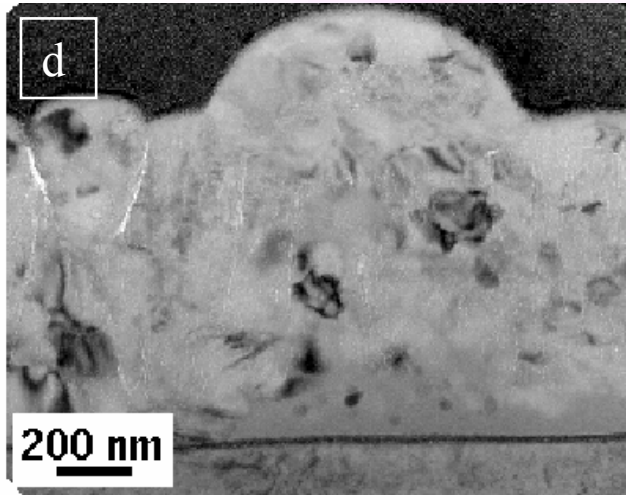
- OCP: porous, tree-like morphology



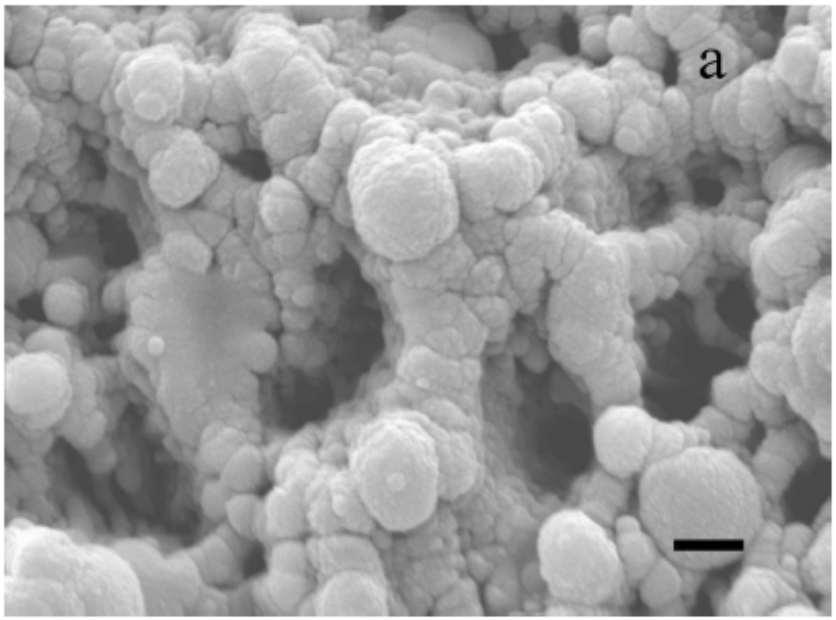


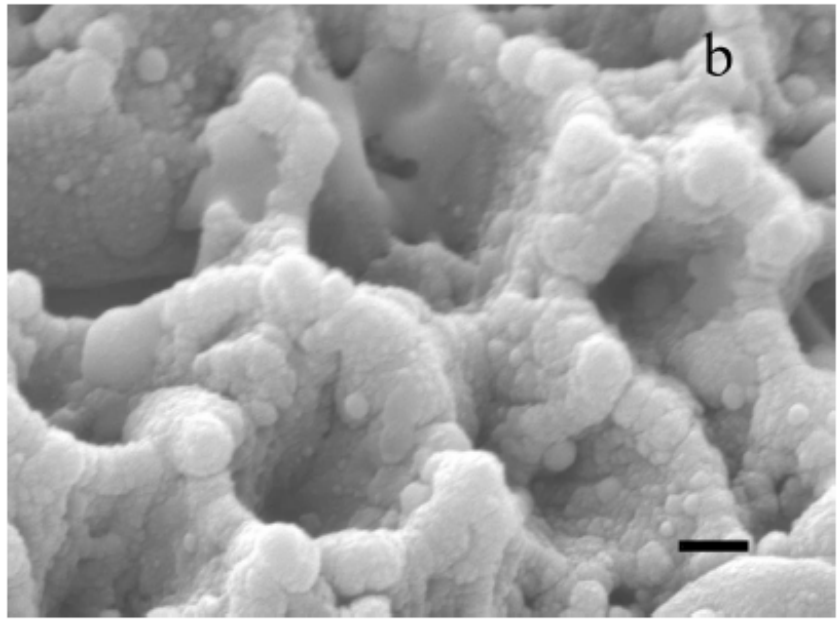
- Mn-CHA: granular, more compact morphology





Sr-HA STRUCTURES EXHIBIT RATHER POROUS MORPHOLOGIES

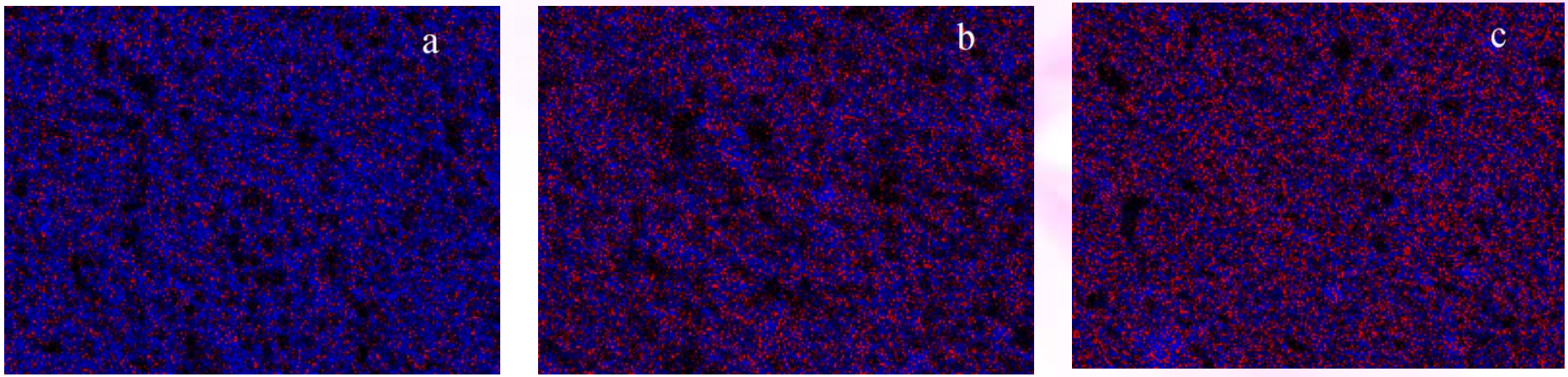




SEM micrographs of thin films deposited from (a) Sr0; (b) Sr10 samples.
Scale bars = 1 μm

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

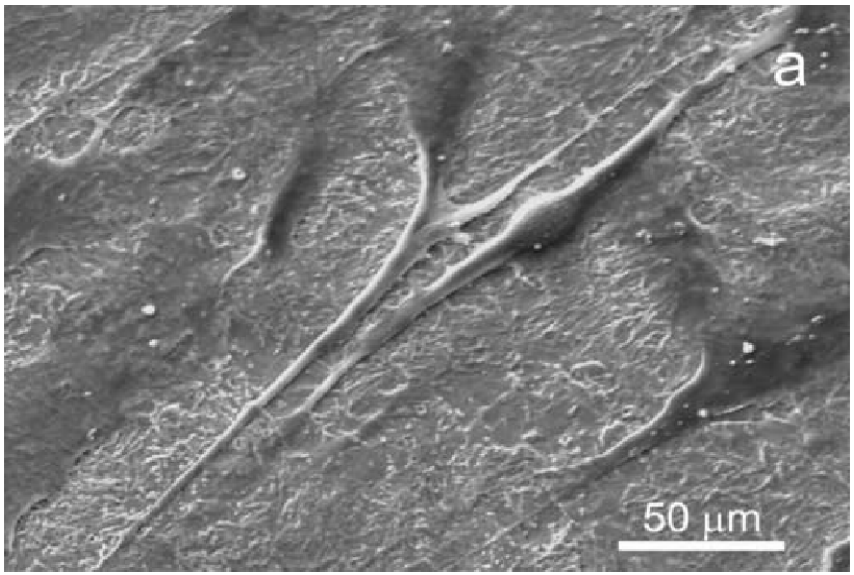
Sr-red, HA blue.

