Marie Skłodowska-Curie Actions (MSCA)
Innovative Training Networks (ITN)
H2020-MSCA-ITN-2017

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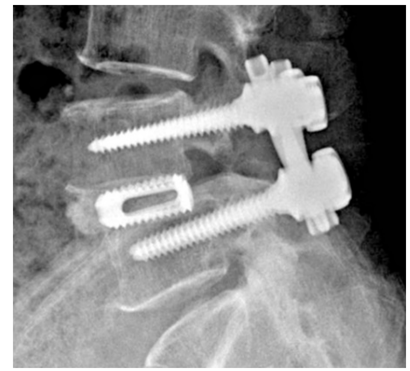
# Development of osteoinductive coatings for spinal implants (fusion cages)

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 766012

# Introduction

Stock photo



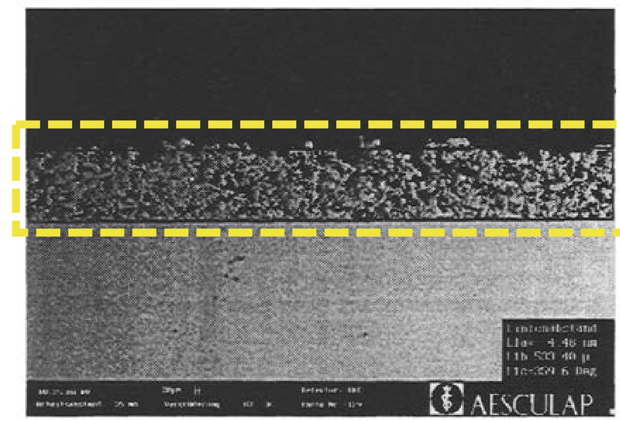
DOI: 10.4184/jkss.2012.19.4.123



**1 in 5 patients need revision!**

# Introduction

**Coating:** Surface modification by deposition of different material.




DOI: 10.1063/1.58204

## For Orthopaedic implants:

- Used to improve osteointegration
- Other possible objectives:
  - Wear Resistance
  - Antibacterial properties

## Standard compositions:

- Titanium
- Calcium Phosphates – **Hydroxyapatite (HAP)**

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DOI: 10.1111/j.1708-8208.2012.00469.x

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# Aims

Development of $TiO_2$/sHAP coatings

Low temperature crystallization of $TiO_2$

Synthesis of Mg and Sr substituted hydroxyapatite

Serum-free Osteogenic Differentiation of Mesenchymal Stem Cell Line

# Design of Experiments

Statistics-based experimental method that aims to extract the most information from a minimum number of conditions.

- Identifies how individual factors affect experimental results
- Identifies how factors interact with each other
- Creates mathematical model that identifies optimal experimental conditions/range of conditions

Well designed DoE results in well organized, more useful and more precise data.

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# Design of Experiments

## How to validate a model

**1. $R^2 – Q^2 < 30\%$**

**2. $Q^2 \geq 50\%$**

$$R^2 = 1 - \frac{SS_{res}}{SS}$$

$$Q^2 = 1 - \frac{PRESS}{SS}$$

**3. Validity > 25%**

$$Val = 1 + 0.57647\log(p_{lof})$$

**4. Reproducibility > 50%**

$$Rep = 1 - \frac{MS_{total\ error}}{MS_{total\ SS\ corrected}}$$

**Model is only valid if it passes all four criteria!**

# Low temperature crystallization of $TiO_2$

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# Objectives

Objectives

- Low temperature crystallization of $TiO_2$ – Design of Experiments
- $TiO_2$ synthesis optimization

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# Background

## Why $TiO_2$:

- Naturally occurs on Ti-based implants
- Enhances implant osteointegration

## Why crystallized $TiO_2$:

- Further enhances osteointegration

## Why Low-temperature Crystallization:

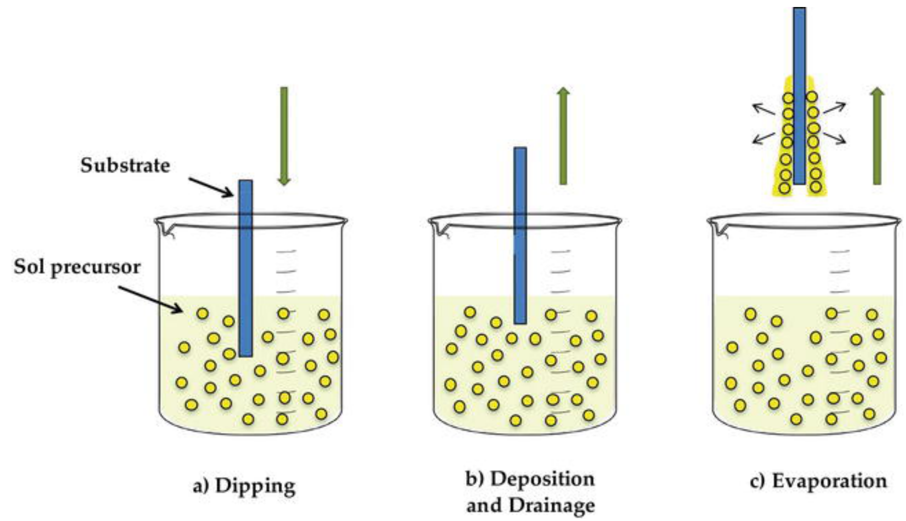
- $TiO_2$ crystallizes at 400 – 450 °C
- Service temperature of PEEK at 250 – 260 °C
- Synthesis conditions can lower crystallization temperature

# Background

# Sol-Gel Chemistry

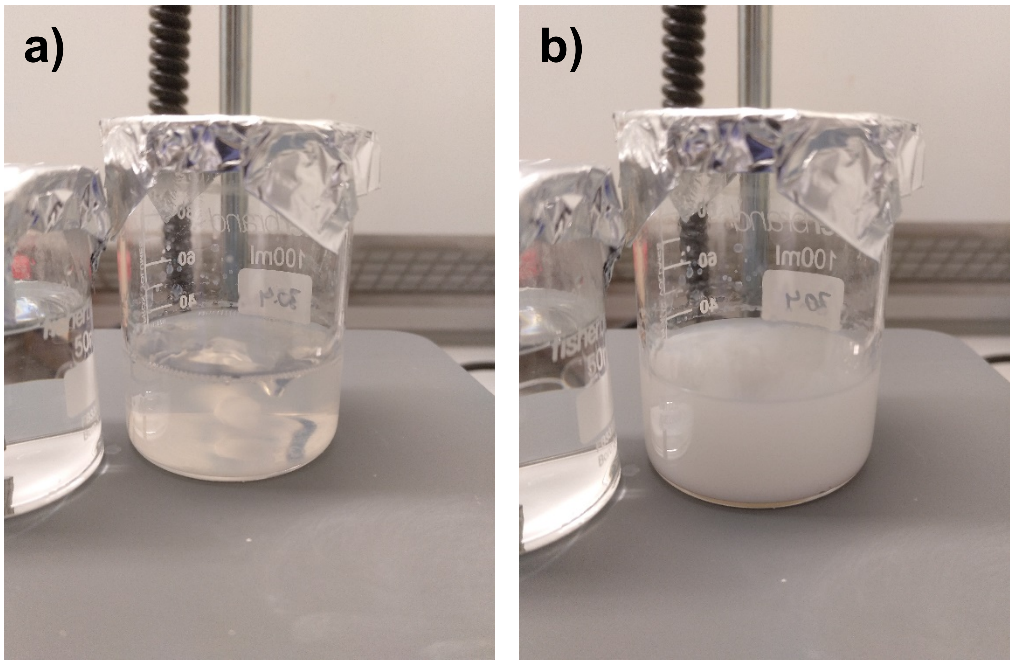
## Why Sol-Gel:

- Simple, inexpensive technique
- High quality coatings
- High Purity
- Excellent Bioactivity
- Appropriate for complex geometries




Sol-gel coating process.
DOI: 10.5772/67830

# TiO2 Sol-Gel Synthesis




Sol-gel synthesis of TiO2: a) before gelification; b) after gelification.

