Final report of COST STSM MP 1301-29946

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STSM topic: **Investigation of biocompatibility and toxicity properties of modified calcium phosphate coatings**

Introduction

Titanium and Ti alloys are commonly used in direct bone-contact prostheses design and realization owing to their excellent mechanical properties, together with the ability of a direct adhesion to bone tissues. Moreover, in long term applications, titanium based implants show no allergic or immunogenic reactions and a good biostability. Titanium and its alloys can be considered bioinert materials and do not show osteoconductive properties. In the last 20 years many attempts were made to fabricate materials actively promoting bone growth. These studies were mainly directed to reduce the healing time and to contribute to improve clinical performance of implanted devices. Coating porous titanium with calcium phosphate (CaP) is an effective way to enhance titanium's osteoinduction capability. The CaP layer can also be doped by biomaterials such as Zn, Mg, Sr to promote bone cell growth and also Ag particles to prevent post-operative infections.

Using electrochemical process to deposit CaP and modified CaP coatings is more cost effective compared to other physical deposition methods (CVD, PVD). Moreover, the other advantage of electrochemical methods that homogenous layer can be deposited on 3D materials with complex geometry. During electrochemical deposition the pH at the cathode/electrolyte interface can be controlled. In an aqueous electrolyte, the following reactions occur at the surface of the cathode (reduction of water, proton discharge, reduction of dissolved oxygen):

$2H_2O + 2e^- \rightarrow H_2 + 2OH^-$

$2H_3O^+ + 2e^- \rightarrow H_2 + 2H_2O$

$O_2 + H_3O^+ + 4e^- \rightarrow 3OH^-$

This results in the formation of hydroxyl ions and hence alkalization, close to the surface.

The main electrochemical reactions at the electrode surfaces might be as follows:

$Ca^{2+} + HPO_4^{2-} \rightarrow CaHPO_4$

$Ca^{2+} + HPO_4^{2-} + 2H_2O \rightarrow CaHPO_4.\ 2H_2O$

$10\ CaHPO_4 + 12OH^- \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 4PO_4^{3-} + 10H_2O$

Experimental

- Electrochemical deposition of CaP and modified CaP coated implant materials

All coatings were prepared by pulse current deposition in a conventional two-electrode cell, where the cathode was the implant material and the anode was platina wire. The applied pulse parameters were 5 ms on-time, 5 ms off-time and current density of 0.4 Acm^{-2}. Each electrolysis process lasted for 30 minutes at 70 °C.

Table 1: Electrochemical parameters for CaP and modified CaP coating deposition onto Ti6Al4V surfaces.

Samples	Electrolyte composition	Treatment after electrochemical deposition
Ti6Al4V	commercially available implant material discs prepared and sterilized by manufacturer Protetim Ltd used for reference, discs with diameter of 19 mm, surface area of 2.83 cm^2	
P3 series	0.49M $Ca(NO_3)_2$, 0.29M $NH_4H_2PO_4$, 1ml H_2O_2	Heat treatment at 900 °C for 30 minutes
P4 series	0.245M $Ca(NO_3)_2$, 0.145M $NH_4H_2PO_4$, 1ml H_2O_2	Treatment in 1M NaOH solution at 70 °C for 2 hours.
P5 series	0.49M $Ca(NO_3)_2$, 9.67 mg/l $Zn(NO_3)_2$, 1.65 mg/l $AgNO_3$, 15 mg/l $Sr(NO_3)_2$ 0.29M $NH_4H_2PO_4$, 1ml H_2O_2	Heat treatment at 900 °C for 30 minutes
P6 series	0.49M $Ca(NO_3)_2$, 8.05 mg/l $Zn(NO_3)_2$, 1.65 mg/l $AgNO_3$, 16.2 mg/l $Sr(NO_3)_2$, 0.29M $NH_4H_2PO_4$, 1ml H_2O_2	• Heat treatment at 900 °C for 30 minutes • Spin coating of 2.75 mg/l Mg-stearate in ethanol solution
P7 series	0.49M $Ca(NO_3)_2$, 0.29M	• Treatment in 1M NaOH solution at 70 °C for 2 hours.

	$NH_4H_2PO_4$, 1ml H_2O_2	• Spin coating of solution with following composition: 7.25 mg/l $Zn(NO_3)_2$, 1.60 mg/l $AgNO_3$ 6.40 mg/l $Sr(NO_3)_2$ in distilled water. + 2.75 mg/l Mg-stearate in ethanol solution • Heat treatment at 250 °C for 2 hours.
P8 series	0.245M $Ca(NO_3)_2$, 0.145M $NH_4H_2PO_4$, 1ml H_2O_2	• Spin coating of solution with following composition: 7.25 mg/l $Zn(NO_3)_2$, 1.60 mg/l $AgNO_3$ 6.40 mg/l $Sr(NO_3)_2$ in distilled water + 2.75 mg/l Mg-stearate in ethanol solution • Heat treatment at 650 °C for 2 hours

- Biocompatible tests

Experiment 1.

