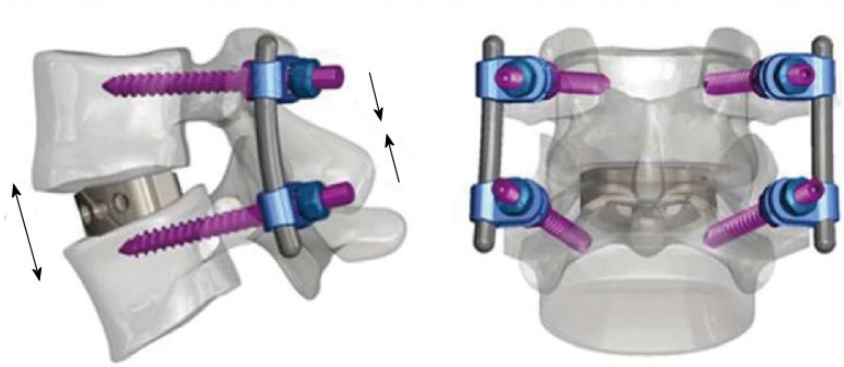
Establishing optimal substitution degrees of hydroxyapatite (HAP) with magnesium and strontium using experimental and statistical tools

ESR1: Denata Syla

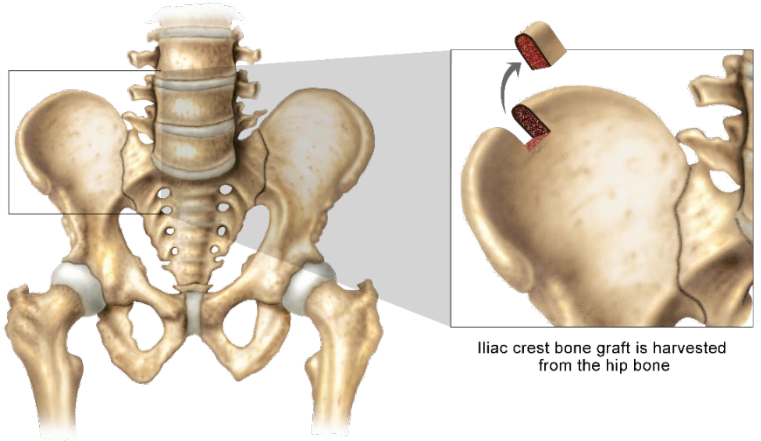
Background: Spinal fusion

- Surgical procedure to treat back pain
- Discectomy of diseased intervertebral disc
- Insertion of an implant (spacer/cage) in intervertebral space