## Method:

- Add Ethanol to 100 ml beaker
- Add 3.9 ml of Titanium Isopropoxide
- Stir for 10 minutes
- Add Acetic Acid
- Stir for 10 minutes
- Add ultrapure water
- Set reaction temperature
- Stir for 24 hours covered in punctured foil
- Sinter at 250 °C

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# Low temperature crystallization of $TiO_2$ — Design of Experiments

## Methods:

- Definitive screening design
- Analysis:
  - TGA
  - XRD
- Responses:
  - Org %
  - Crystallinity %

| Standard Order | Run Order | A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|
| **1** | 12 | 0 | + | + | + | + | + |
| **2** | 1 | 0 | - | - | - | - | - |
| **3** | 6 | + | 0 | + | - | - | + |
| **4** | 3 | - | 0 | - | + | + | - |
| **5** | 8 | + | + | 0 | + | - | - |
| **6** | 11 | - | - | 0 | - | + | + |
| **7** | 7 | + | - | + | 0 | + | - |
| **8** | 5 | - | + | - | 0 | - | + |
| **9** | 4 | + | - | - | + | 0 | + |
| **10** | 9 | - | + | + | - | 0 | - |
| **11** | 13 | + | + | - | - | + | 0 |
| **12** | 2 | - | - | + | + | - | 0 |
| **13** | 10 | 0 | 0 | 0 | 0 | 0 | 0 |

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| **A** | Reaction temperature (°C) | 50 | 60 | 70 |
| **B** | Ethanol volume (ml) | 15.2 | 22.8 | 30.4 |
| **C** | Ultrapure water volume (ml) | 1 | 2 | 3 |
| **D** | Acetic acid volume (ml) | 0.7 | 2.8 | 4.1 |
| **E** | Heating rate (°C/min) | 4 | 12 | 20 |
| **F** | Sintering time (hours) | 1 | 3.5 | 6 |

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# Low temperature crystallization of $TiO_2$ — Design of Experiments

## Results:

| Standard Order | A | B | C | D | E | F | Org % | Crystallinity % |
|---|---|---|---|---|---|---|---|---|
| 1 | 0 | + | + | + | + | + | 8.10 | 63.91 |
| 2 | 0 | - | - | - | - | - | 11.17 | 0.00 |
| 3 | + | 0 | + | - | - | + | 7.04 | 73.23 |
| 4 | - | 0 | - | + | + | - | 13.34 | 0.00 |
| 5 | + | + | 0 | + | - | - | 12.31 | 52.25 |
| 6 | - | - | 0 | - | + | + | 12.24 | 36.88 |
| 7 | + | - | + | 0 | + | - | 7.71 | 71.34 |
| 8 | - | + | - | 0 | - | + | 7.10 | 0.00 |
| 9 | + | - | - | + | 0 | + | 7.18 | 45.99 |
| 10 | - | + | + | - | 0 | - | 10.92 | 0.00 |
| 11 | + | + | - | - | + | 0 | 7.11 | 0.00 |
| 12 | - | - | + | + | - | 0 | 9.74 | 67.56 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 9.35 | 57.43 |

| Factor | Description |
|---|---|
| A | Reaction temperature (°C) |
| B | Ethanol volume (ml) |
| C | Ultrapure water volume (ml) |
| D | Acetic acid volume (ml) |
| E | Heating rate (°C/min) |
| F | Sintering time (hours) |

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# Low temperature crystallization of $TiO_2$ — Design of Experiments

## Models:

$$-\boldsymbol{Org}^{-1} = -0.1054 - 0.0141A - 0.0004C + 0.0024D - 0.0160F - 0.0306C^2 + 0.0231D^2$$

$$\boldsymbol{Crystallinity}\ (\%) = 53.00 + 13.84A - 10.56B + 23.00C + 11.96D + 9.64F - 22.04C^2 - 6.22AD$$

| Factor | Description |
|---|---|
| A | Reaction temperature (°C) |
| B | Ethanol volume (ml) |
| C | Ultrapure water volume (ml) |
| D | Acetic acid volume (ml) |
| E | Heating rate (°C/min) |
| F | Sintering time (hours) |

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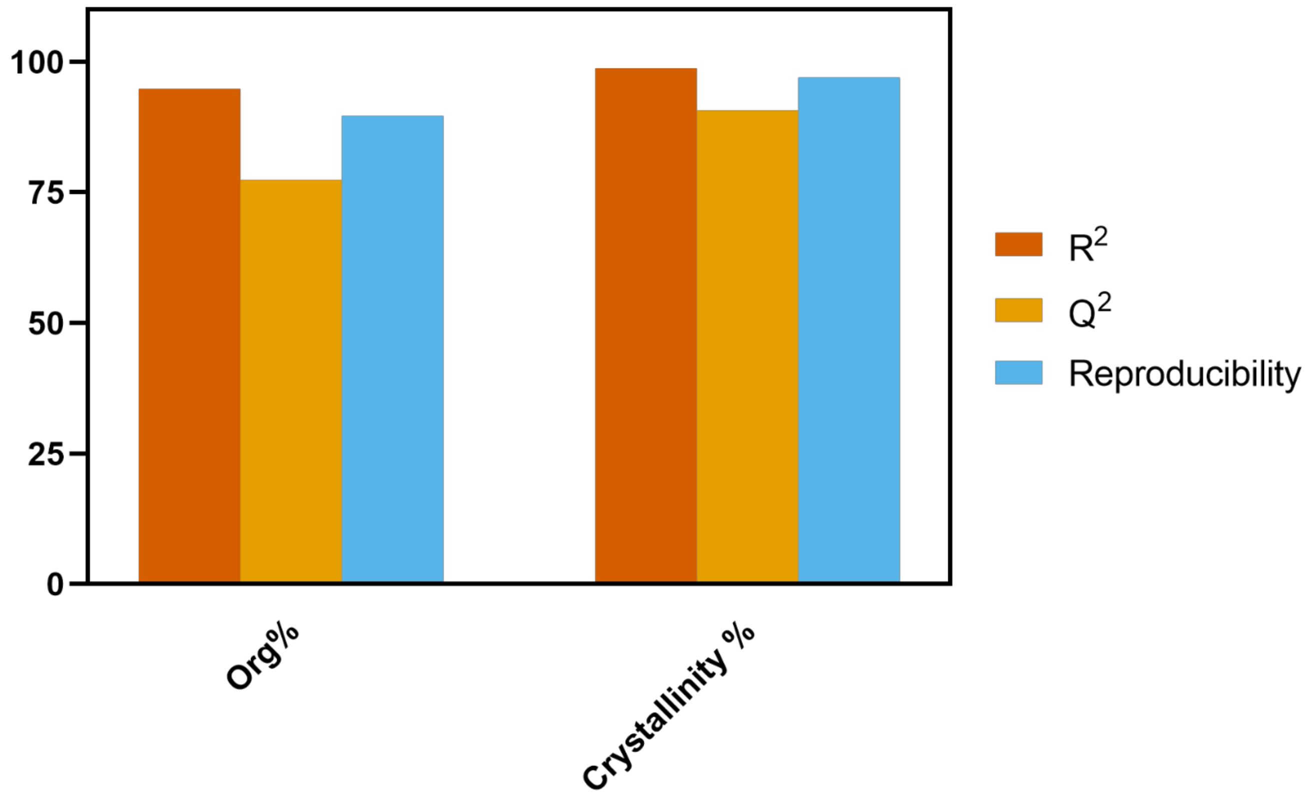
# Low temperature crystallization of $TiO_2$ – Design of Experiments

## Models: Summary of Fit



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# $TiO_2$ synthesis optimization

| Response | Factor | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| **Org %** | 1 | N/A | -1 | 0 | N/A | 1 |
| **Crystallinity %** | 1 | -1 | 0.52 | 1 | N/A | 1 |
| **Optimized settings** | 1 | -1 | **?** | **?** | 1 | 1 |

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| **A** | Reaction temperature (°C) | 50 | 60 | 70 |
| **B** | Ethanol volume (ml) | 15.2 | 22.8 | 30.4 |
| **C** | Ultrapure water volume (ml) | 1 | 2 | 3 |
| **D** | Acetic acid volume (ml) | 0.7 | 2.8 | 4.1 |
| **E** | Heating rate (°C/min) | 4 | 12 | 20 |
| **F** | Sintering time (hours) | 1 | 3.5 | 6 |