In our experiments the samples, whose diameter were 19 mm, were put in 6-well plates (well area: 9.6 cm^2). Cells used for the experiments are represented by the MG-63 cell line, which is a line of human osteoblast-like cells. Cells were grown on two 75 cm^2 flasks and were detached by tripsin. We counted the detached cells in a Neubauer chamber. The count of cells was 1532.5 cells/µl and 1532500/ml. We seeded the cells at a density of $1.5x10^5$ cells/well in 3 ml medium for each well. Medium was DMEM (Dulbecco's Modified Eagles Medium) with 10% of FBS (Fetal Bovine Serum, containing growth factors and nutrients to support cell growth) and 100 U/ml penicillin and 100 µg/ml streptomycin to minimize the risk of infections. The cultures were maintained at 37°C, 5% CO_2 in a humidified atmosphere in incubator. The cytotoxicity (LDH assay) and WST-1 values were measured after 24 hours and 3 days.

Experiment 2.

Samples were extensively washed with PBS for two days before cell seeding. For measurements we used 12-well plate in which the diameters of each well were 22mm, in order to limit the movement of the samples within the wells.
Cells were detached and counted. The mean cell count was 32.5. This means 3250000 cell/ml. We plated 10^5 cells in a volume of 150 µl on the surface of samples in each well. After the cells adhered to the material (~2h after cell seeding), 1.5 ml of DMEM 10 %FBS were added to each well. The cultures were maintained at 37°C, 5% CO_2 in a humidified atmosphere in incubator (). The cytotoxicity (LDH assay) and WST-1 values were measured after 3 days.

Experiment 3.

In order to perform gene expression analyses, MG-63 were seeded on the sample surface at a density of 82000 cells/sample, in a volume of 150 µl. After the cells adhered to the material (~2h after cell seeding), 1.5 ml of DMEM 10% FBS, with 7 mM β-glycerophosphate, 50 µg/ml ascorbic acid 2-phosphate and 0.1 mM dexamethasone, were added to each well. This modified DMEM stimulates the expression of bone-specific proteins. The expression of bone-specific genes was measured after 5 days.

- Cell proliferation tests with reagent WST-1

Type: colorimetric, MPT format
Quantitation of cell viability, proliferation or cytotoxicity
Samples: adherent or suspension cell cultures
Incubation of cells was carried out with WST-1, followed by spectrophotometric assay of coloured product for 3.5 hours.
Significance of reagent:
The cell proliferation reagent WST-1 is a ready to use substrate which measures the metabolic activity of viable cells. The WST-1 assay is non-radioactive and can be performed entirely in a microtiter plate (MTP). It is suitable for measuring cell proliferation, cell viability and cytotoxicity.
Test principle: The assay is based on the reduction of WST-1 by viable cells. The reduction produces a soluble formazan salt. The procedure involves culturing the cells in a 6-well

microtiter plate then incubating them with WST-1 for 3.5 hours. During this incubation period viable cells convert WST-1 to a water soluble formazan dye. Quantitating the formazan dye in the MTP can be done with an ELISA plate reader. The absorbance directly correlates with the cell number.

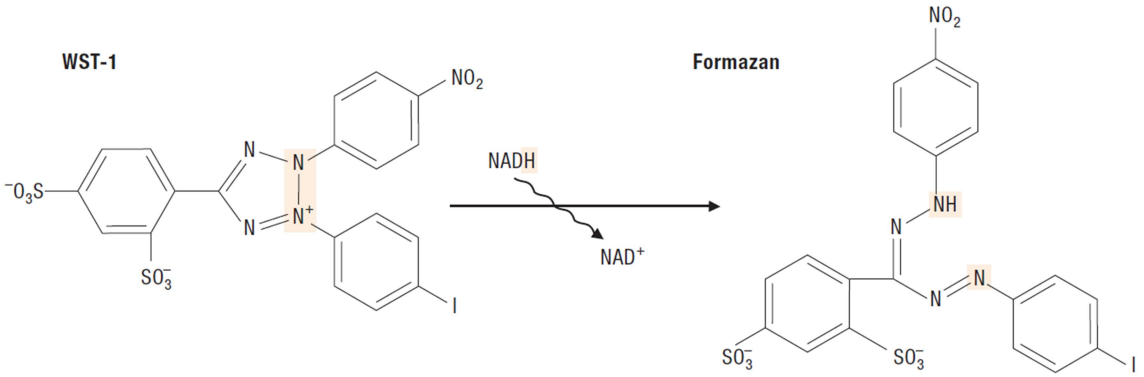


Fig. 1: Molecular structure of WST-1 and its corresponding reaction product

Since proliferating cells are metabolically more active than non-proliferating (resting) cells, the assays are suitable not only for the determination of cell viability and factor-mediated cytotoxicity but also for the determination of cell activation and proliferation. However, under nonideal cell culture conditions (such as the pH and D-glucose concentration in culture medium), the MTT response may vary greatly in viable cells due to the metabolic state of the cells (e.g., cellular concentration of pyridine nucleotides.