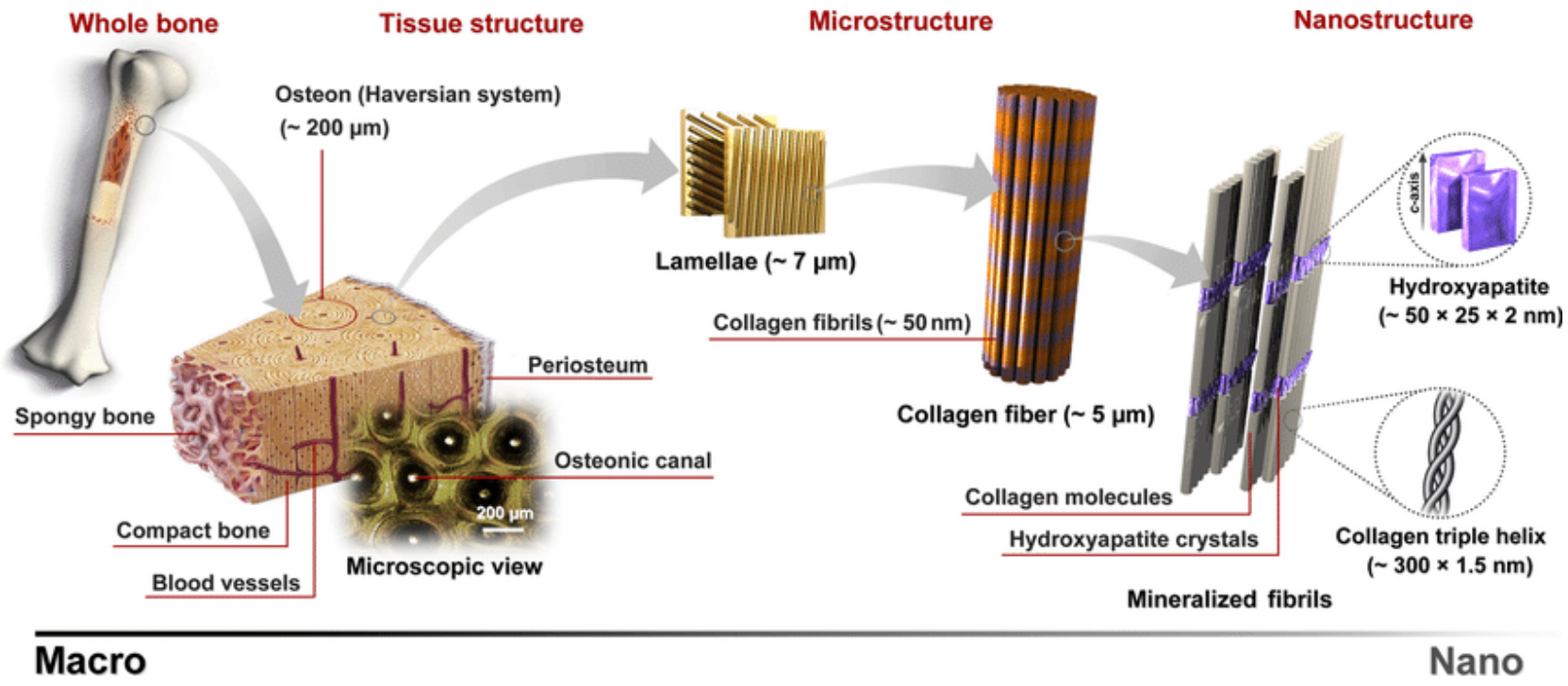


Background: Spinal fusion

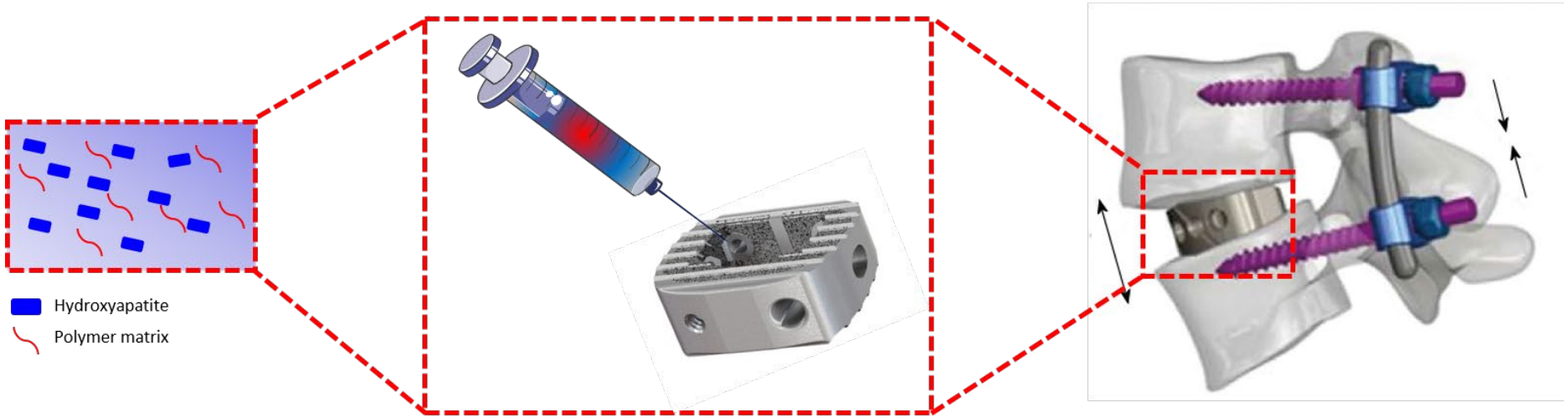
- Surgical procedure to treat back pain
- Discectomy of diseased intervertebral disc
- Insertion of an implant (spacer/cage) in intervertebral space
- Filling of implant: with autograft as current standard
- Fixation with screws