# $TiO_2$ synthesis optimization

## Optimizing Factor D (Acetic acid volume)

| Settings | Factors | | | |
|---|---|---|---|---|
| | A | B | C | F |
| | 1 | -1 | 1 | 1 |

$$-Org^{-1} = -10539 - 0.01412A - 0.00044C + 0.00239D - 0.01603F - 0.03058C^2 + 0.02314D^2$$

$$Crystallinity\ (\%) = 53.00 + 13.84A - 10.56B + 23.00C + 11.96D + 9.64F - 22.04C^2 - 6.22AD$$

| D | Org % | Crystallinity % |
|---|---|---|
| 0 | 6.00 | 88.00 |
| 0.5 | 6.27 | 90.87 |
| 1 | 7.09 | 93.74 |

# $TiO_2$ synthesis optimization

## Response optimization

| Response | Factor | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | F |
| Org % | 1 | N/A | -1 | 0 | N/A | 1 |
| Crystallinity % | 1 | -1 | 0.52 | 1 | N/A | 1 |
| **Optimized settings** | 1 | -1 | **1** | **0.5** | 1 | 1 |

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| A | Reaction temperature (°C) | 50 | 60 | 70 |
| B | Ethanol volume (ml) | 15.2 | 22.8 | 30.4 |
| C | Ultrapure water volume (ml) | 1 | 2 | 3 |
| D | Acetic acid volume (ml) | 0.7 | 2.8 | 4.1 |
| E | Heating rate (°C/min) | 4 | 12 | 20 |
| F | Sintering time (hours) | 1 | 3.5 | 6 |

| Response | Modelled | Real |
|---|---|---|
| Org % | 6.27 | **5.52** |
| Crystallinity % | 90.87 | **77.95** |

# Chapter remarks

- It is possible to decrease the crystallization temperature of $TiO_2$
- Optimal synthesis conditions were assessed using Design of Experiments

- $TiO_2$/sHAP coatings will be developed using optimized synthesis conditions

# Synthesis of Mg and Sr substituted hydroxyapatite (sHAP)

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# Objectives

Objectives

Mg and Sr sHAP Synthesis
–
Preliminary Work

Mg and Sr sHAP Synthesis
–
Design of Experiments

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# Background

## Why sHAP:

- HAP is the basis of bone mineral
- Biologic HAP is not stochiometric
- sHAP closer to biologic HAP – **better biological performance**

## Why Mg:

- Stimulates osteoblasts
- **Stimulates bone mineralization**

## Why Sr:

- Inhibits osteoclasts
- **Inhibits bone resorption**

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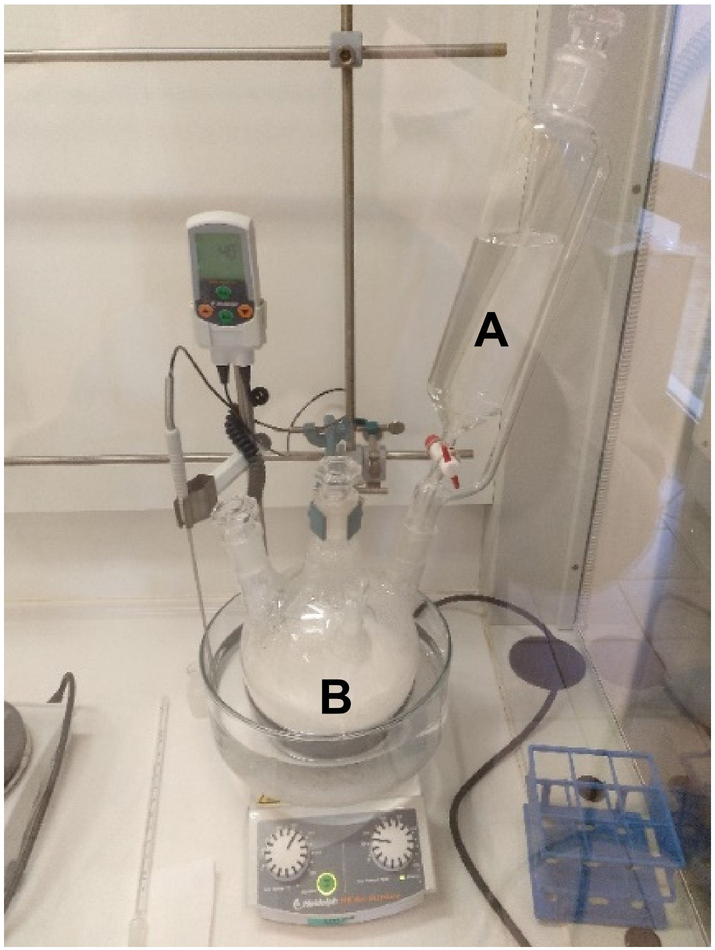
# Wet Chemical Precipitation




**A** – $H_3PO_4$ solution
**B** – Basic solution
- $CaOH_2$
- $MgCl_2$
- $Sr(NO_3)_2$
- Ammonia solution 28%

## Method:

- Set B to 40 °C;
- Add A to B, under stirring, 1 drop/sec;
- Leave overnight under stirring at 37 °C;
- Wash, dry overnight and grind to powder.

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# Formulations studied

| Formulation | Basic solution | | |
|---|---|---|---|
| | Ca - $Ca(OH)_2$ | Mg - $MgCl_2 \cdot 6H_2O$ | Sr - $Sr(NO_3)_2$ |
| | mol% | mol% | mol% |
| **C0** | 100 | 0 | 0 |
| **C4** | 90 | 5 | 5 |
| **C5** | 85 | 5 | 10 |
| **C6** | 85 | 10 | 5 |
| **C7** | 80 | 10 | 10 |
| **C8** | 85 | 7.5 | 7.5 |

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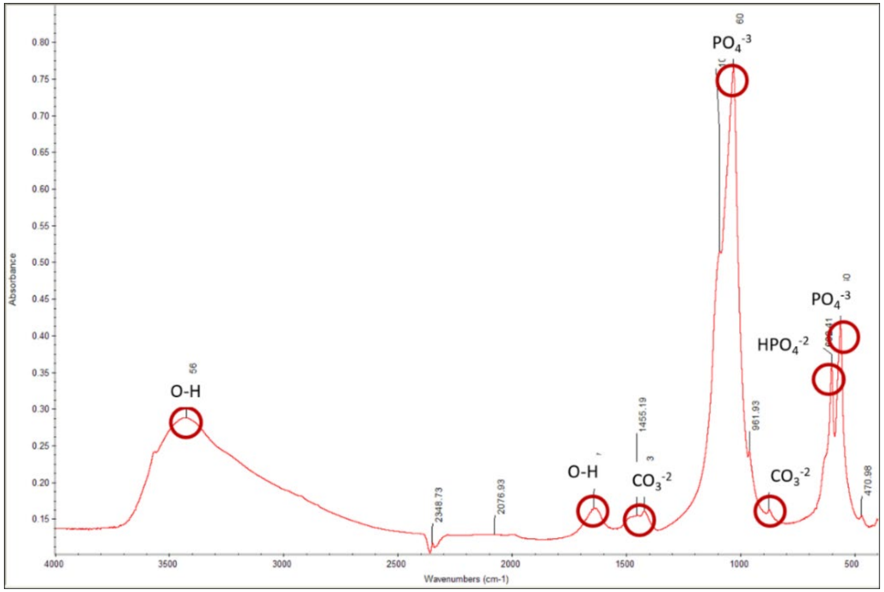
# Mg and Sr sHAP Synthesis – Preliminary Work

## Methods:

- All formulations synthesised
- No ammonia solution added
- All samples analysed by FTIR




FTIR spectrum of pure HAP (C0), with relevant peaks identified.