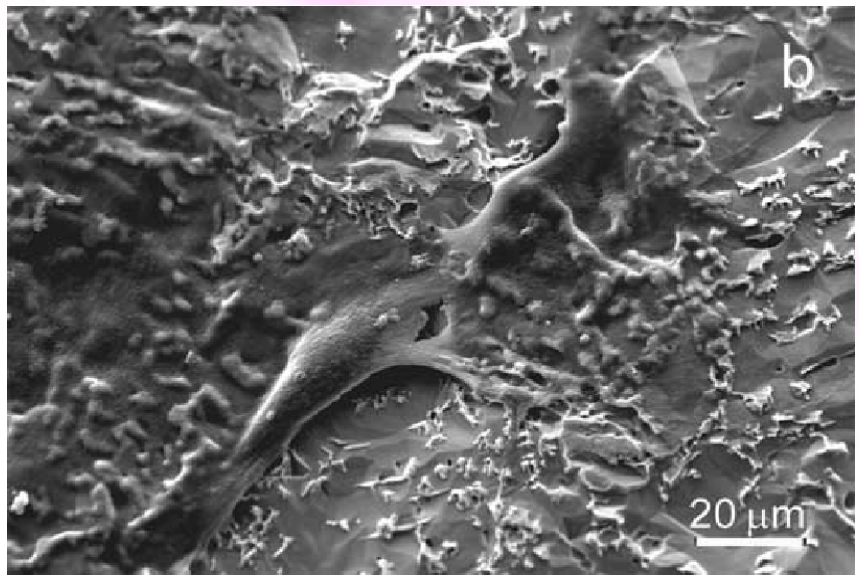
Biocompatibility Tests - Cell Cultures (Proliferation and Viability)