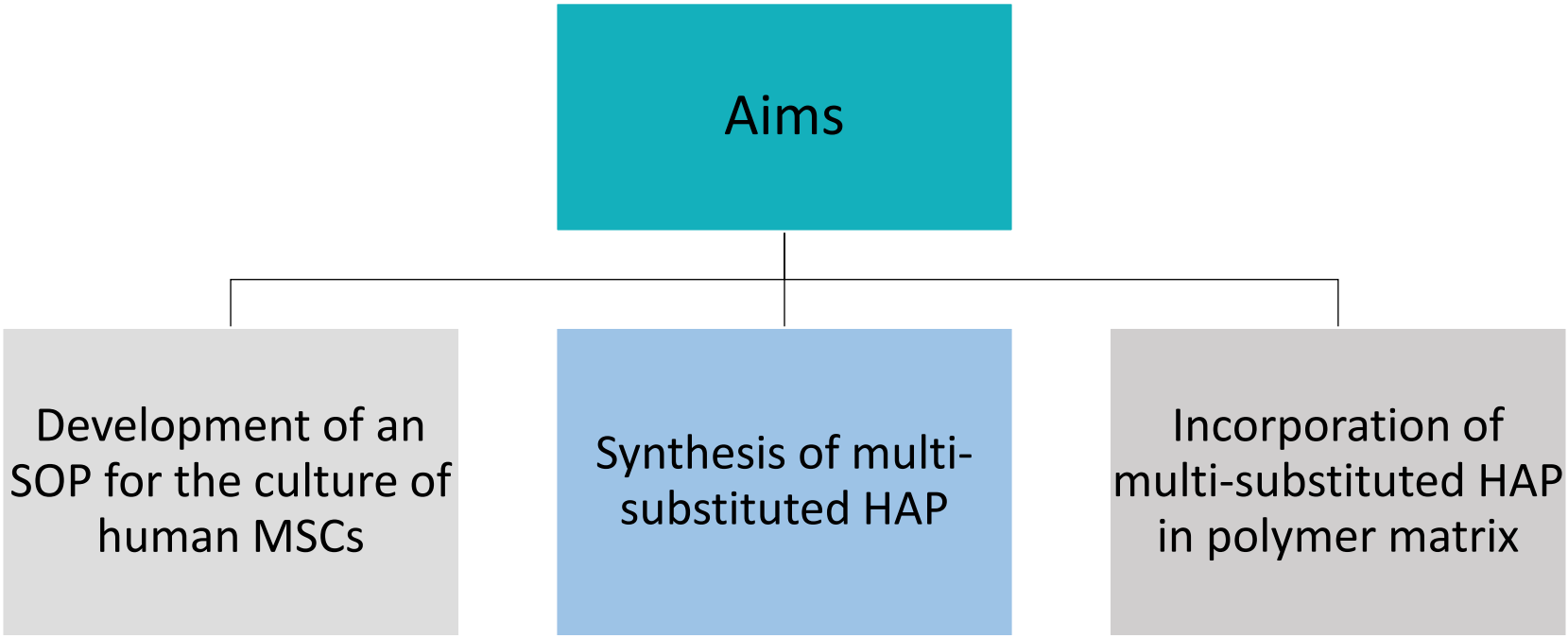
Background: Bone structure



Project Overview



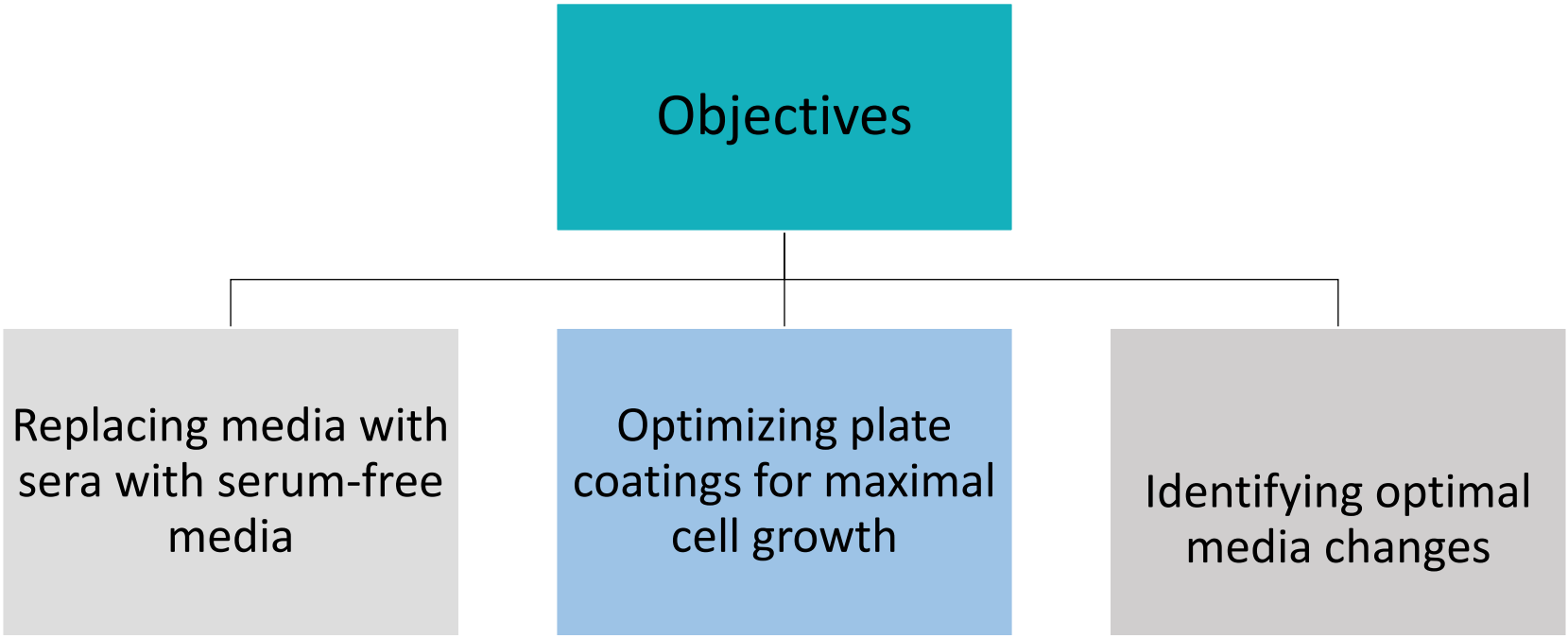
Aims


Aims
Development of an SOP for the culture of human MSCs
Synthesis of multi-substituted HAP
Incorporation of multi-substituted HAP in polymer matrix

Developing an SOP for the growth of Y201 in serum and xeno free conditions

Chapter#1

Objectives


Objectives
Replacing media with sera with serum-free media
Optimizing plate coatings for maximal cell growth
Identifying optimal media changes

Background

- The lack of uniformity in sera compositions → variable and inconsistent *in-vitro* cell behaviour
- Serum-free media → more consistent performance and avoid masking of biological tests
- Lower risk of contamination and disease transmission
- Ethical consideration

Methods

- Immortalized human mesenchymal stem cell line (hTERT-MSC Y201) used at density of 4000 cells/cm^2
- Well-plates were non-coated or coated with different substrates
- Media changes varied in different groups
- Cells were analysed with light microscopy and metabolic resazurin reduction assay on day 1, day 3 and day 7

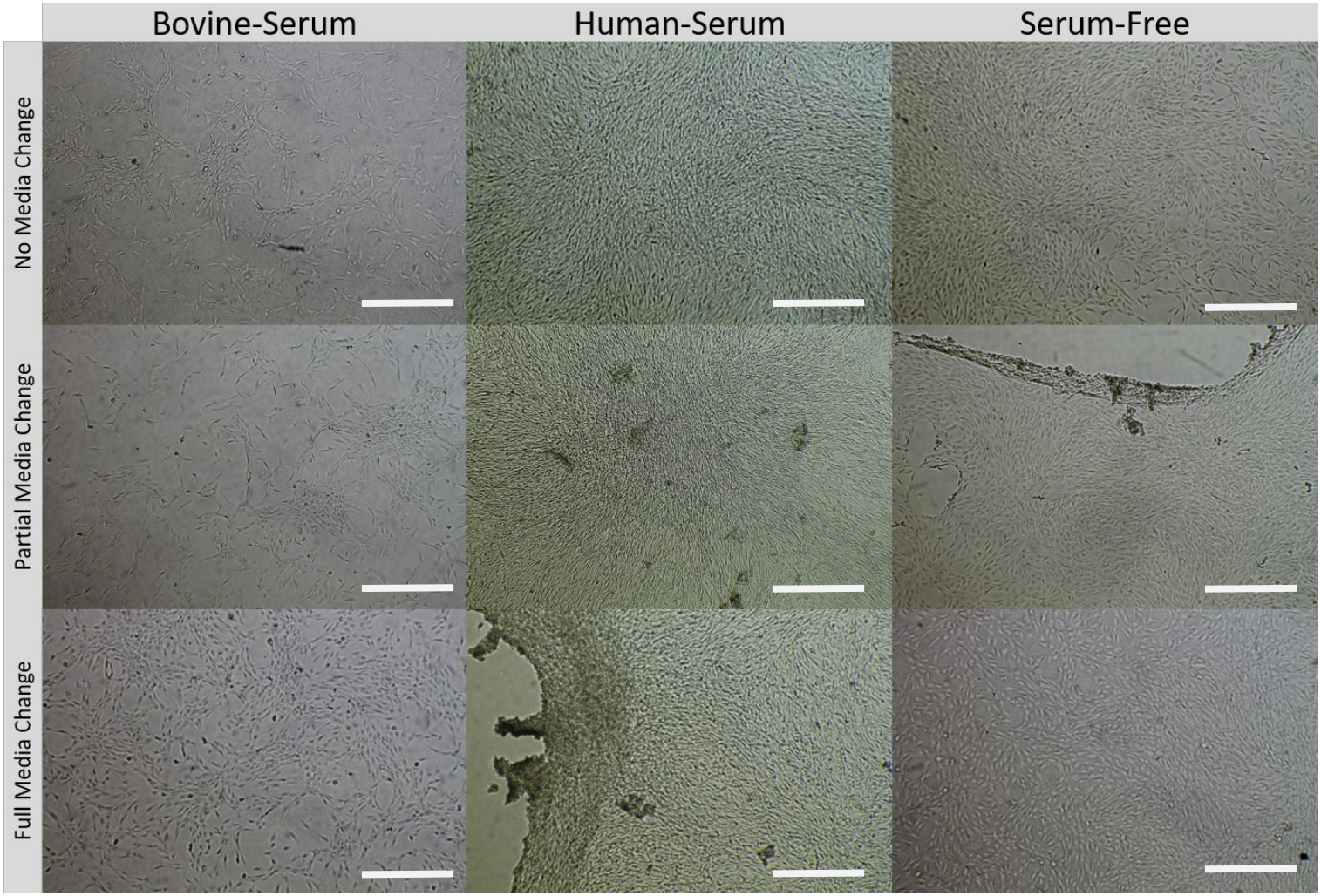
Methods

Media	Composition	Sera Supplements
BM3	DMEM (GIBCO) + 10% FBS (GIBCO)	Bovine Serum
CD1	StemMACSTM MSC Expansion Media Kit XF, human (Miltenyi Biotec), serum-free and xeno-free	Serum free
HSM	Human Meschencymal-XF Expansion Medium (Merck), human-serum	Human Serum