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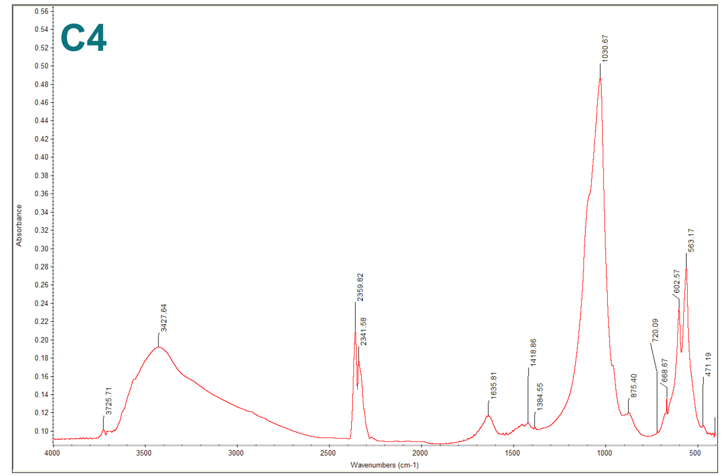
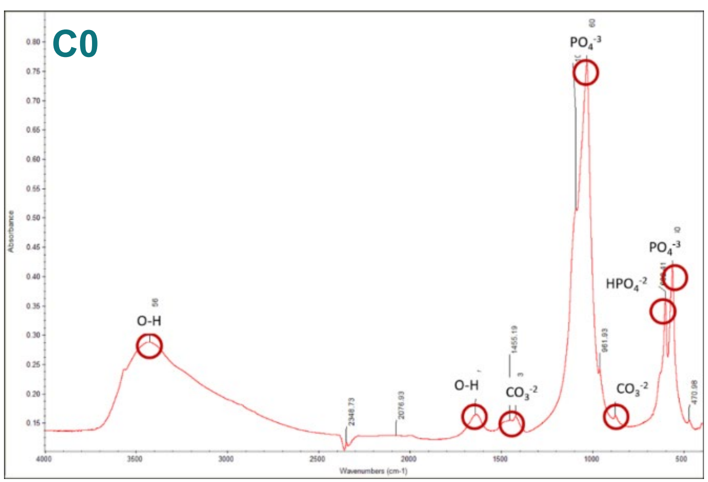
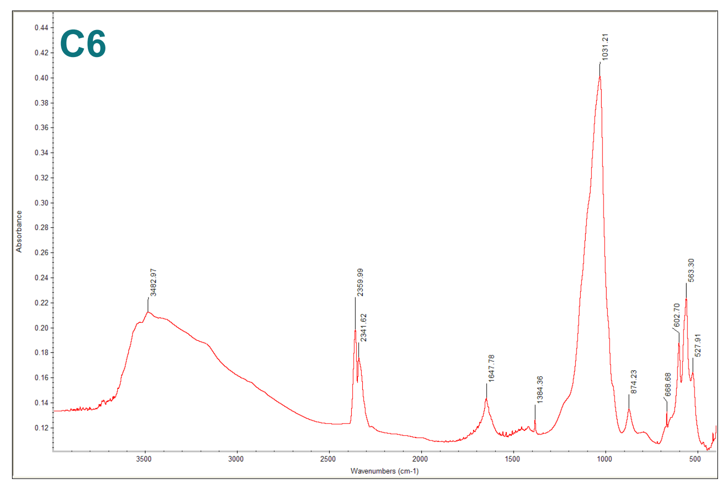
# Mg and Sr sHAP Synthesis – Preliminary Work

## Results:

- C4 spectrum similar to C0 spectrum;
- Spectra for C5 to C8 do not resemble C0 spectrum;



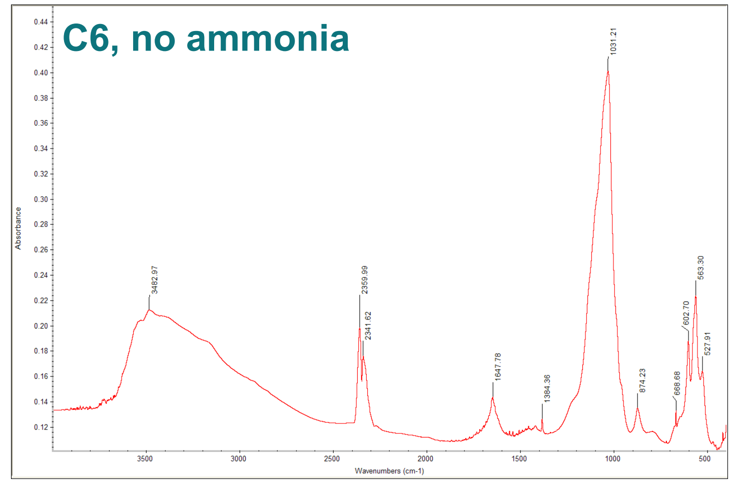



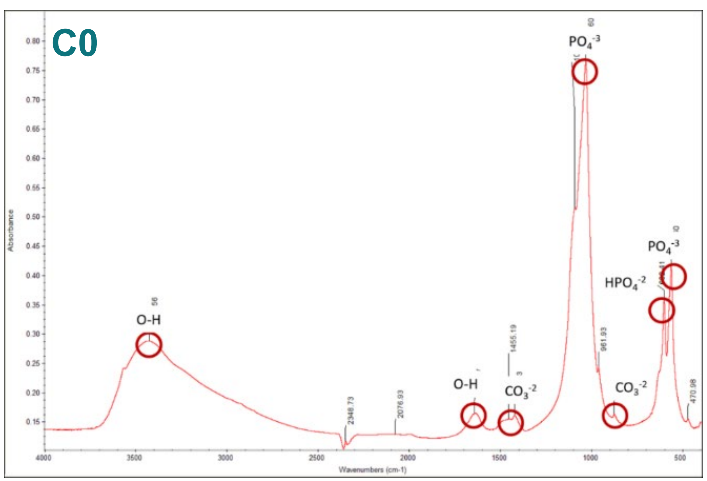



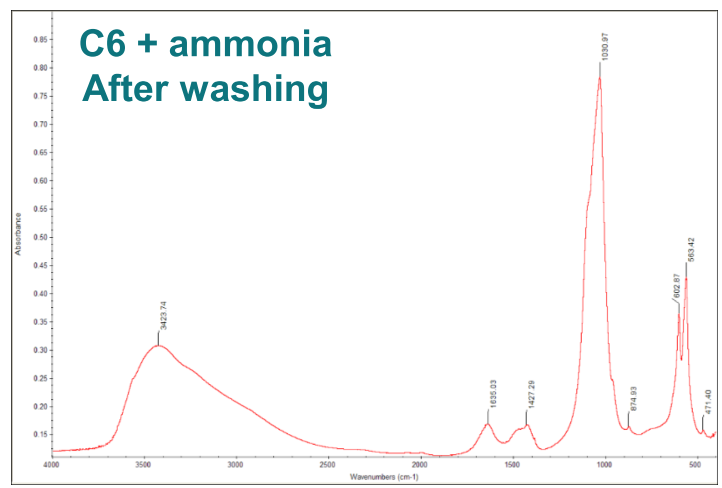
# Mg and Sr sHAP Synthesis – Preliminary Work

## Results:

- C6 spectrum now similar to C0
- Ammonia traces before washing



# Mg and Sr sHAP Synthesis – Design of Experiments

## Methods:

- Full factorial design + three centre points
- Analysis:
    - ICP-OES
    - XRD
- Responses:
    - Ca/P
    - (Ca+Mg+Sr)/P
    - Mg %
    - Sr %
    - HAP phase %

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| A | Mg substitution degree (%) | 5 | 7.5 | 10 |
| B | Sr substitution degree (%) | 5 | 7.5 | 10 |
| C | Ammonia solution 28% volume (ml) | 15 | 32.5 | 50 |

| Standard Order | Run Order | A | B | C |
|---|---|---|---|---|
| 1 | 7 | - | - | - |
| 2 | 4 | + | - | - |
| 3 | 8 | - | + | - |
| 4 | 10 | + | + | - |
| 5 | 1 | - | - | + |
| 6 | 5 | + | - | + |
| 7 | 3 | - | + | + |
| 8 | 2 | + | + | + |
| 9 | 9 | 0 | 0 | 0 |
| 10 | 6 | 0 | 0 | 0 |
| 11 | 11 | 0 | 0 | 0 |

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# Mg and Sr sHAP Synthesis – Design of Experiments

## Models:

Effects of main factors and interactions, and summary of fit of optimized DoE models
(* Box-Cox transformation of exponent λ)

| Model | $\beta_0$ | A | B | C | AB | AC | BC |
|---|---|---|---|---|---|---|---|
| Ca/P | 1.3618 | -0.0525 | -0.0550 | +0.015 | -- | -- | -- |
| (Ca+Mg+Sr)/P | 1.5736 | -0.0300 | -0.3500 | +0.055 | -- | -- | -- |
| Mg % * λ = 4 | 3.8030 | +3.1090 | -- | +3.171 | -- | +2.632 | -- |
| Sr % * λ = 1 | 6.8609 | -- | +1.9575 | -- | -- | -- | -- |
| HAP phase % * λ = 9 | 11.676 | -4.8110 | -1.313 | +1.566 | -- | -- | -- |