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

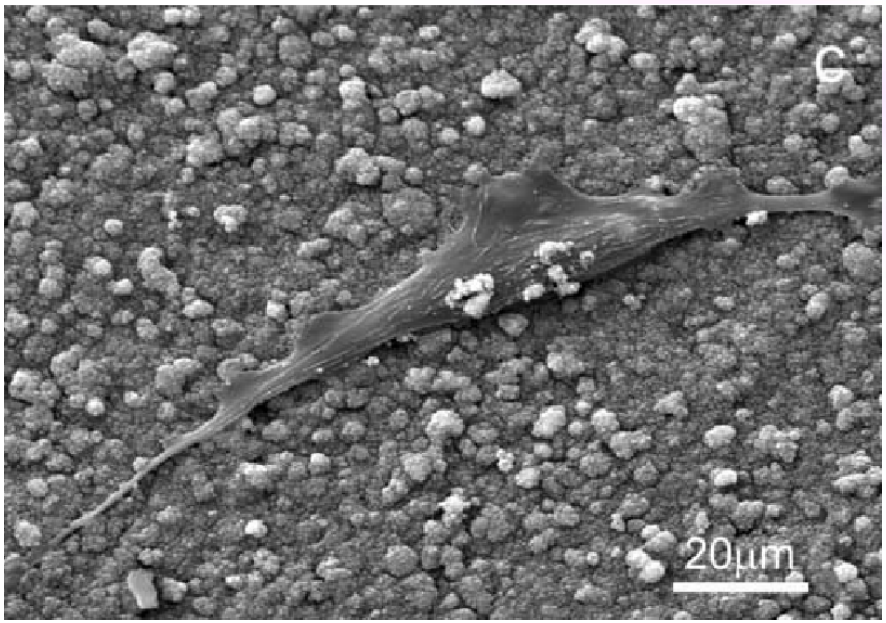


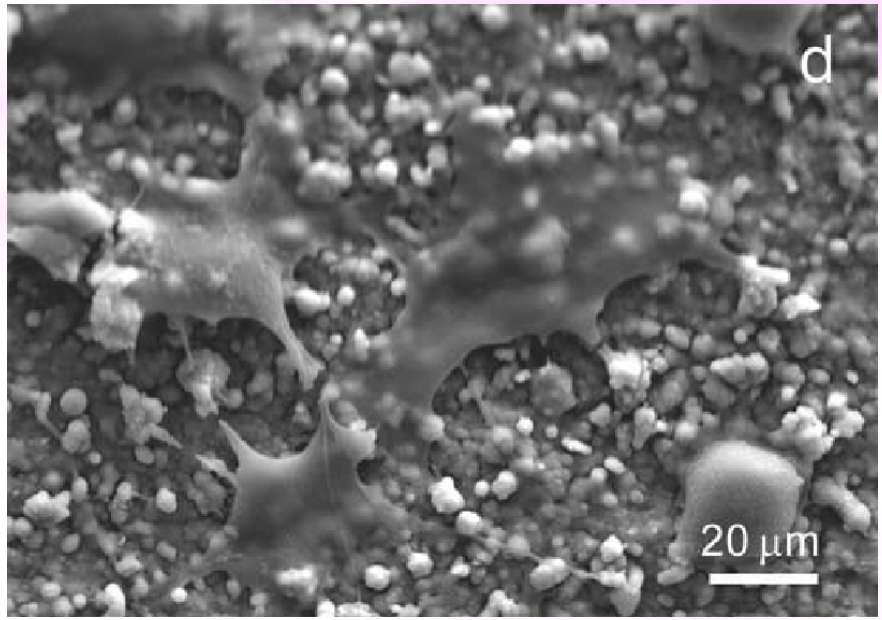


Elongated, rod-like morphology

hOB response: SEM micrographs

- **OCP coatings**: (c) after 7 days, (d) after 21 days



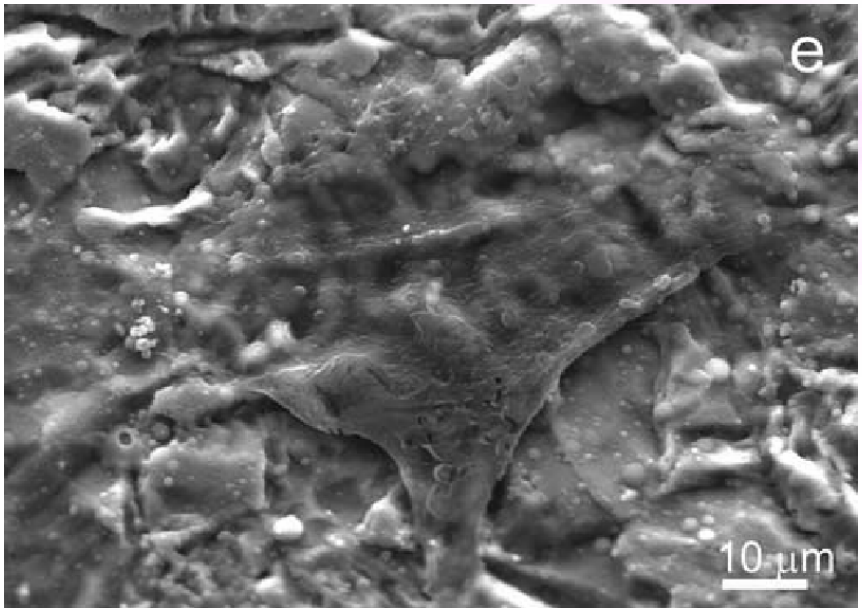


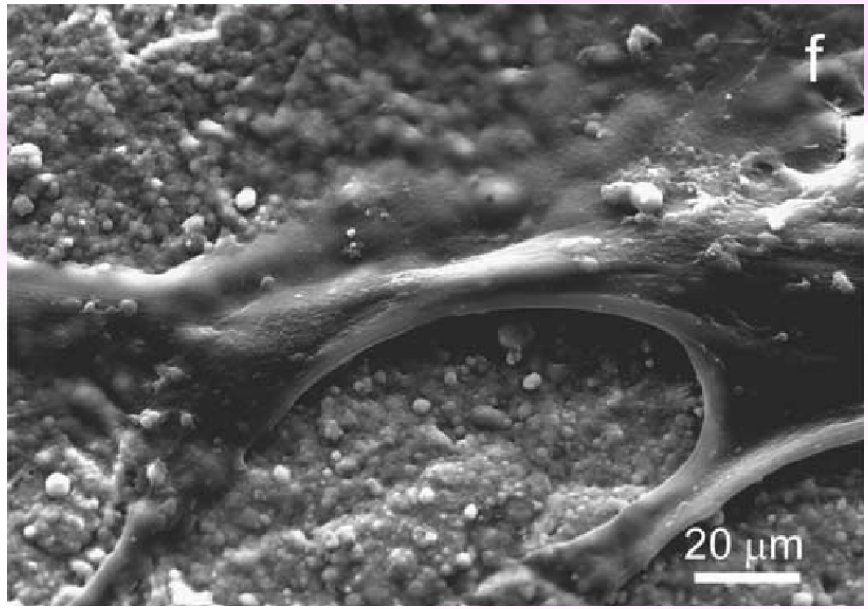
➢ Over time, the cells spread and expand with flattened, polyhedral-morphology.

➢ Numerous cytoplasmatic extensions → firm attachment

hOB response: SEM micrographs

- **Mn-CHA coatings**: (e) after 7 days, (f) after 21 days

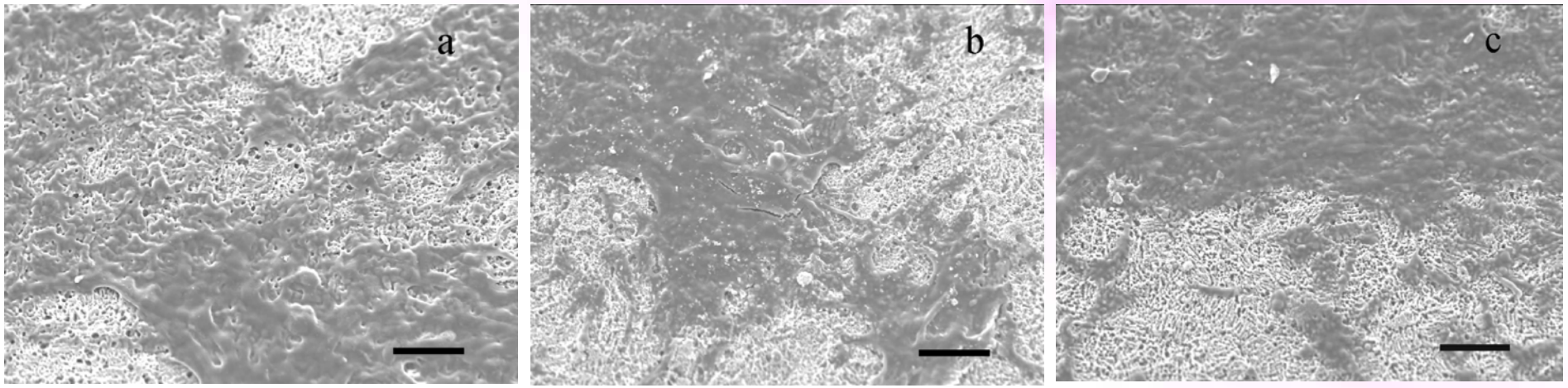




➢The cells spread and expand overtime, showing a flattened, polyhedral-morphology;
➢Fewer cytoplasmatic extensions