Code	Media Change
N	None
P	Partial
F	Full

Code	Coatings
NS	No Coating
PS	Fibronectin Coating
FS	Gelatin Coating

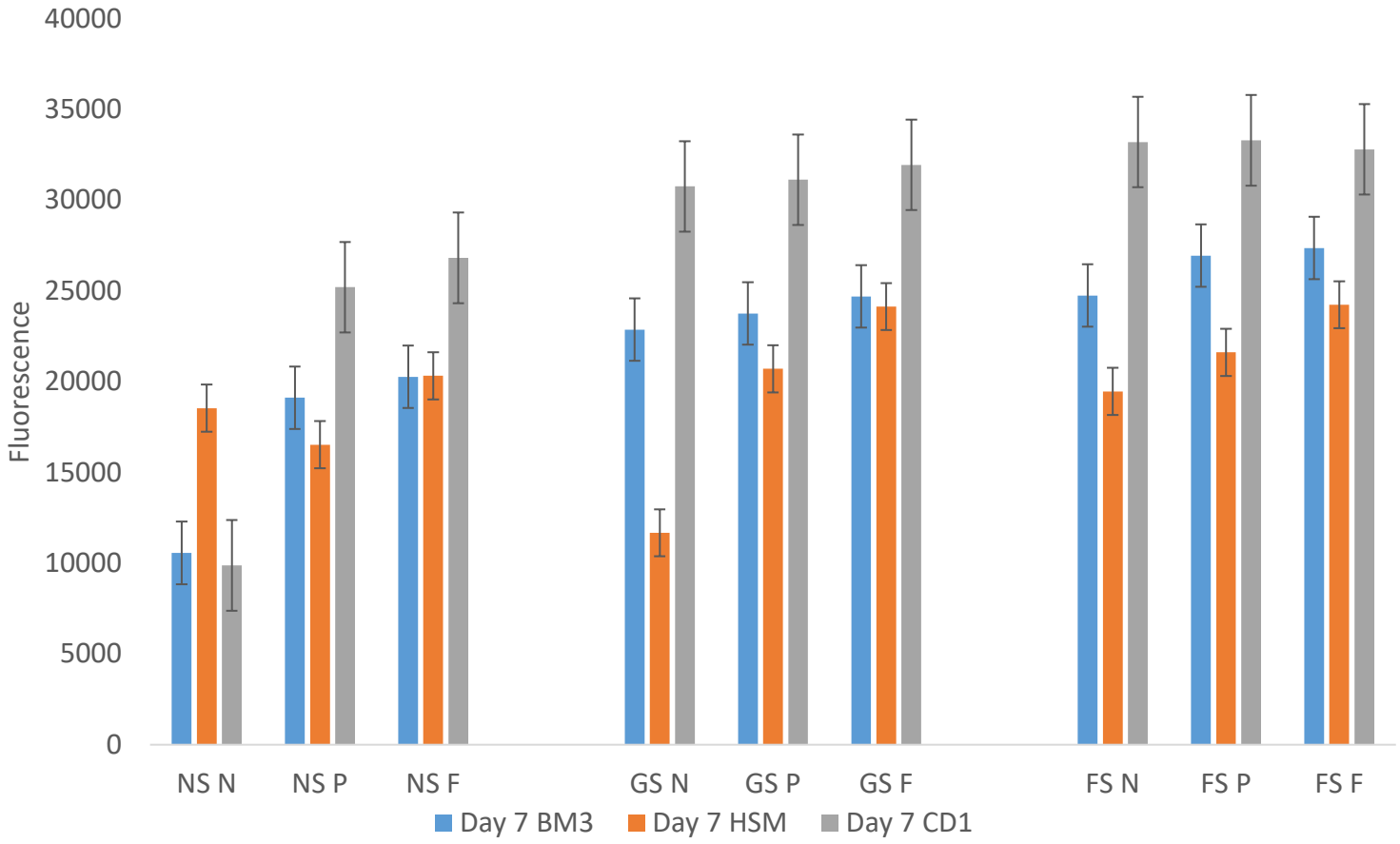
Results



Human MSCs (Y201) on day 7 Scale bar = 500 µm.