WST-1 test results

Experiment 1:

Cell proliferation values after 1 day in culture with the materials:

WST-1	**CTR**	**Ti**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
	1.564	0.866	0.351	0.272	0.331	0.588	0.398	0.400
	1.569	0.850	0.343	0.300	0.352	0.580	0.413	0.415
	1.654	0.902	0.459	0.276	0.348	0.358	0.331	0.399
	1.597	0.887	0.470	0.275	0.353	0.336	0.333	0.391
mean	**1.596**	**0.876**	**0.406**	**0.281**	**0.346**	**0.466**	**0.369**	**0.401**
std dev	**0.041**	**0.023**	**0.068**	**0.013**	**0.010**	**0.137**	**0.043**	**0.010**

% viability

	CTR	Ti	P3	P4	P5	P6	P7	P8
	97.995	54.261	21.992	17.043	20.739	36.842	24.937	25.063
	98.308	53.258	21.491	18.797	22.055	36.341	25.877	26.003
	103.634	56.516	28.759	17.293	21.805	22.431	20.739	25.000
	100.063	55.576	29.449	17.231	22.118	21.053	20.865	24.499
mean	**100.000**	**54.903**	**25.423**	**17.591**	**21.679**	**29.167**	**23.105**	**25.141**
std dev	**2.588**	**1.435**	**4.265**	**0.811**	**0.641**	**8.594**	**2.687**	**0.627**

Cell proliferation values after 3 days in culture with the materials:

WST-1	CTR	2	3	4	5	6	7	8
	2.024	1.043	0.219	0.377	0.239	0.227	0.306	0.668
	2.038	1.021	0.229	0.365	0.239	0.222	0.311	0.681
	1.892	1.029	0.247	0.385	0.261	1.788	0.412	0.431
	1.897	0.981	0.237	0.412	0.270	1.761	0.430	0.438
mean	**1.963**	**1.019**	**0.233**	**0.385**	**0.252**	**1.000**	**0.365**	**0.555**
std dev	**0.079**	**0.027**	**0.012**	**0.020**	**0.016**	**0.895**	**0.065**	**0.139**

% viability

	CTR	Ti	P3	P4	P5	P6	P7	P8
	103.121	53.140	11.158	19.208	12.177	11.565	15.590	34.034
	103.834	52.019	11.667	18.596	12.177	11.311	15.845	34.696
	96.395	52.426	12.584	19.615	13.298	91.097	20.991	21.959
	96.650	49.981	12.075	20.991	13.756	89.721	21.908	22.316
mean	**100.000**	**51.891**	**11.871**	**19.603**	**12.852**	**50.923**	**18.584**	**28.251**
std dev	**4.027**	**1.355**	**0.606**	**1.016**	**0.802**	**45.597**	**3.332**	**7.066**

Experiment 2

Cell proliferation values after 3 days in culture with the materials:

WST1

	CTR	Ti	P3	P4	P5	P6	P7	P8
	1.956	2.364	1.180	2.013	0.213	0.225	0.159	0.764
	2.103	2.345	1.202	1.978	0.216	0.226	0.155	0.786
	2.098	2.171	1.193	1.019	0.210	0.179	0.162	0.771
	2.161	2.242	1.192	1.034	0.211	0.182	0.174	0.789
mean	**2.080**	**2.281**	**1.192**	**1.511**	**0.213**	**0.203**	**0.163**	**0.778**
std dev	**0.087**	**0.091**	**0.009**	**0.560**	**0.003**	**0.026**	**0.008**	**0.012**

% viability

	CTR	Ti	P3	P4	P5	P6	P7	P8
	94.061	113.681	56.744	96.802	10.243	10.820	7.646	36.740
	101.130	112.767	57.802	95.119	10.387	10.868	7.454	37.798
	100.890	104.400	57.370	49.002	10.099	8.608	7.790	37.076
	103.919	107.814	57.321	49.723	10.147	8.752	8.367	37.942
mean	**100.000**	**109.666**	**57.309**	**72.662**	**10.219**	**9.762**	**7.814**	**37.389**
std dev	**4.191**	**4.355**	**0.434**	**26.914**	**0.127**	**1.251**	**0.394**	**0.575**

- LDH test for cytotoxicity

Most current assays for measuring cytotoxicity are based on alterations of plasma membrane permeability and the consequent release (leakage) of components into the supernatant or the uptake of dyes, normally excluded by viable cells (dye exclusion method"). A serious disadvantage of such permeability assays is that the initial sites of damage of many, if not most cytotoxic agents are intracellular. Therefore, cells may be irreversibly damaged and committed to die and the plasma membrane is still intact. Thus, these assays tend to underestimate cellular damage when compared to other methods. Despite this fact, some permeability assays have been widely accepted for the measurement of cytotoxicity.