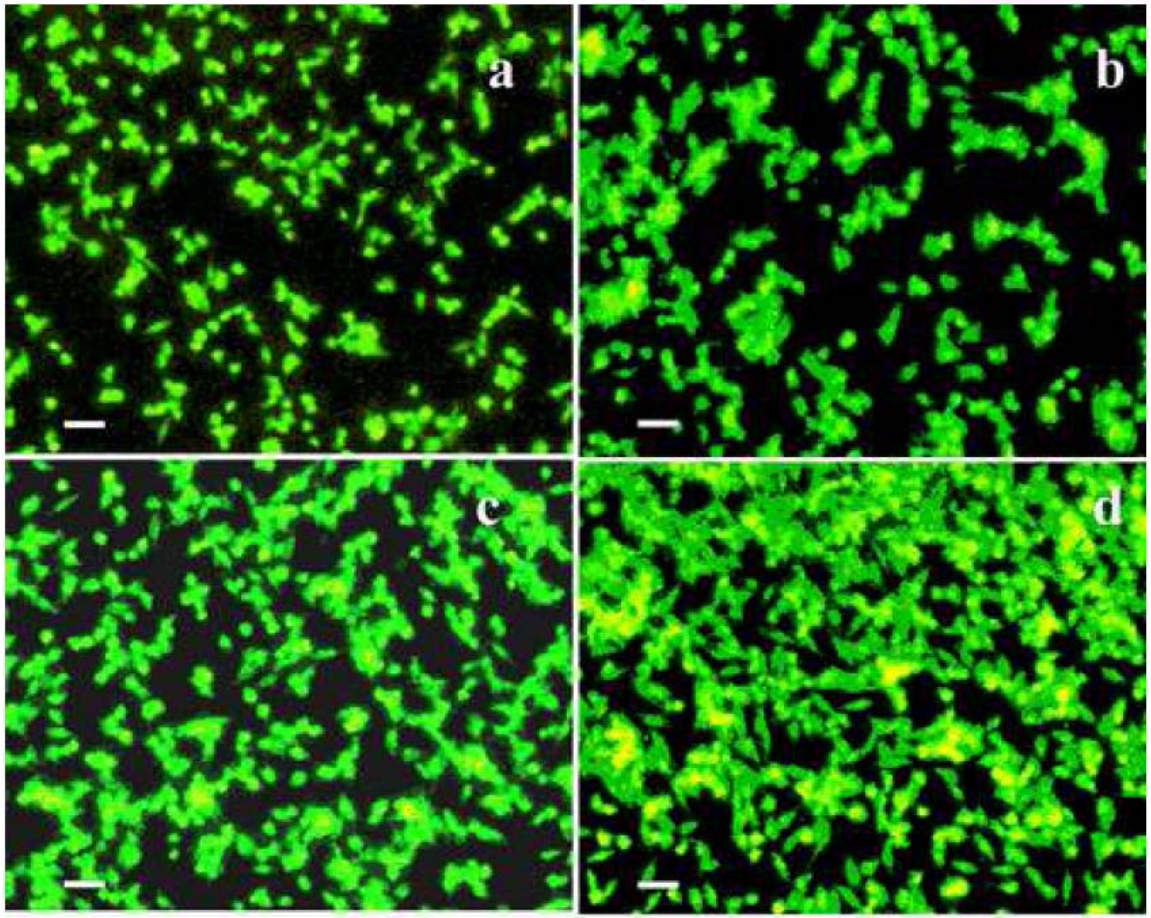
hOB response: SEM micrographs

- **Sr-HA coatings**: SEM micrographs of osteoblasts after 21 days of culture on: (a) TiHA; (b) TiSr5; and (c) TiSr10. Scale bars = 50 μm.



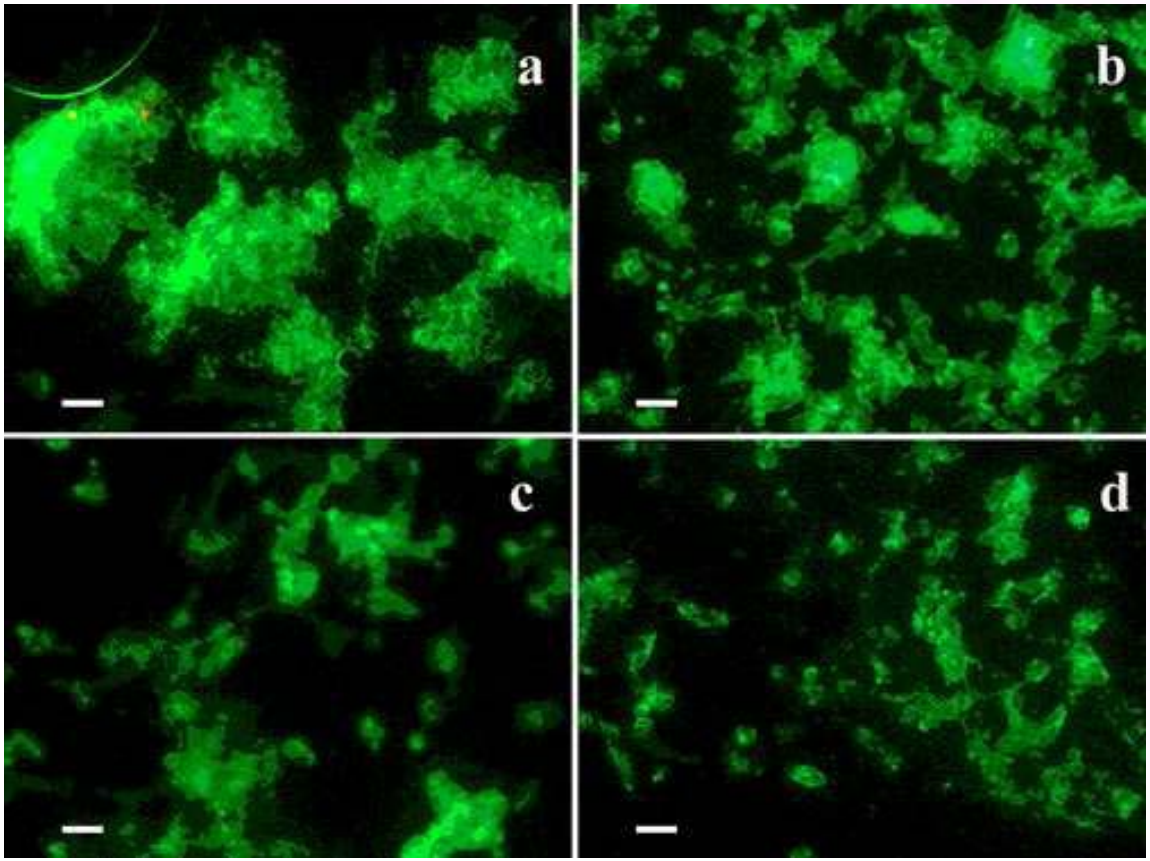
→ Ti/HA: hOB were flattened, with polygonal configuration and dorsal ruffles; well attached to the substrate by cellular extensions.
→ Ti/Sr doped HA: hOB appear much more flattened and better spread across the surface.

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.

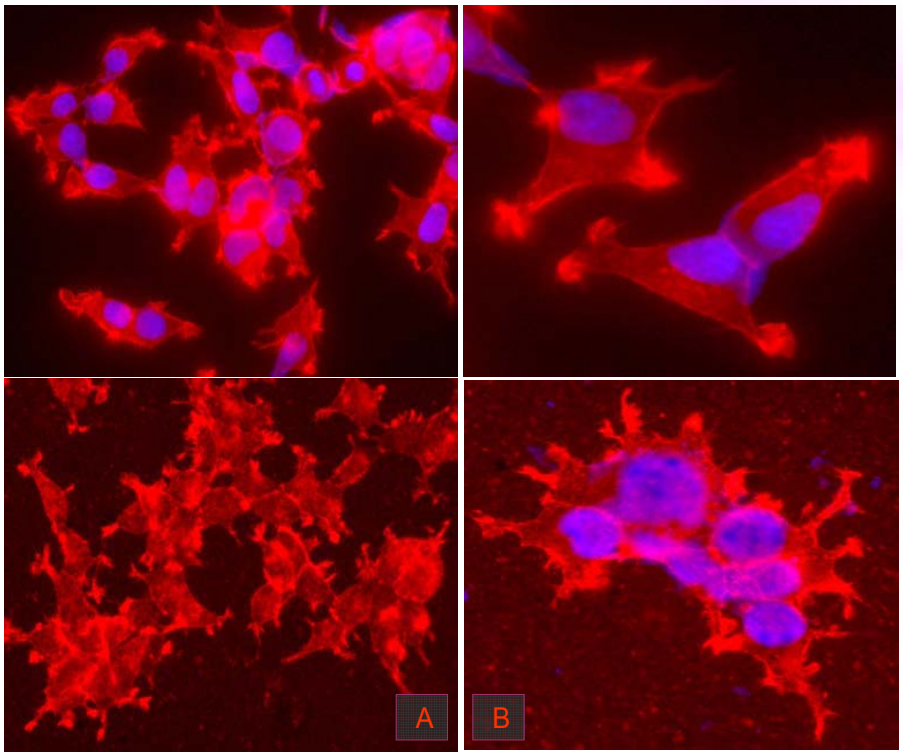
Proliferation of osteoclast (hOC) culture on Sr-HA coatings: 21 days