Results

Human MSCs metabolic activity on day 7



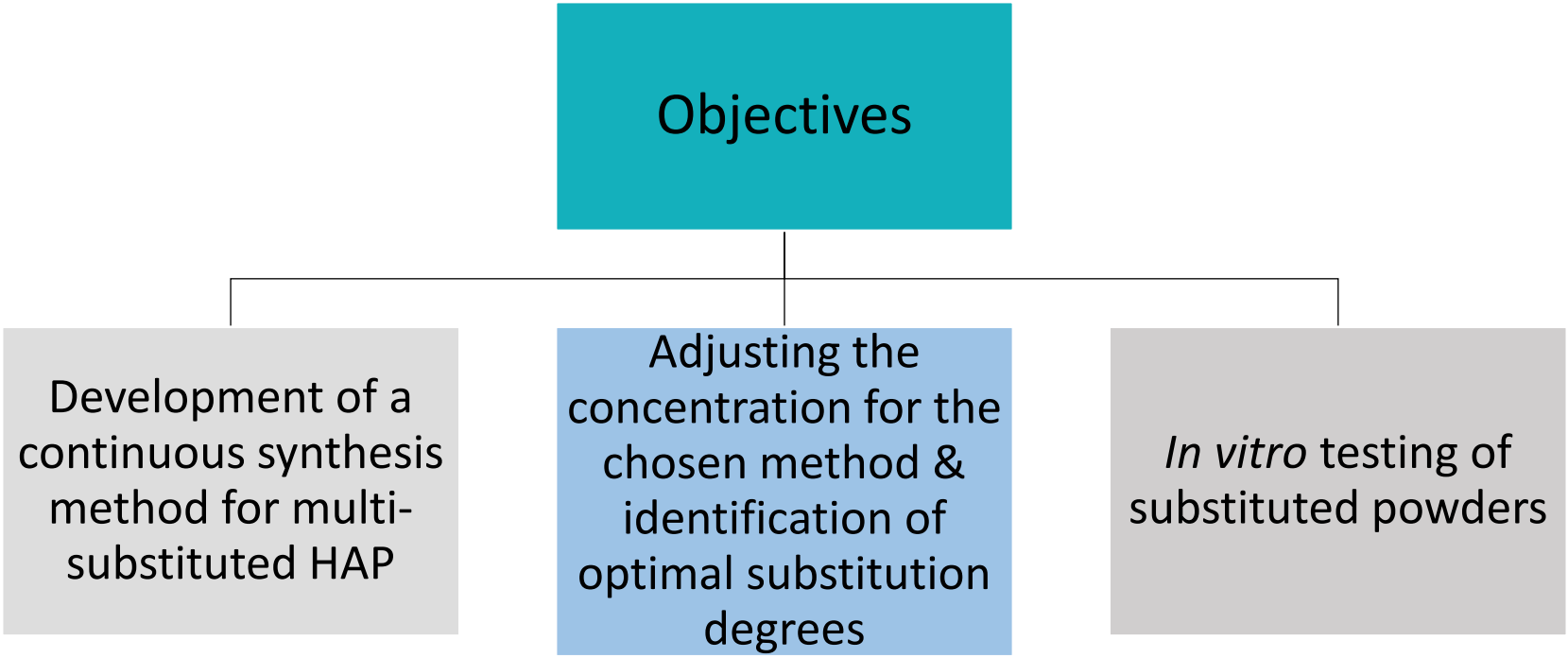
Conclusion

- Serum free medium sustains high cell growth
- No coatings are necessary for cell growth
- Partial media change seems is the best condition for cell growth

Establishing optimal substitution degrees of hydroxyapatite with magnesium and strontium using experimental and statistical tools

Chapter#2

Objectives


Objectives
Development of a continuous synthesis method for multi-substituted HAP
Adjusting the concentration for the chosen method & identification of optimal substitution degrees
In vitro testing of substituted powders

Background: What is hydroxyapatite

Physiological "HAP"	Stoichiometric HAP
• Physiological hydroxyapatite (HAP) is a bone mineral • Makes 70% of bone volume with other inorganic components • Gives bone its hardness and rigidity • Molar ratio Ca/P: 1.5 - 1.67 • Naturally substituted	• Synthetic nanocrystal • High biocompatibility and osteoinductive properties *in vitro* • Molar ratio Ca/P: 1.67 • Can be doped (=substituted) with various atoms

Background: Substituents for hydroxyapatite

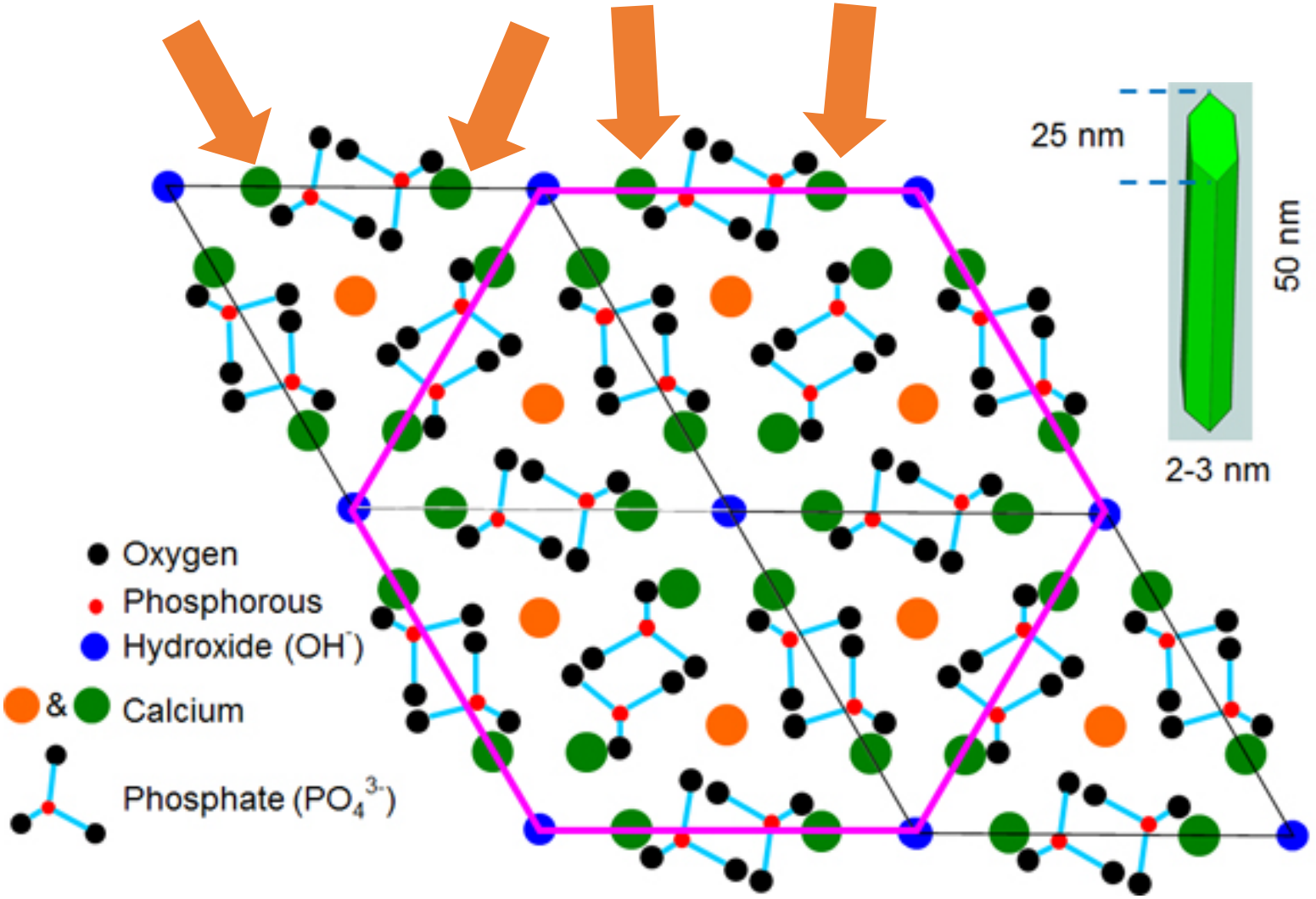
❖Magnesium:

- Involved in bone metabolism
- Lack of magnesium in in vivo models leads to reduction of osteoblast and osteocyte activity, osteopenia and bone fragility and bone growth impairment
- Increases solubility of HAP

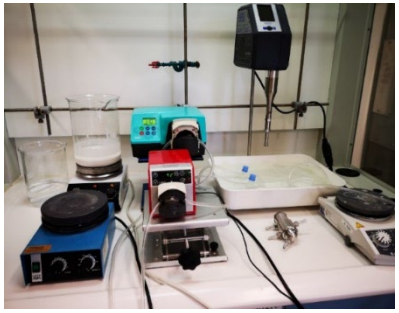
❖Strontium:

- Sr physiologically presented in bone
- Increases bone formation and bone mineral density
- In vitro used for the treatment of osteoporosis in low amounts
- Increases solubility of HAP

Background: Structure of stoichiometric HAP

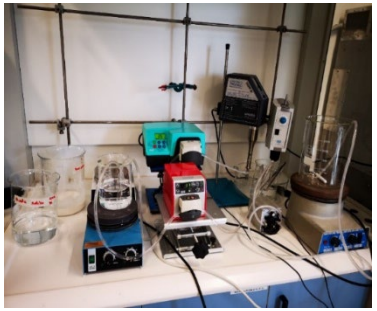


Methods: Previous synthesis systems



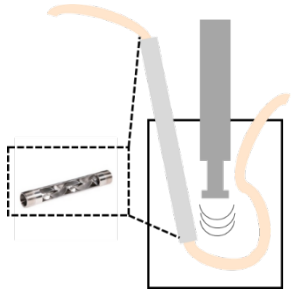
Mixing System via glass Y-tube:

- Material clogs → Synthesis does not work



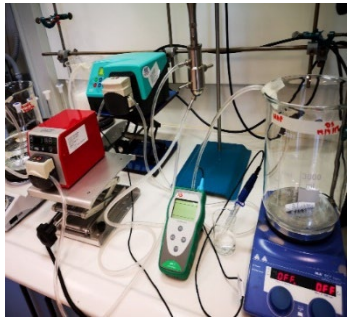
Manual Mixing in a Beaker:

- Material clogs → Synthesis does not work
- Mixing is not sufficient
- Phase separation



Column: Small

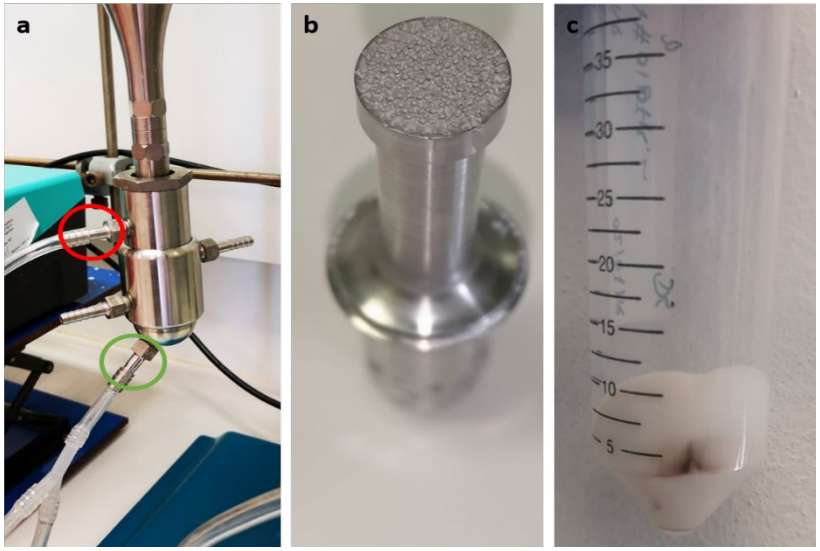
- Material clogs → Synthesis does not work
- Mixing is not sufficient
- Phase separation



Flow cell method

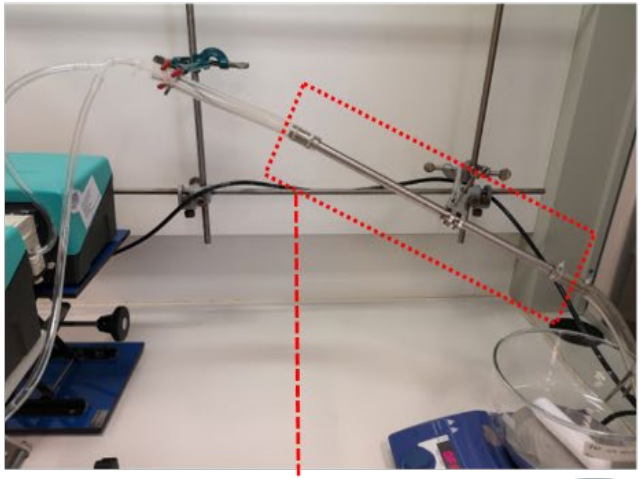
- Synthesis successful BUT particle release

Methods: Previous synthesis systems

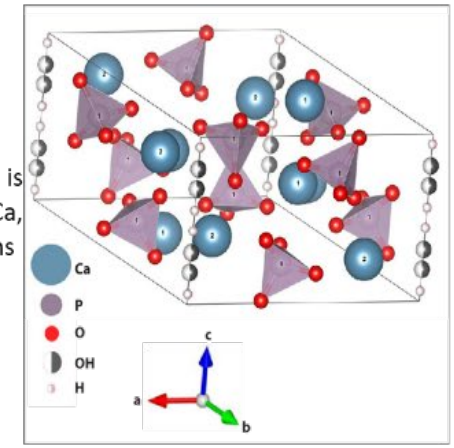


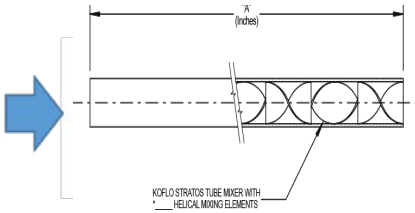
Synthesis successful BUT particle release