Assays that measure plasma membrane leakage

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant when the plasma membrane is damaged. With the Cytotoxicity Detection Kit, LDH activity can easily be measured in culture supernatants by a single point assay. The use of a spectrophotometric microplate reader (ELISA plate reader) allows the simultaneous measurement of multiple probes and thereby guarantees the easy processing of a large number of samples (Figure 2)

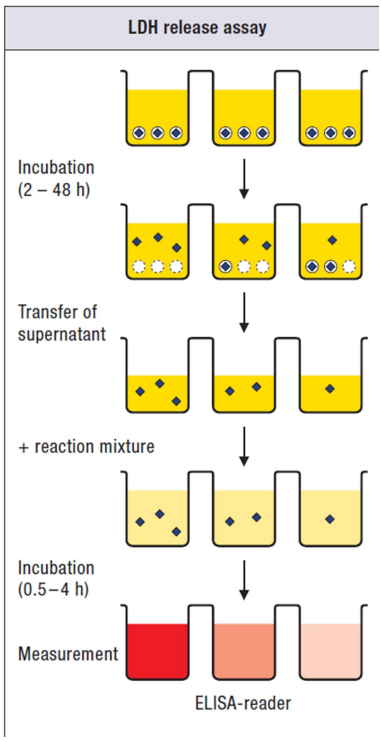


Fig. 2: Measurement of LDH activity using the microplate format

LDH test results

Experiment 1

Cytotoxicity values of samples after 1 day in culture with the materials:

LDH

	CTR	**Ti**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
	1.538	1.508	1.409	2.050	1.061	1.469	1.876	1.753
	1.601	1.688	1.518	2.088	1.152	1.551	1.921	1.661
	1.637	1.638	1.464	2.714	1.483	1.570	1.278	1.694
	1.647	1.607	1.187	2.803	1.588	1.537	1.363	1.803
mean	**1.606**	**1.610**	**1.395**	**2.414**	**1.321**	**1.532**	**1.610**	**1.728**

std dev	**0.049**	**0.076**	**0.145**	**0.400**	**0.254**	**0.044**	**0.336**	**0.063**

% Cytotox.

	CTR	**Ti**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
	5.014	7.234	14.561	-32.877	40.315	10.120	-20.000	-10.897
	0.352	-6.087	6.494	-35.689	33.580	4.052	-23.330	-4.089
	-2.313	-2.387	10.490	-82.017	9.084	2.646	24.255	-6.531
	-3.053	-0.093	30.990	-88.603	1.314	5.088	17.965	-14.598
mean	**0.000**	**-0.333**	**15.634**	**-59.796**	**21.073**	**5.476**	**-0.278**	**-9.029**
std dev	**3.648**	**5.617**	**10.754**	**29.605**	**18.804**	**3.254**	**24.867**	**4.660**

Cytotoxicity values of samples after 3 days in culture with the materials:

	CTR	**Ti**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
	1.906	1.870	1.716	2.311	1.905	1.508	1.675	1.705
	1.856	2.106	1.678	2.308	1.840	1.529	1.781	1.856
	1.936	2.175	1.526	2.559	1.505	2.071	1.687	1.386
	1.935	2.275	1.579	2.691	1.521	2.109	1.688	1.331
mean	**1.908**	**2.107**	**1.625**	**2.467**	**1.693**	**1.804**	**1.708**	**1.570**
std dev	**0.038**	**0.172**	**0.088**	**0.190**	**0.209**	**0.330**	**0.049**	**0.252**

% Cytotox.

	CTR	**Ti**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**
	0.125	2.122	10.667	-22.347	0.180	22.208	12.942	11.278
	2.899	-10.972	12.776	-22.181	3.787	21.043	7.061	2.899
	-1.540	-14.801	21.210	-36.108	22.375	-9.030	12.276	28.978
	-1.484	-20.350	18.269	-43.432	21.487	-11.139	12.221	32.029
mean	**0.000**	**-11.000**	**15.730**	**-31.017**	**11.957**	**5.771**	**11.125**	**18.796**
std dev	**2.081**	**9.558**	**4.859**	**10.540**	**11.616**	**18.334**	**2.729**	**14.000**

Experiment 2

Cytotoxicity values of samples after 3 days in culture with the materials:

LDH

	CTR	2	3	4	5	6	7	8
	1.587	1.563	1.410	1.737	0.985	0.815	0.301	1.514
	1.624	1.564	1.394	1.720	1.050	0.817	0.311	1.535
	1.628	1.634	1.212	1.710	1.082	0.268	0.308	1.612
	1.690	1.642	1.216	1.757	1.097	0.262	0.304	1.560
mean	**1.632**	**1.601**	**1.308**	**1.731**	**1.054**	**0.541**	**0.306**	**1.555**
std dev	**0.043**	**0.043**	**0.109**	**0.021**	**0.050**	**0.318**	**0.004**	**0.042**

% Cytotox.

	CTR	2	3	4	5	6	7	8
	2.965	4.537	14.562	-6.863	42.408	53.546	87.224	7.748
	0.541	4.472	15.610	-5.749	38.149	53.415	86.568	6.372
	0.278	-0.115	27.535	-5.094	36.052	89.386	86.765	1.327
	-3.784	-0.639	27.273	-8.174	35.070	89.779	87.027	4.734
mean	**0.000**	**2.064**	**21.245**	**-6.470**	**37.920**	**71.532**	**86.896**	**5.045**
std dev	**2.797**	**2.826**	**7.125**	**1.350**	**3.256**	**20.844**	**0.288**	**2.768**