(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



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

Human embryonic kidney (HEK293) cells

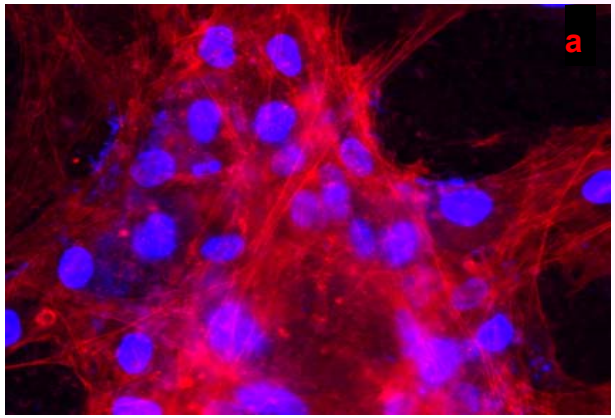
- cell morphology: polyhedral
- good spreading, establish cell-cell contacts, tend to occupy the culture surface

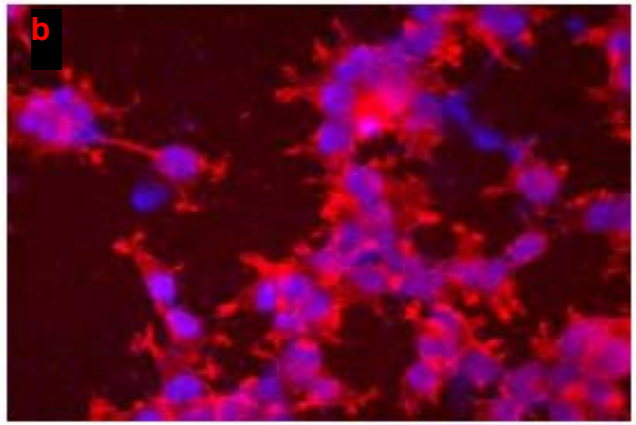
The actin filament pattern of **cytoskeleton** of cells on HA+MP → indicative of **biocompatibility**

➢ Prominent focal adhesions: firmly ancorate cells to the substrates → good adhesion;

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

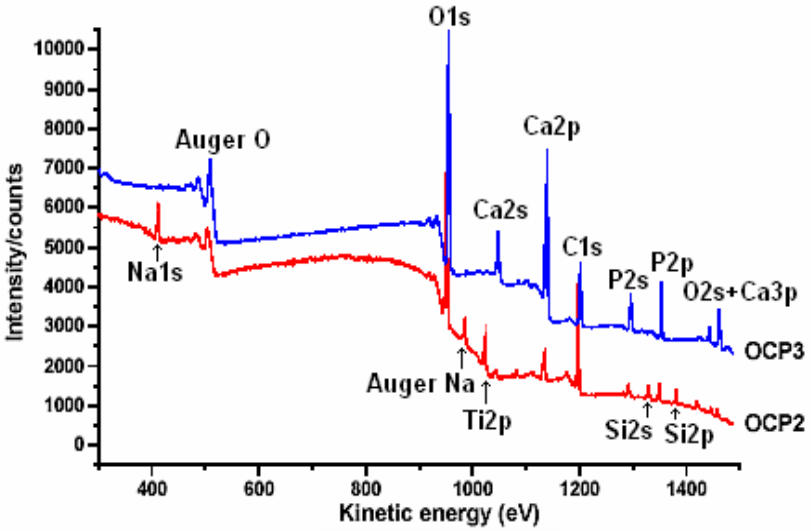
DEGRADATION TESTS

SBF composition	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HPO_4^{2-}	SO_4^{2-}	HCO_3^-
concentration mM	142	5	1.5	2.5	103	1	0.5	27

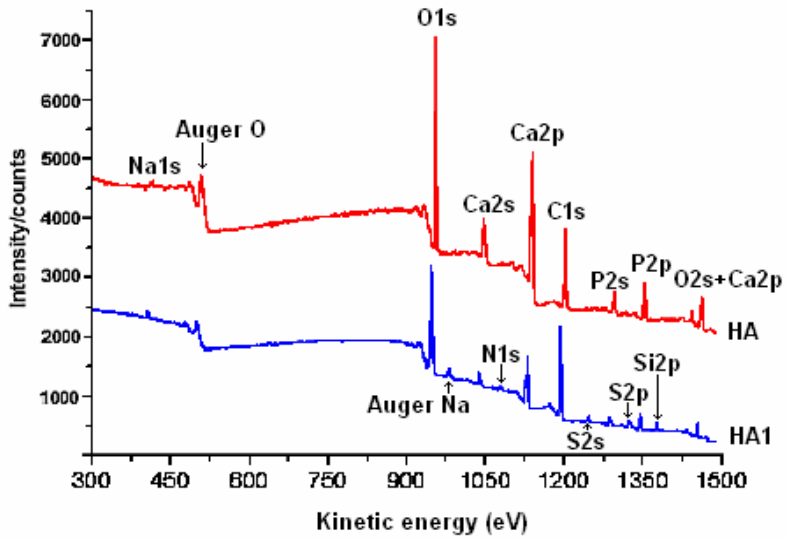
OCP coatings dissolve and disappear almost totally after 7 days of immersion in SBF.

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

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



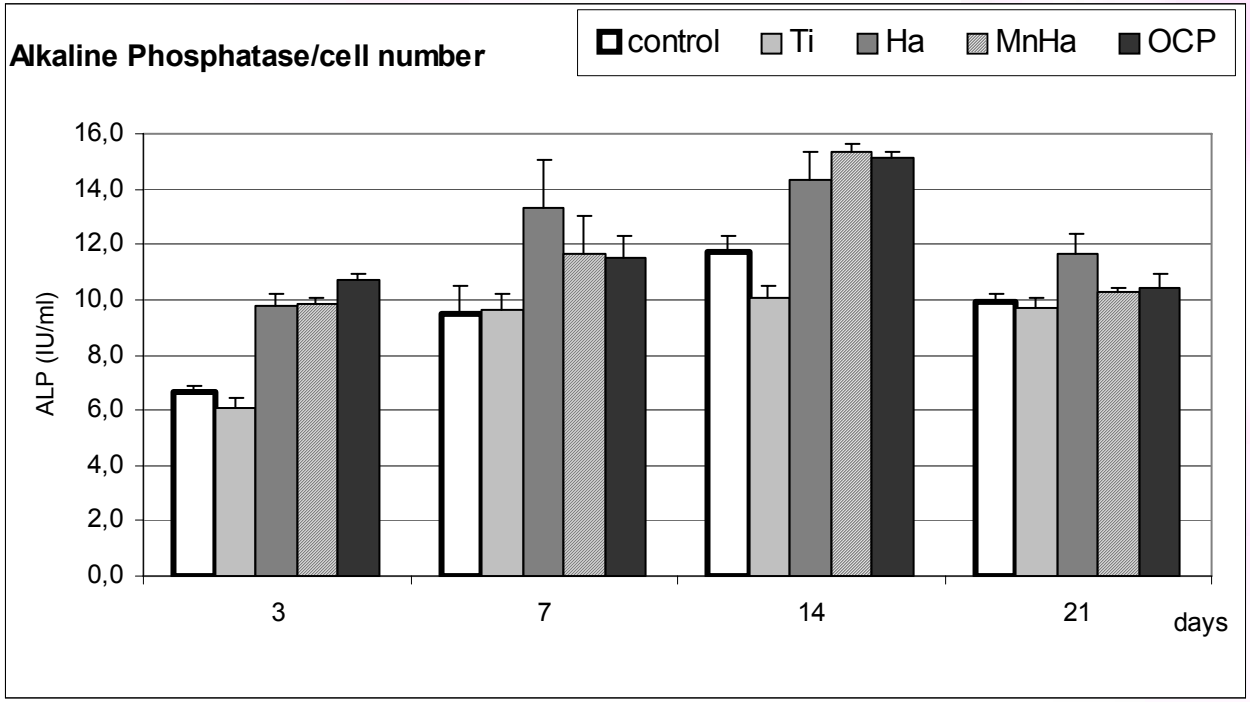
XPS spectrum of Mn-CHA before (HA) and after (HA1) degradation tests