Continuous flow method for HAP synthesis



Natural hydroxyapatite is composed of Ca, P, OH and H ions

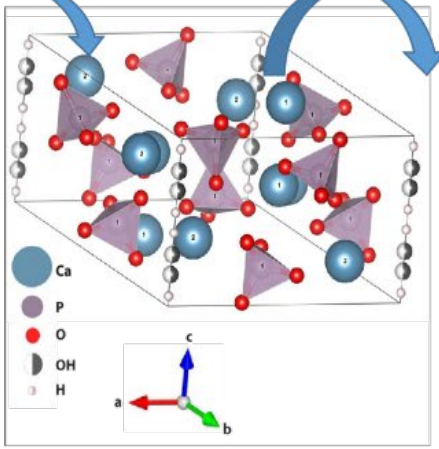




Laminar flow in the column leads to precipitation of substituted hydroxyapatite

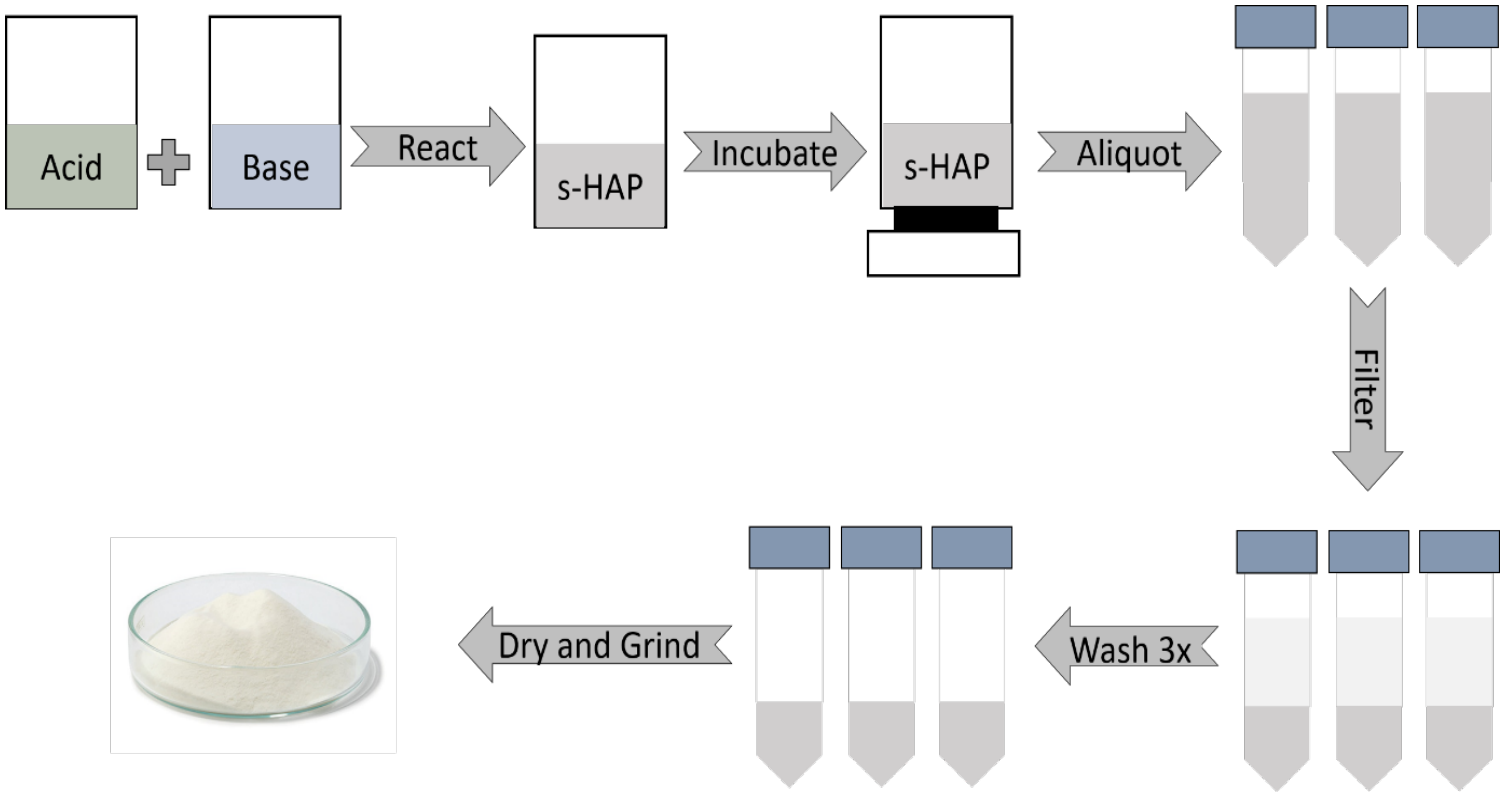
Sr +Mg

Ca



In the chemical structure of hydroxyapatite, Ca is removed and substituted with Mg and Sr ions

Methods: HAP processing



S-HAP = substituted hydroxyapatite

Methods: Ca molar concentrations & substitution degrees

Molar concentration of Ca [mol/L]	Problem	Outcome
1.35	High viscosity	Rejected
1.15	Different apatites produced	Rejected
1.01	Very high viscosity and high pressure	Rejected
0.54	Lower throughput	Accepted

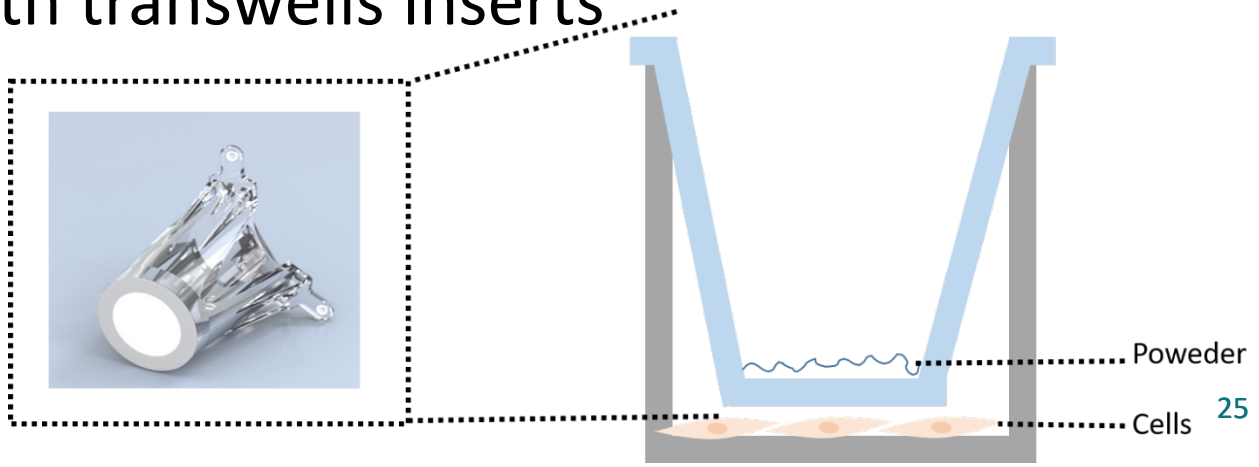
Composition [mol%]	Formulation			
	1	2	3	4
Ca	60	75	75	90
Mg	20	5	20	5
Sr	20	20	5	5