Summary of cytotoxicological and cell viability measurements

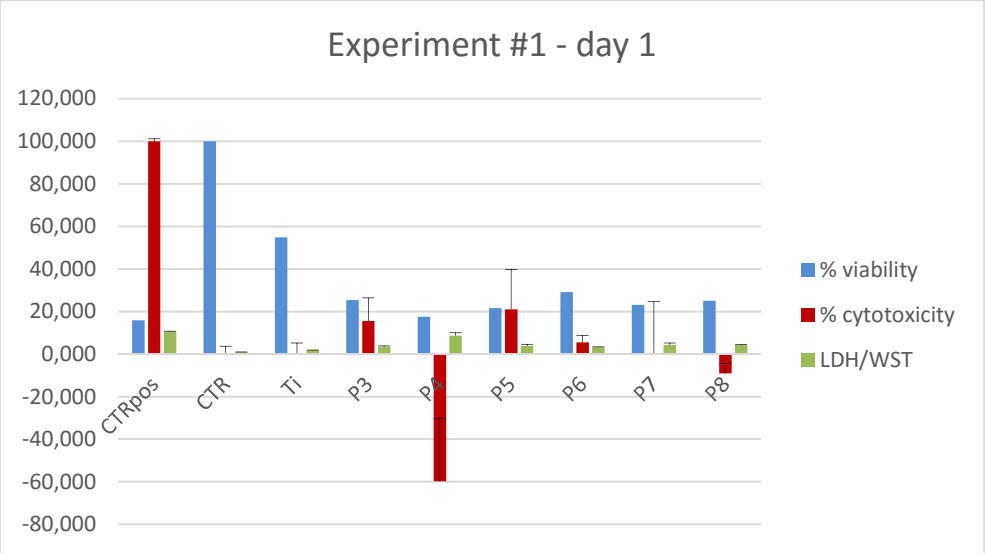


Fig. 3: WST-1, LDH as well as LDH/ WST-1 percentages of Ti and CaP coatings compared to positive and negative control after 24 hours of immersion. (positive control: dead cells, negative control: cells only in medium)

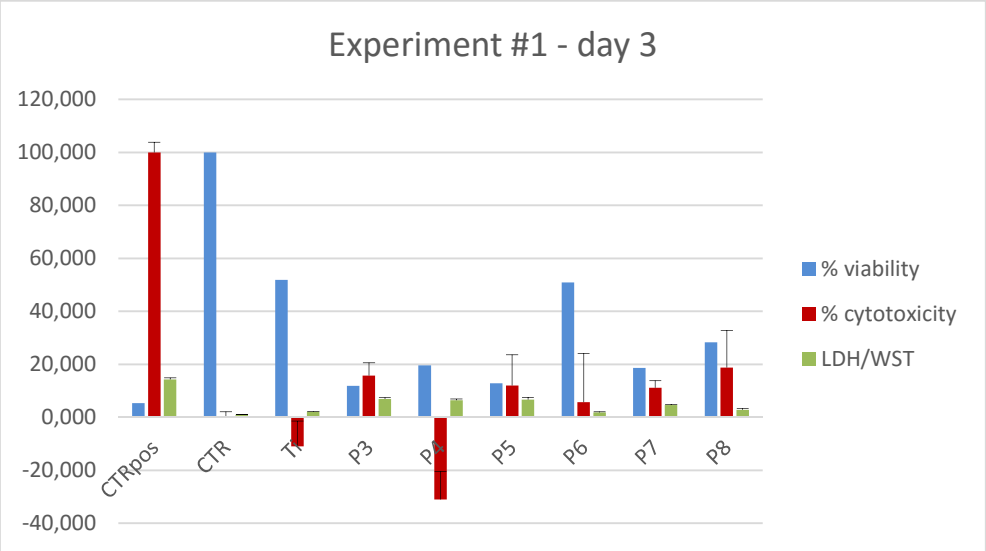


Fig. 4: WST-1, LDH as well as LDH/ WST-1 percentages of Ti and CaP coatings compared to positive and negative control after 72 hours of immersion. (positive control: dead cells, negative control: cells only in medium)

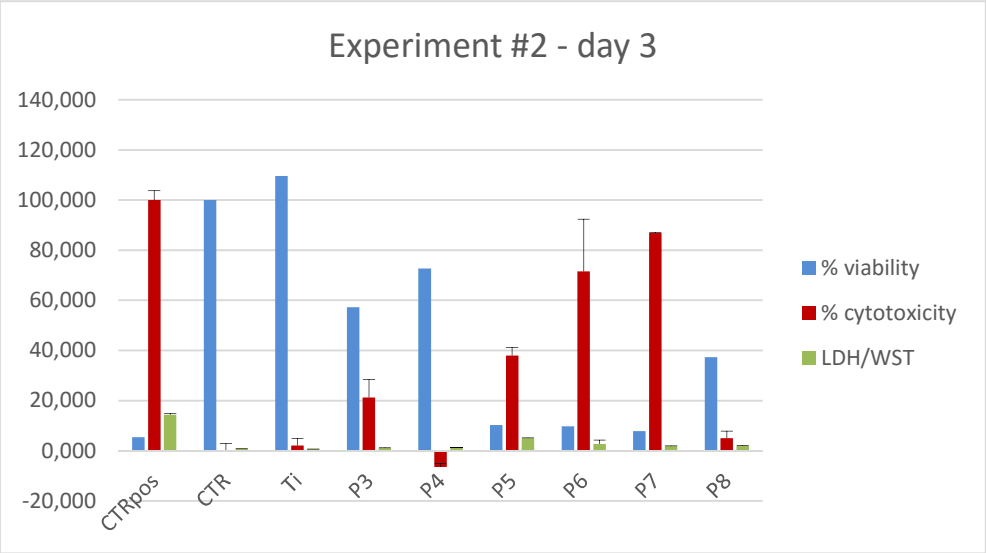


Fig. 5: WST-1, LDH as well as LDH/ WST-1 percentages of Ti and CaP coatings compared to positive and negative control after 24 hours of immersion. (positive control: dead cells, negative control: cells only in medium) second experiment.