BIOACTIVITY TESTS

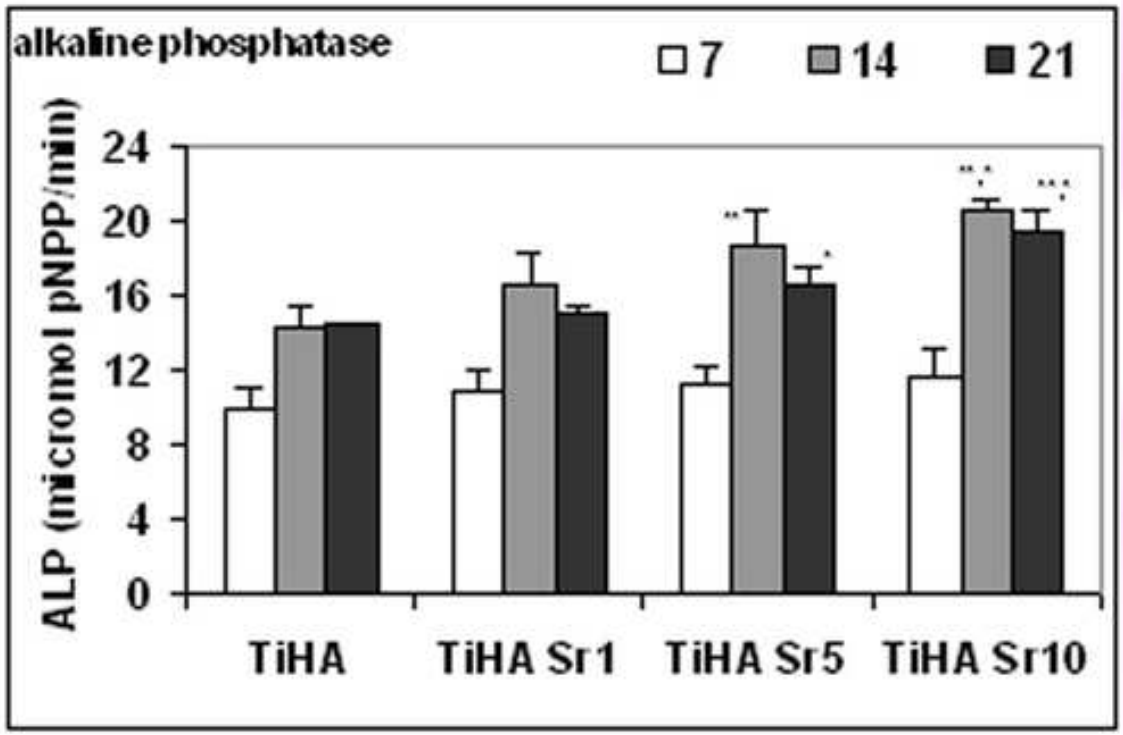
ALKALINE PHOSPHATASE ACTIVITY (ALP)

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**

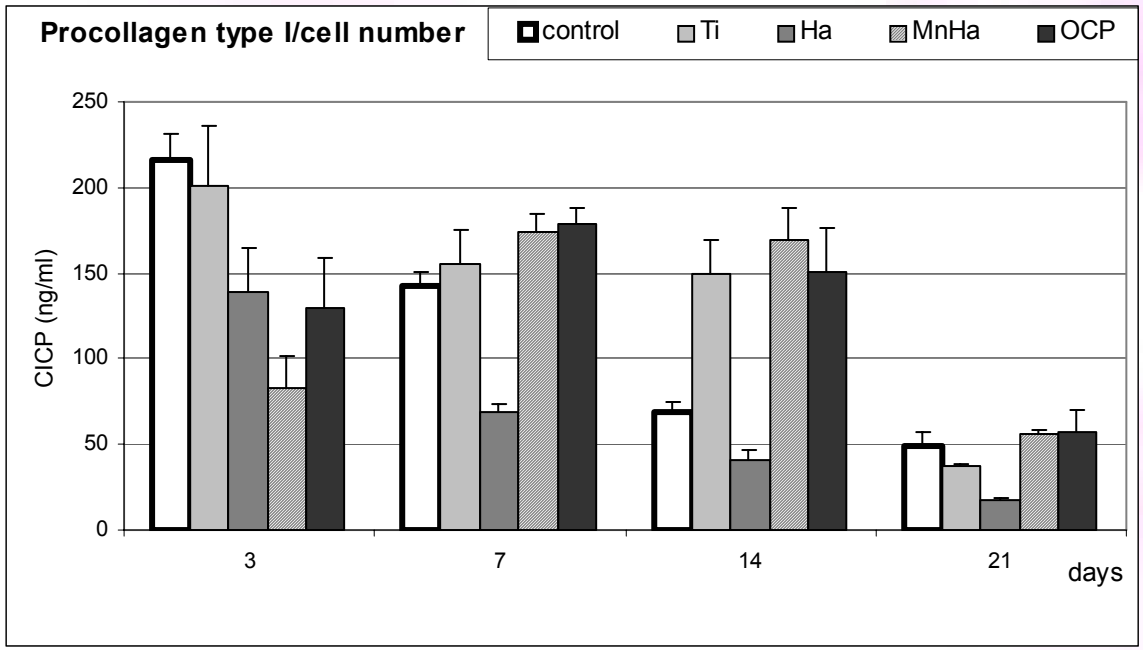
Osteoblast proliferation and activity after 7, 14, and 21 days of culture on Sr:HA, ALP test



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):

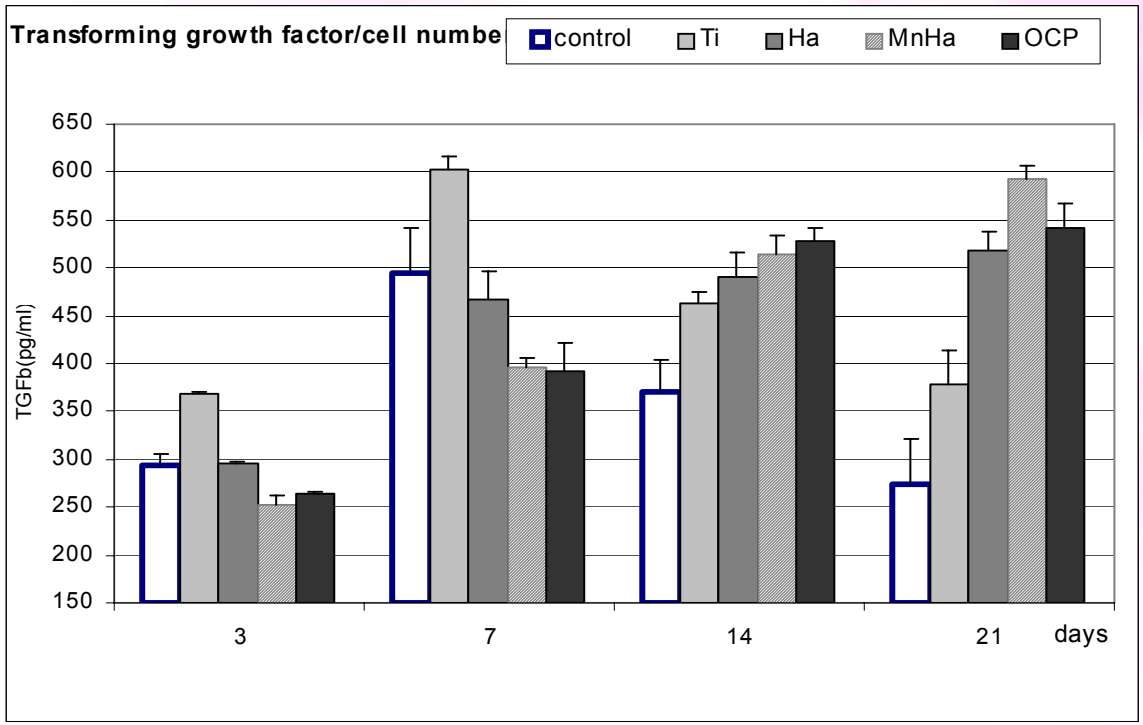
Collagen type I is synthesized by osteoblasts as the major organic macromolecule in the extracellular bone matrix.