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| A | Mg substitution degree (%) | 5 | 7.5 | 10 |
| B | Sr substitution degree (%) | 5 | 7.5 | 10 |
| C | Ammonia solution 28% volume (ml) | 15 | 32.5 | 50 |

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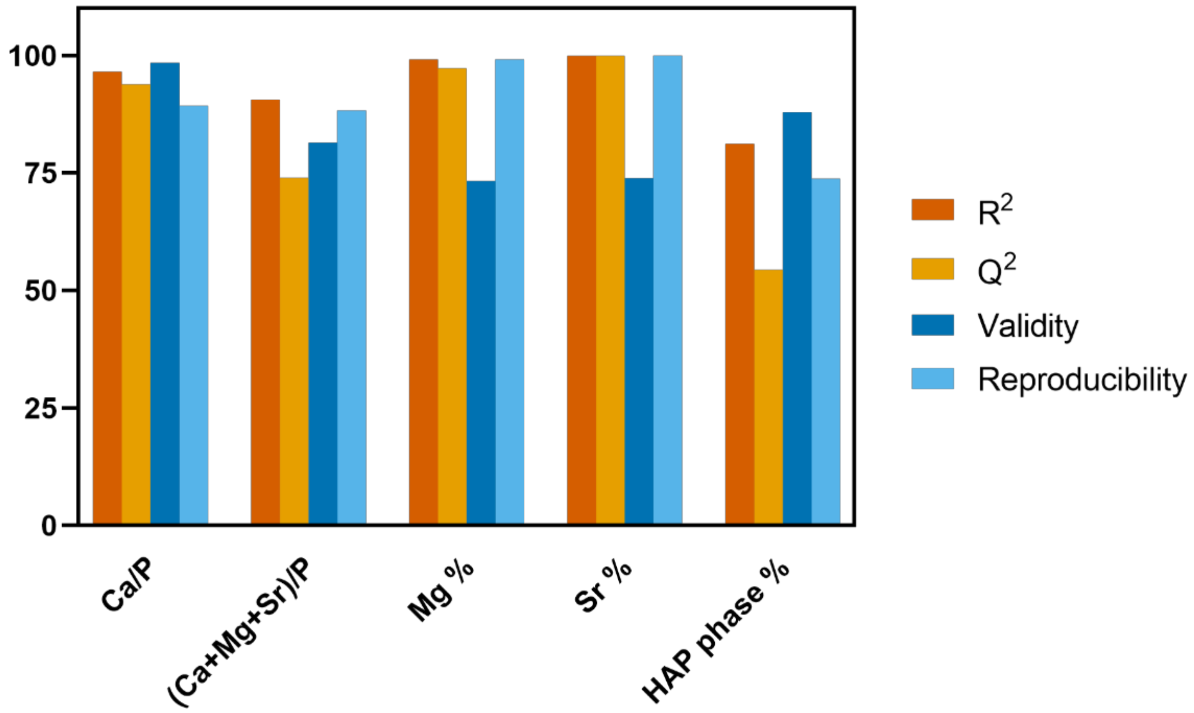
# Mg and Sr sHAP Synthesis – Design of Experiments

## Models: Summary of Fit



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# Chapter remarks

- It was possible to synthesise sHAP with different substitution degrees
- It was possible to model how synthesis conditions affect the composition and quality of sHAP

- Optimal synthesis conditions will be assessed after *in-vitro* testing
- Optimal sHAP will be used to develop sHAP/$TiO_2$ coatings

# Serum-free Osteogenic Differentiation of Mesenchymal Stem Cell Line

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# Objectives

Objectives

Comparing serum-free and serum-containing media

Assessing best osteogenic culture conditions

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# Background

## Why serum-free:

- Different sera have different compositions – **inconsistent results**
- Serum-free media have consistent compositions – **consistent results**
- Less ethical issues

## Why cell line:

- More consistent results
- Easier to culture
- Easier to passage without losing properties

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# Comparing serum-free and serum-containing media

## Methods:

- hTERT- MSCs Y201
  - Cell density: 4000 cells/cm$^2$
- Three different media
  - Volume: 300 µl
- Three media change protocols
  - O – 1 full change/week
  - H – 3 half changes/week
  - F – 3 full changes/week
- Three supplementation profiles

Cell culture media

| | |
|---|---|
| **BM3** | DMEM (GIBCO)<br>+ 10% FBS (GIBCO) |
| **CD1** | StemMACS™ MSC Expansion Media Kit XF, human (Miltenyi Biotec), serum-free and xeno-free |
| **HSM** | Human Mesenchymal-XF Expansion Medium (Merck), human-serum |

Supplementation profiles

| | |
|---|---|
| **EM** | No supplements |
| **OM1** | 1st media change – AA2P<br>2nd media change – βGP, Dex |
| **OM2** | 1st media change – AA2P, βGP, Dex |
| AA2P | L-Ascorbic Acid 2-phosphate (5 mg/ml) |
| βGP | Beta-glycerophosphate (0.5 M) |
| Dex | Dexamethasone (10 µM) |

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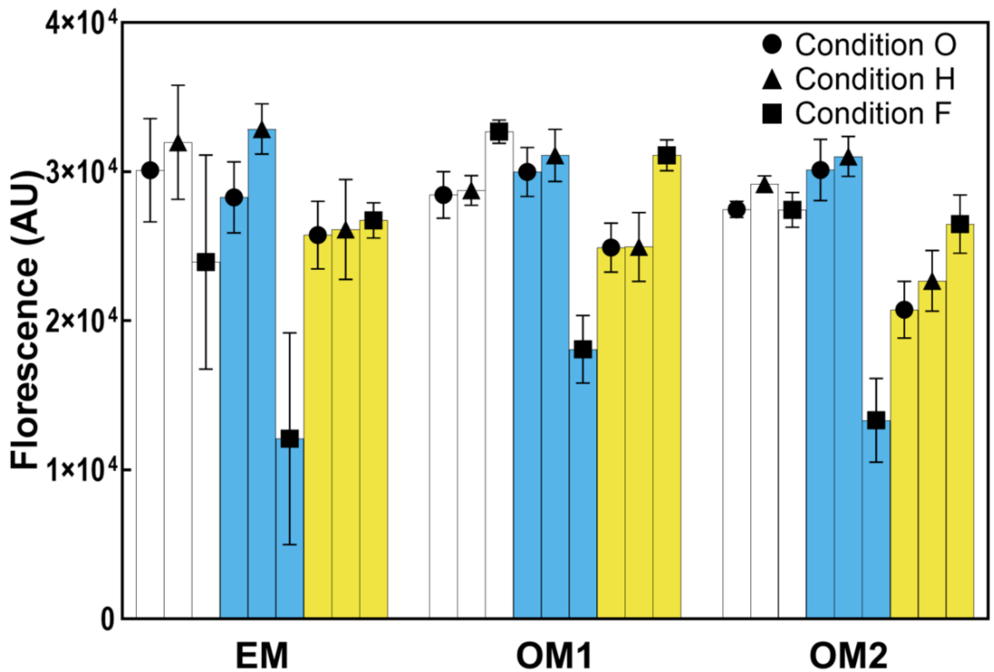
# Comparing serum-free and serum-containing media