It is visible on Figures 3-5 that the cell viability values do not increase significantly in the cases of each coatings compared to bare implant material and the standard deviation of all values are high. However, for LDH measurements, in some cases the coated samples show significantly lower cytotoxity than that for Ti alloy implants. These samples are mainly in P4 and P8 series but in experiment 1, the P6 series demonstrate relatively high cell proliferation and low cytotoxicity. In this case the cell proliferation value is higher than that for bare implant material.

- Live/Dead cell staining experiments

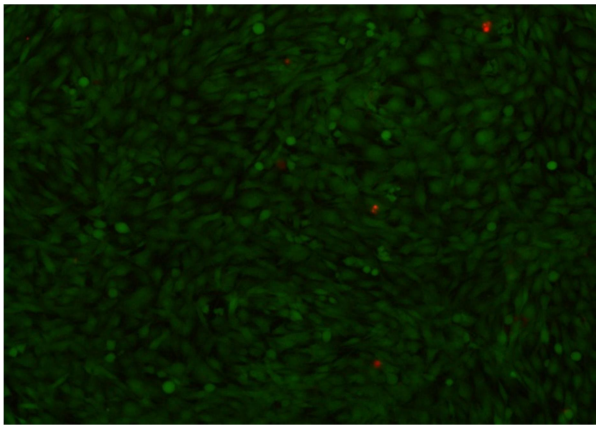
Cell viability is an important component of any in-vitro cell based assay. Culture conditions and experimental treatments can affect cell viability by directly or indirectly inducing cytotoxicity, apoptosis and/or necrosis. Cell viability itself can be an important experimental endpoint. In addition, it is important to be cognizant of cell viability when interpreting any experimental results since low cell viability can confound data interpretation. Live cells are identified on the basis of intracellular esterase activity (generating green fluorescence) and exclusion of the red dye. Dead cells are identified by the lack of esterase activity and non-intact plasma membrane which allows red dye staining.

The Live/Dead assay stain solution is a mixture of two highly fluorescent dyes that differentially label live and dead cells:

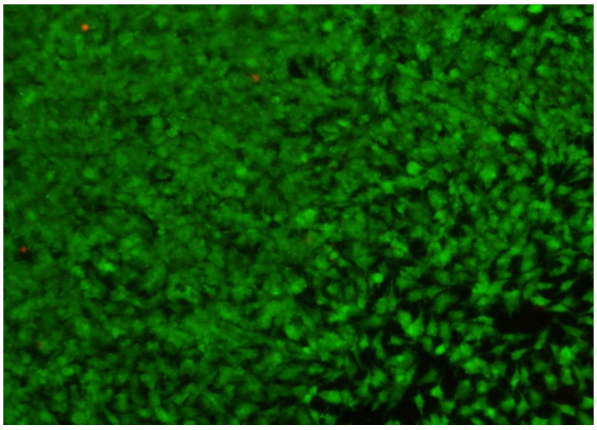
•The Live cell dye labels intact, viable cells green. It is membrane permeant and non-fluorescent until ubiquitous intracellular esterases remove ester groups and render the molecule fluorescent. The Excitation (max) and Emission (max) are 494nm and 515nm, respectively (similar to FITC).

•The Dead cell dye labels cells with compromised plasma membranes red. It is membrane-impermeant and binds to DNA with high affinity. Once bound to DNA, the fluorescence increases >30-fold. The Excitation (max) and Emission (max) are 528nm and 617nm, respectively.

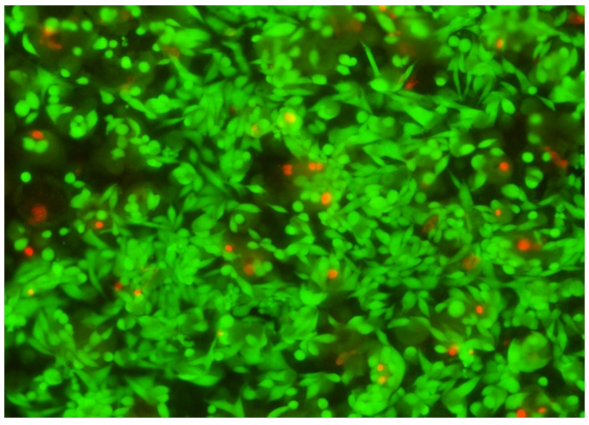
Results after 4 days in culture with the materials:



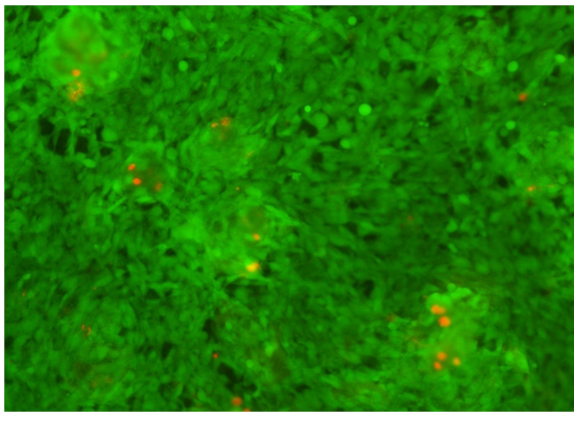
CTR, MG63 osteoblasts on culture plate



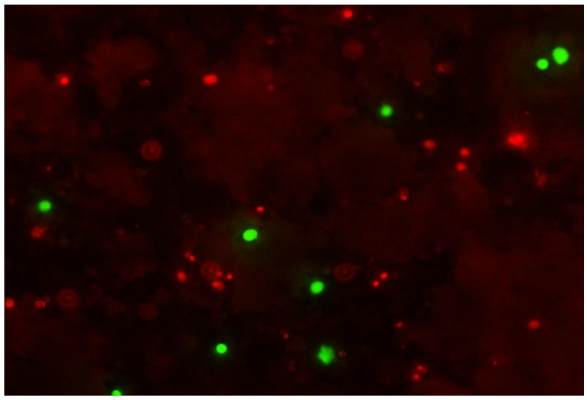
Ti, a layer of living cells, very few dead, normal morphology



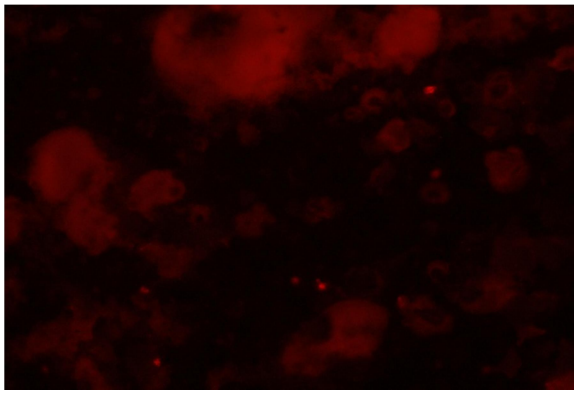
P3 a layer of living cells, some dead, normal morphology



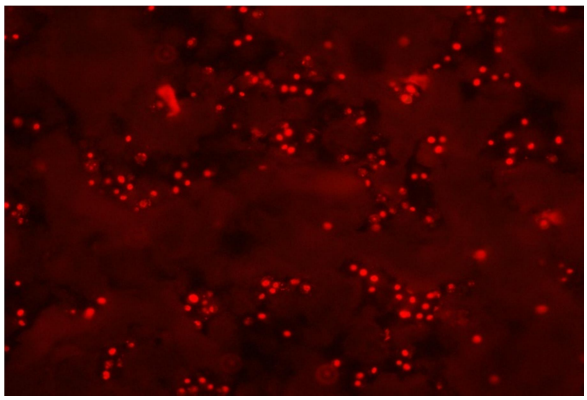
P4 confluent monostrate of living cells, very few dead, normal morphology



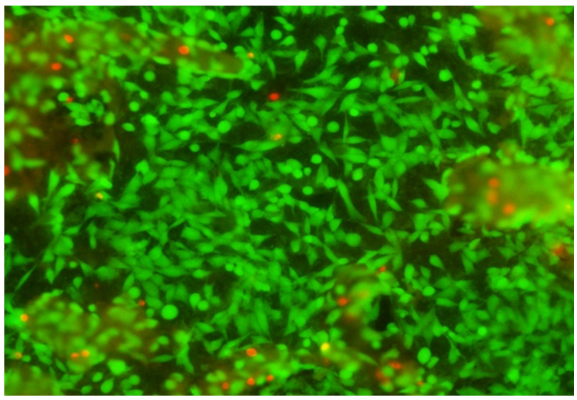
P5, few living cells but round shape, some dead



P6, no visible cells, the reason might be that the cells were washed off of the coating during cell seeding procedure.



P7, high number of dead cells



P8, a layer of living cells, some dead, normal morphology

Fig. 6: Live/ Dead cell staining measurements on MG63 osteoblast cells, pure Ti alloy implant materials as well as pure and modified CaP layers. (see coating preparation parameters on Table 1)

From the Live/Dead cell staining measurement, it is visible that on series P3, P4 and P8 there are mainly living cells with only a few dead cells which also prove their biocompatibility. The amount of living cells on these coatings is close to that of pure titanium implant.

Gene expression analysis:

- Principle on the analysis of gene expression.

The analysis of gene expression by Real-Time PCR is a powerful technique to determine the expression of specific genes in cells grown under specific stimuli. In the context of the project, the expression of bone-specific genes was evaluated in cells grown on the different materials, in order to understand whether they may stimulate an osteogenic differentiation.