- **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 (TGF β1):

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.

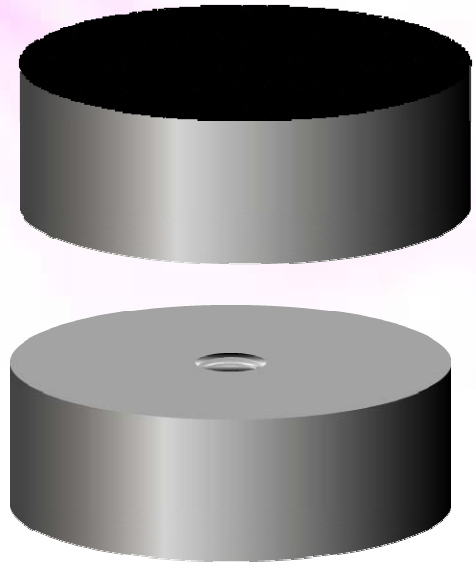
• New Zealand White adult female rabbits, 8 months, 3000-3500 g weight

• Moderate Ti substrate roughness was chosen:

- **High** enough to stimulate bone repair and growth ; but

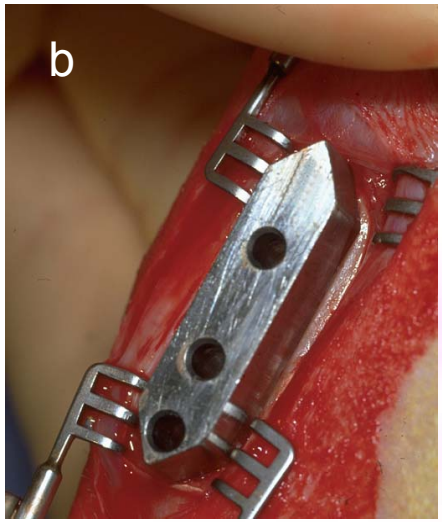
- low enough to allow separation of biological effects;

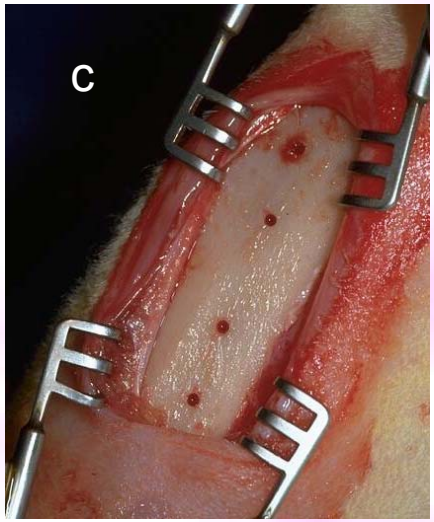
- threated on reverse side

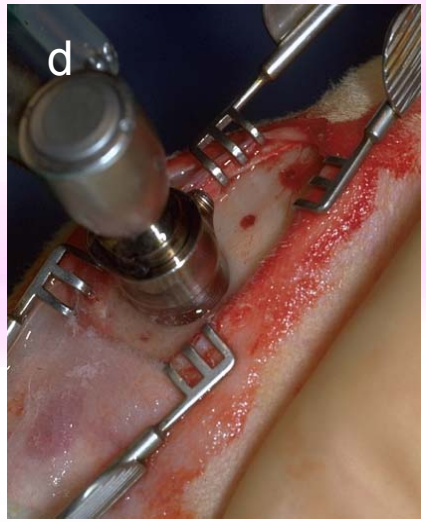


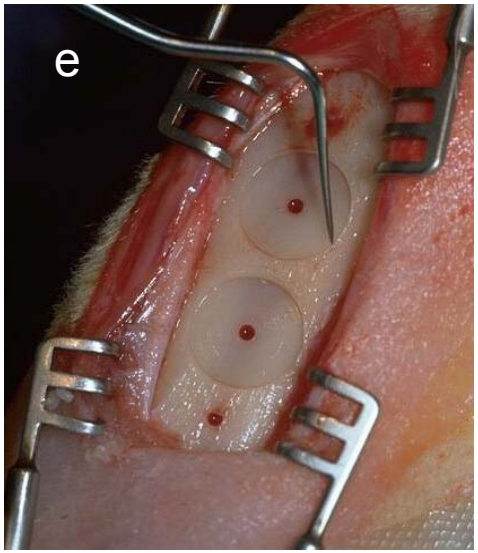
PULL OUT PROCEDURE (1)

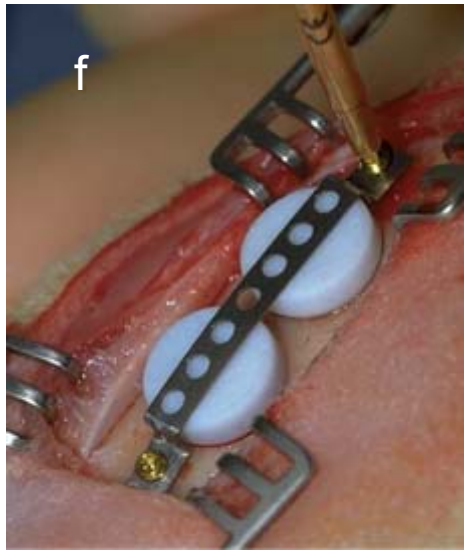
Surgical procedures (a - g)