## Results:

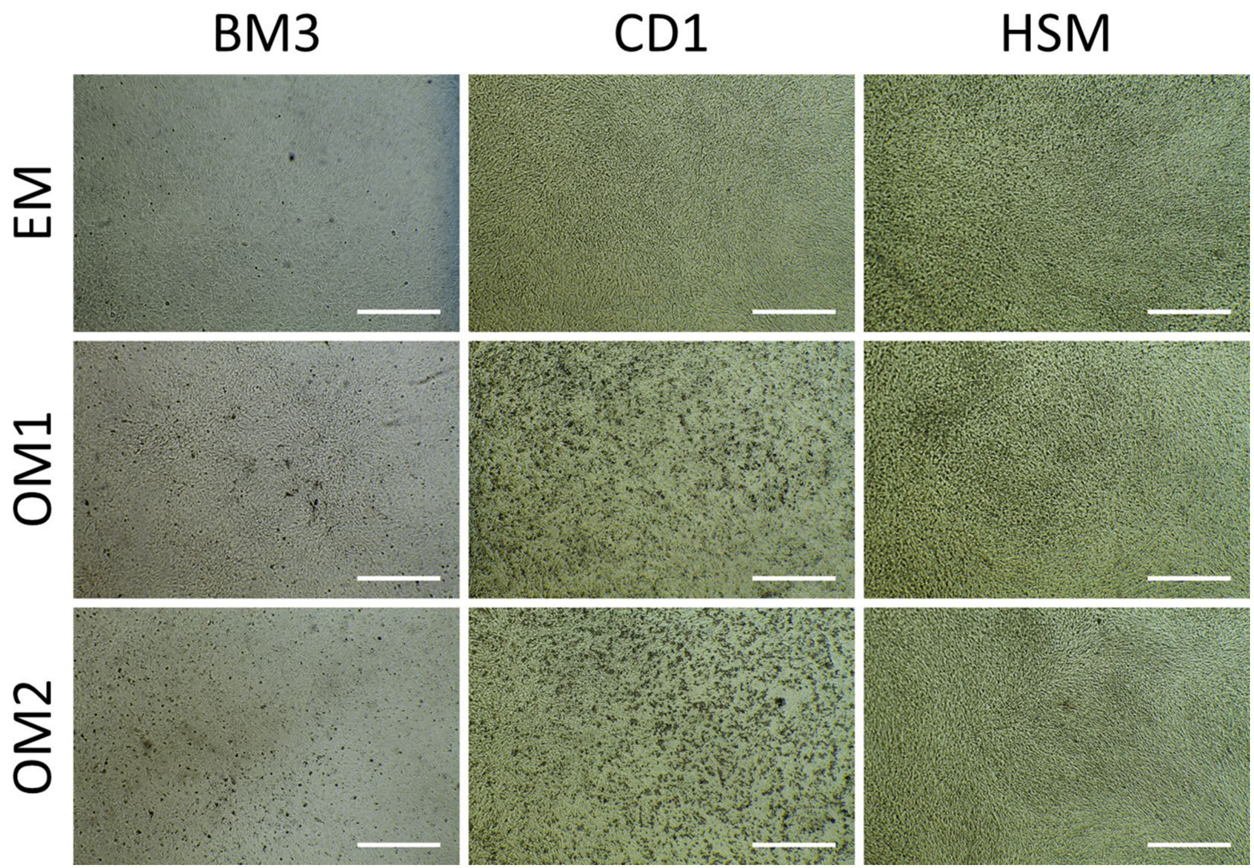



Metabolic activity of Y201 cells on day 21.

| | |
|---|---|
| **BM3** | DMEM (GIBCO)<br>+ 10% FBS (GIBCO) |
| **CD1** | StemMACS™ MSC Expansion Media Kit XF, human (Miltenyi Biotec), serum-free and xeno-free |
| **HSM** | Human Mesenchymal-XF Expansion Medium (Merck), human-serum |

# Comparing serum-free and serum-containing media

## Results:




Y201 cell at day 21, 1 media change/week. Scale bar = 500 μm.

# Assessing best osteogenic culture conditions

## Methods:

- hTERT- MSCs Y201
  - Cell density: 4000 cells/cm$^2$
- Media:
  - StemMACS™ MSC Expansion Media Kit XF, human (Miltenyi Biotec)
  - Volume: 400 µl
- Three supplementation profiles
- Two media change protocols

Supplementation profiles

| | |
|---|---|
| **EM** | No supplements |
| **OM1** | 1st media change – AA2P<br>2nd media change – βGP, Dex |
| **OM2** | 1st media change – AA2P, βGP, Dex |
| AA2P | L-Ascorbic Acid 2-phosphate (5 mg/ml) |
| βGP | Beta-glycerophosphate (0.5 M) |
| Dex | Dexamethasone (10 µM) |

Media change protocols

| | |
|---|---|
| **O** | 1 full media change/week |
| **H** | 3 half media changes/week |

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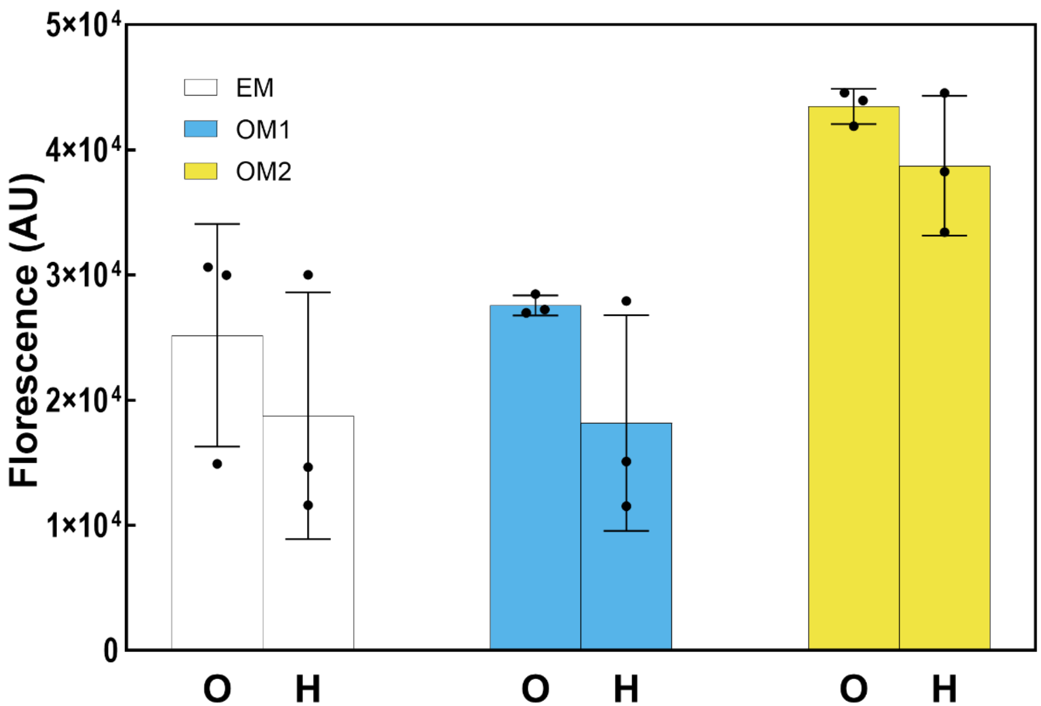
# Assessing best osteogenic culture conditions

## Results:



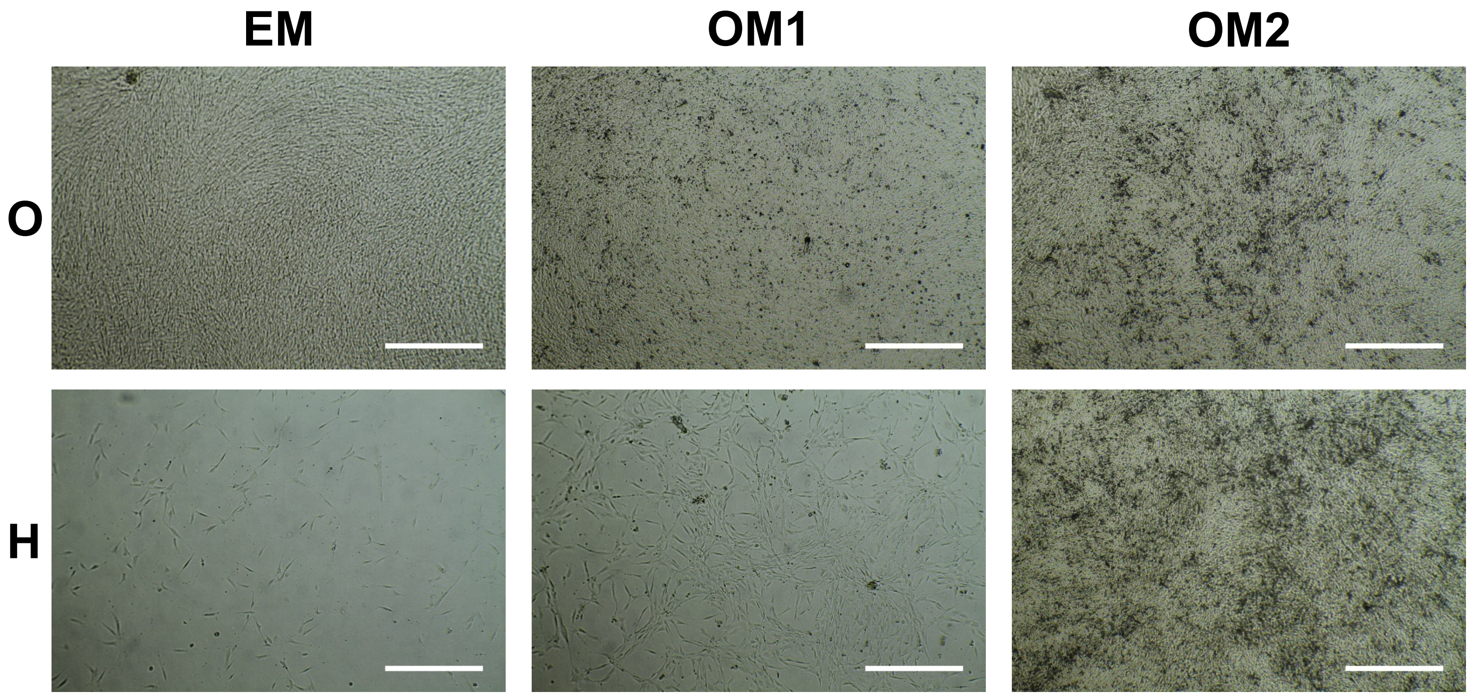

Metabolic activity of Y201 cells on day 21.