The first stage in gene expression analysis is RNA extraction. There are several techniques which could be employed, the most used is a phenol/chloroform extraction. We used a silica column-based extraction, where the RNA binds with high affinity. RNA is extensively washed and then recovered by eluting it with RNase-free water.

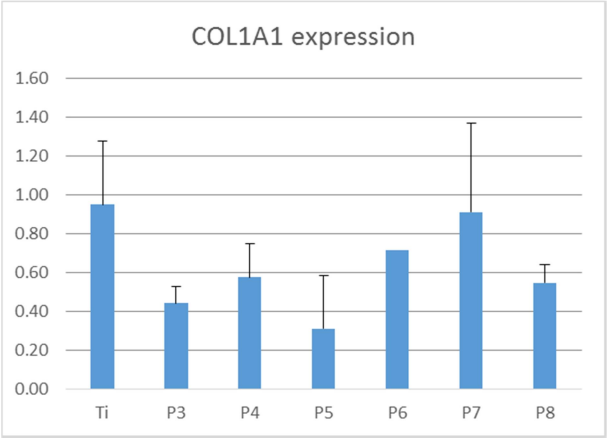
The purified RNA is quantified by the analysis of its absorbance at 260 nm. Subsequently, reverse transcription of RNA into cDNA (complementary DNA) is required for downstream applications. This process requires the use of a specific enzyme called reverse transcriptase: in a reaction mixture comprising nucleotides (dNTPs), primers (random hexamers) and magnesium as a cofactor, the enzyme produces cDNA from the RNA template. cDNA has a higher stability and can be used for quantitative gene expression analysis using Real-Time PCR.

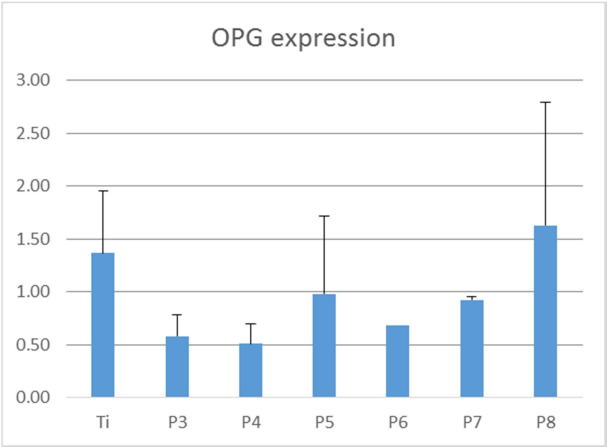
Real Time PCR is a technique based upon the classic Polymerase Chain Reaction (PCR), with the difference that signal amplification is monitored in real time by the use of DNA intercalants, which are dyes that emit fluorescence upon binding to double strand DNA.

In an optimized reaction, the target quantity will approximately double during each amplification cycle. In quantitative PCR (qPCR), the amount of amplified product is linked to fluorescence intensity using a fluorescent reporter molecule.

The raw data of Real-Time PCR are represented by the Ct, i.e. the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold (a fluorescent signal significantly above the background fluorescence). This value is in direct proportion to the initial amount of cDNA.

In order to evaluate the relative expression, it is necessary to perform a double normalization. The first (ΔCt) is the difference between the Ct of the gene of interest and the Ct of an housekeeping gene (a gene whose expression level is constant in all culturing conditions). This step ensures the expression of a particular gene is normalized among samples, avoiding differences in the initial amount of the cells. Then the $\Delta\Delta$Ct has to be calculated, which is the difference between the ΔCt of the test sample (cells with the materials) and the ΔCt of the control samples (cells alone). The fold change in gene expression between the test and the control sample can be calculated as $2^{-\Delta\Delta Ct}$.





The graphs show the fold change in expression of bone-specific genes: COL1A1 (encoding for the alpha-1 chain of collagen I) and OPG (encoding for osteoprotegerin).
The analyses showed no differences in the expression of these genes among the samples. However, the expression levels may vary consistently among the cells grown in different materials, resulting in very high standard deviations.

Conclusion

As a conclusion we can say that the cell viability values did not increase significantly in the cases of each coating compared to bare implant materials and the standard deviation of all values were high. This fact might be attributed to the large thickness of coatings. However, for LDH measurements, in some cases, the coated samples showed significantly lower cytotoxity than that for Ti alloy implants. These samples were mainly in P4 and P8 series, however in experimental 1, the P6 series demonstrated relatively high cell proliferation and low cytotoxicity. In this case the cell proliferation value was higher than that for bare implant material. According to the Live/Dead cell staining experiments, the coatings prepared by P4

and P8 parameters (Table 1) showed similar or even larger quantity of living cells than that of pure implant materials. Therefore, in the future we are planning to prepare the coatings using these parameters. The gene expression analyses showed no differences in the expression of genes studied among the samples. However, the expression levels varied consistently among the cells grown in different materials, resulting in very high standard deviations.

It can be stated that all experiments showed very high standard deviation and varied significantly even in samples prepared with same parameters. This means that it is necessary to improve the reproducibility of preparation procedure and reduce significantly the thickness of coatings during deposition process. Taking all the biocompatible experiments into account we can say that there are promising methods for preparing biocompatible and antimicrobial coatings but further analysis are still needed focusing on modified coatings prepared by methods that gave the best results.