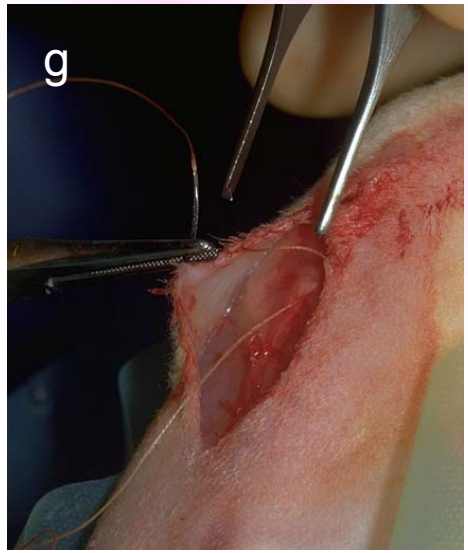








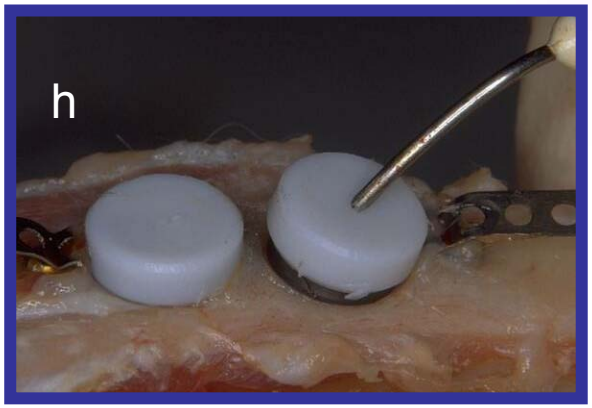


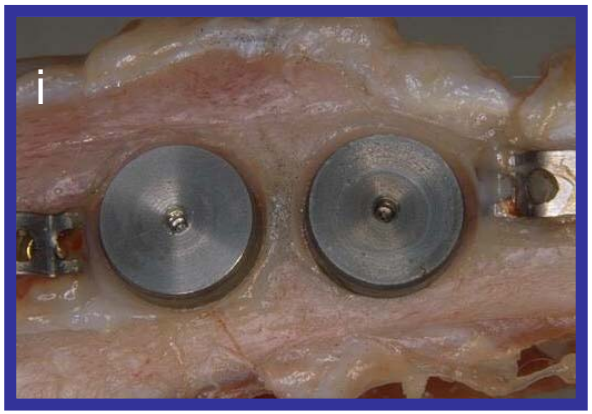


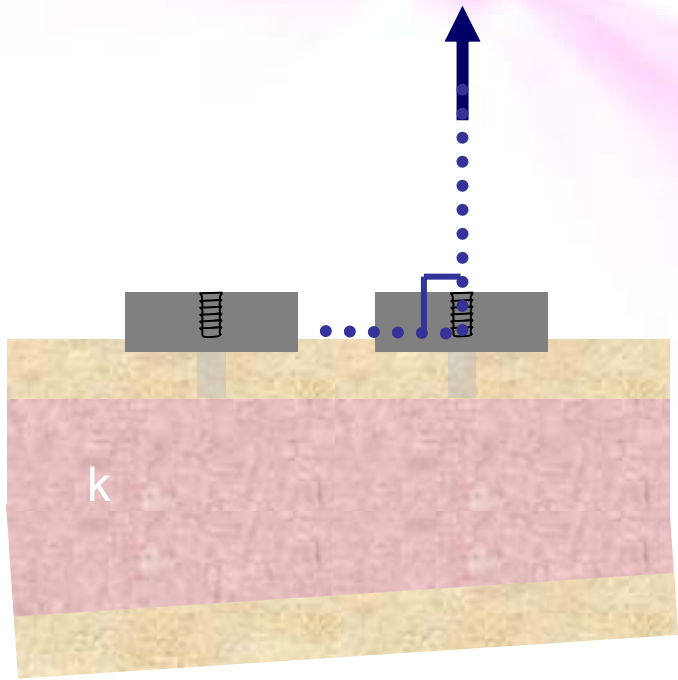
PULL OUT PROCEDURE (2)

Tensile test procedures (h - k)

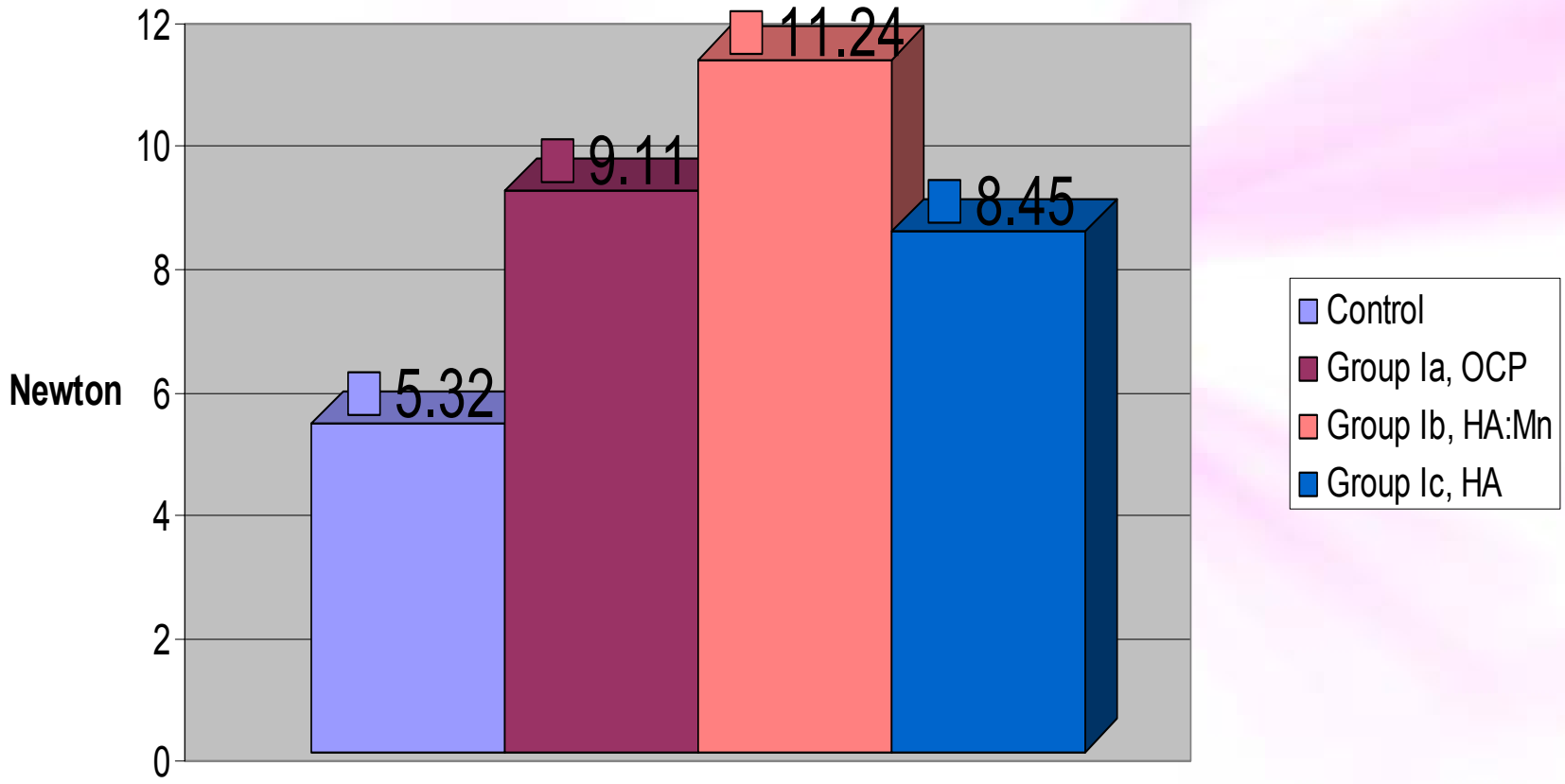
- Pullout test conducted after 8-week healing time;
- Cross head speed was set to 1,0 mm/min







Average pullout force for CaPs vs. control



- **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.

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