Media change protocols

| | |
|---|---|
| **O** | 1 full media change/week |
| **H** | 3 half media changes/week |

# Assessing best osteogenic culture conditions

## Results:




Y201 cell at day 21. Scale bar = 500 μm.

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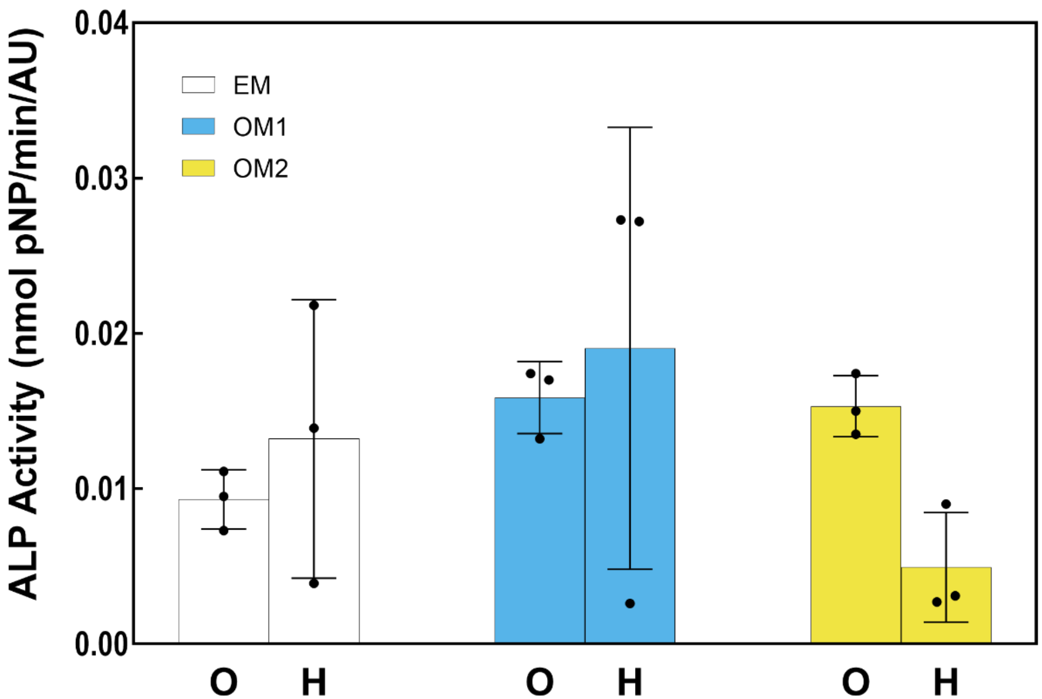
# Assessing best osteogenic culture conditions

## Results:




Media change protocols

| | |
|---|---|
| **O** | 1 full media change/week |
| **H** | 3 half media changes/week |

Alkaline phosphatase activity of Y201 cells on day 14.

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# Chapter remarks

- Serum-free media is a good alternative to media supplemented with serum
- 1 media change/week allows for cells to remain viable with lower risk of cell detachment
- Any supplementation profile studied promotes differentiation of hTERT-MSCs Y201

- CD1 with OM2 supplementation and 1 media change/week will be used as positive control

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# Project Summary

- Sol-Gel synthesis of $TiO_2$ has been optimized
- Synthesis of sHAP has been modelled
- In-vitro testing conditions have been setted

- Test synthesised sHAP *in-vitro*
- Study development of $TiO_2$/sHAP using DoE
- Determine optimal coating conditions for different substrates

# Aknowledgements

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- Claudio De Luca

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- Denata Syla
- Chloé Techens
- Jennifer Fayad
- Marco Sensale
- Cameron James

# THANK YOU

## ANY QUESTIONS?

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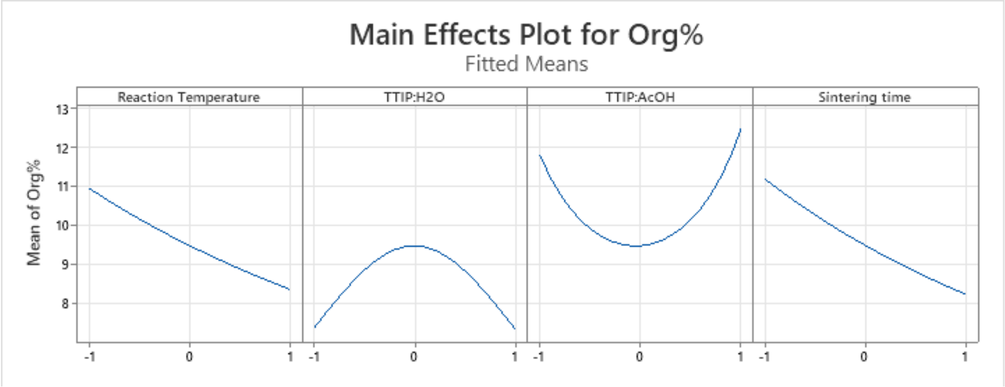
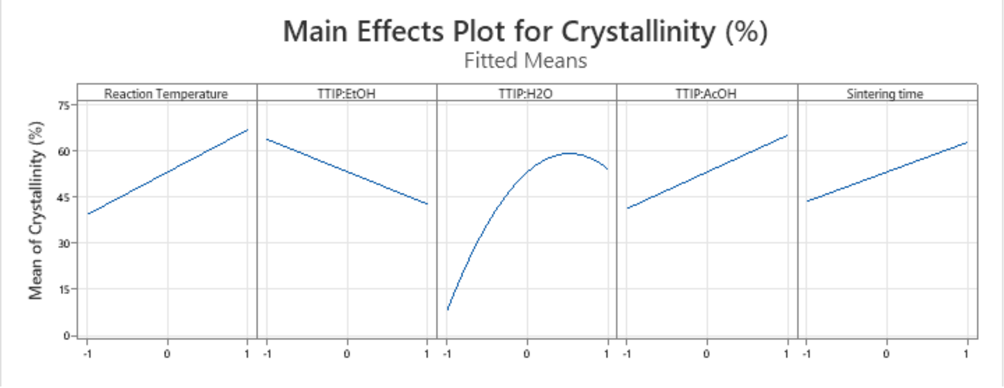
# $TiO_2$ synthesis optimization

## Optimizing factor C (Ultrapure water volume)




### Org (%)

- Lower when C = 1 or C = -1

### Crystallinity (%)

- Higher when C = 0.52
- Still high when C = 0 or C = 1

### Optimized C = 1

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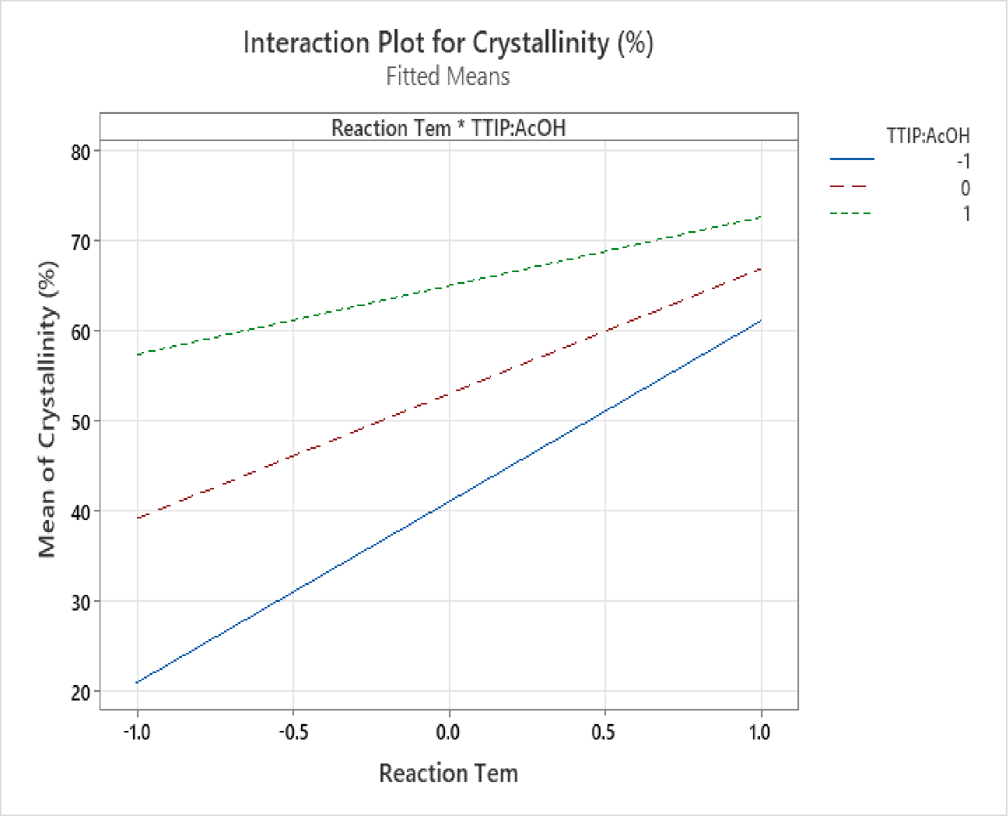
## Optimizing Factor D (Acetic acid volume)




**Organic Residues (%)**

Lower when D = 0

Highest when D =1

**Crystallinity (%)**

Higher when D = 1

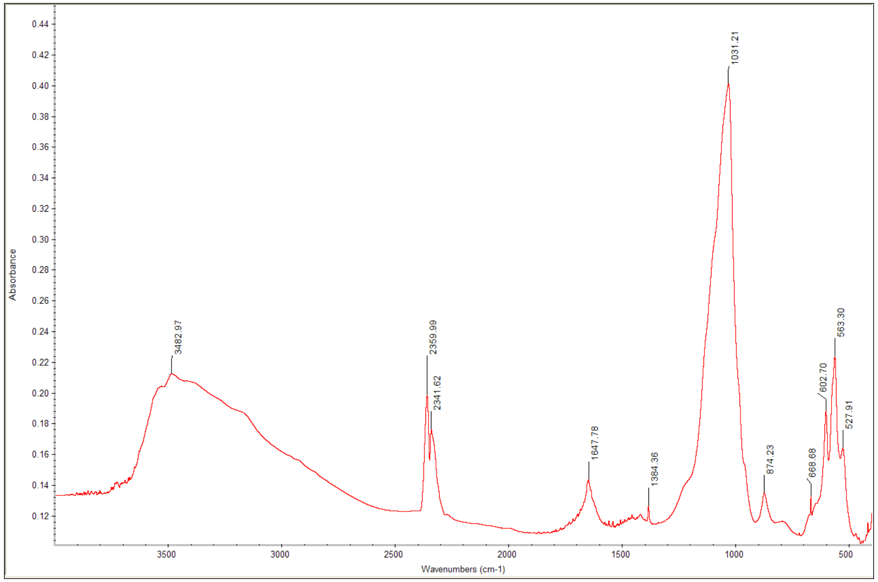
Interaction with A also significant, and higher when D = 1

**Check responses when $0 \leq D \leq 1$**

# sHAP Process optimization

## Methods:

- Synthesised formulation C6
- pH monitored during synthesis
- pH monitored after synthesis for 1 hour
- Ammonia solution 28% added when pH < 10




FTIR spectrum of pure sHAP C6, no ammonia.

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# Mg and Sr sHAP Synthesis – Design of Experiments

## Results:

| Standard Order | A | B | C | Ca/P | Ca+Mg+Sr/P | Mg % | Sr % | HAP Phase % |
|---|---|---|---|---|---|---|---|---|
| 1 | - | - | - | 1.46 | 1.60 | 4.09 | 4.81 | 97.4 |
| 2 | + | - | - | 1.36 | 1.53 | 6.49 | 4.89 | 93.5 |
| 3 | - | + | - | 1.34 | 1.51 | 2.49 | 8.79 | 97.0 |
| 4 | + | + | - | 1.24 | 1.42 | 3.98 | 8.82 | 80.1 |
| 5 | - | - | + | 1.48 | 1.66 | 5.97 | 4.99 | 100.0 |
| 6 | + | - | + | 1.38 | 1.63 | 10.32 | 4.92 | 92.4 |
| 7 | - | + | + | 1.39 | 1.63 | 5.59 | 8.79 | 100.0 |
| 8 | + | + | + | 1.27 | 1.58 | 10.34 | 8.87 | 90.0 |
| 9 | 0 | 0 | 0 | 1.34 | 1.61 | 7.35 | 6.82 | 91.4 |
| 10 | 0 | 0 | 0 | 1.38 | 1.57 | 7.77 | 6.90 | 95.6 |
| 11 | 0 | 0 | 0 | 1.34 | 1.57 | 7.68 | 6.87 | 90.7 |

| Factor | Description | - | 0 | + |
|---|---|---|---|---|
| A | Mg substitution degree (%) | 5 | 7.5 | 10 |
| B | Sr substitution degree (%) | 5 | 7.5 | 10 |
| C | Ammonia solution 28% volume (ml) | 15 | 32.5 | 50 |

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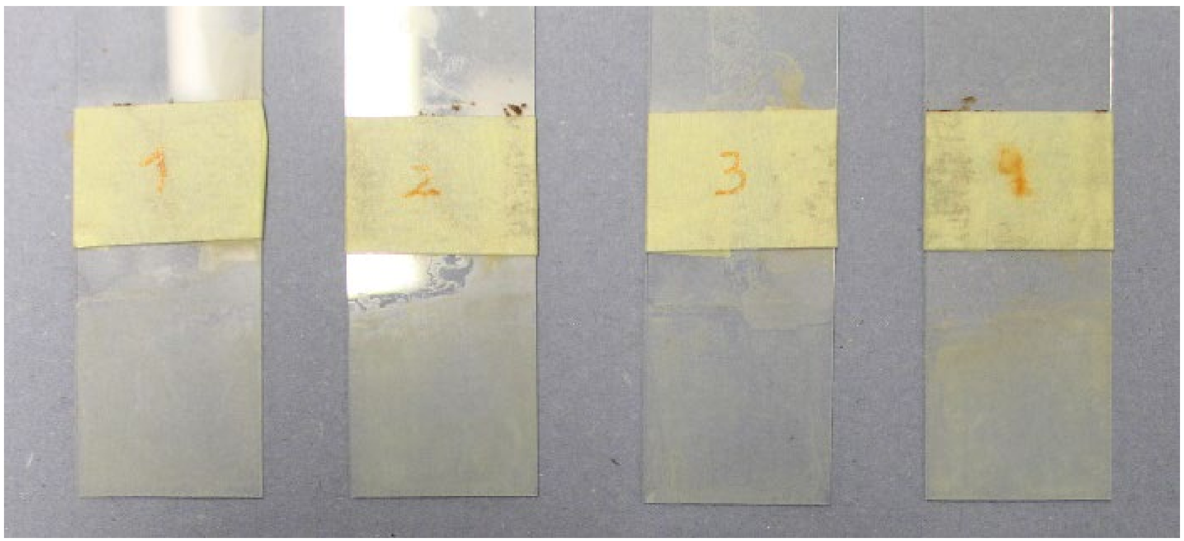
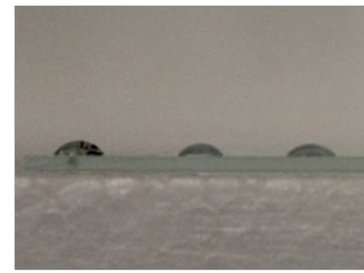
# TiO2/sHAP coatings – Preliminary work




Uncoated Glass



$TiO_2$/sHAP coating