Methods: Testing of powders in vitro

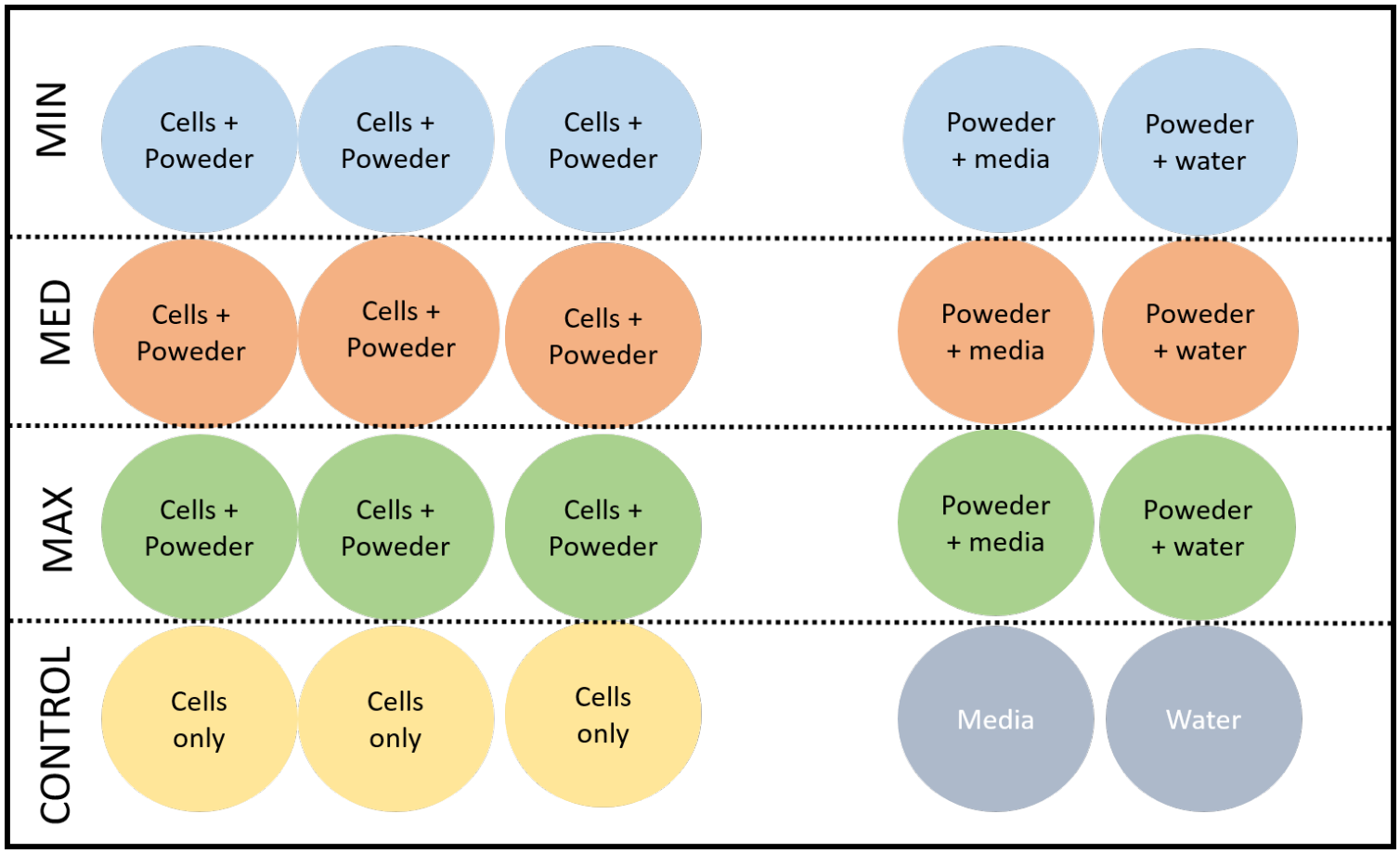
- MgHAP (Sintlife®used)
- Concentrations/ml media:

Levels	Concentration [µg]
Min	100
Med	505
Max	1000
Control	0

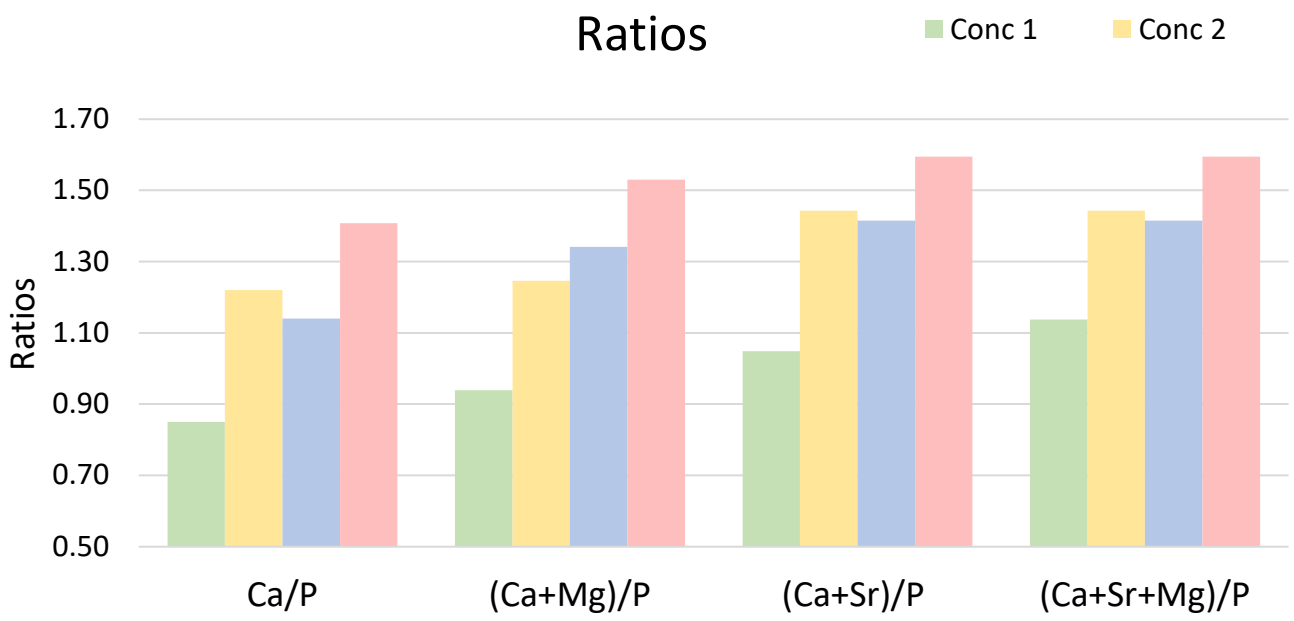
- Testing with transwells inserts



Methods: Testing of powders in vitro



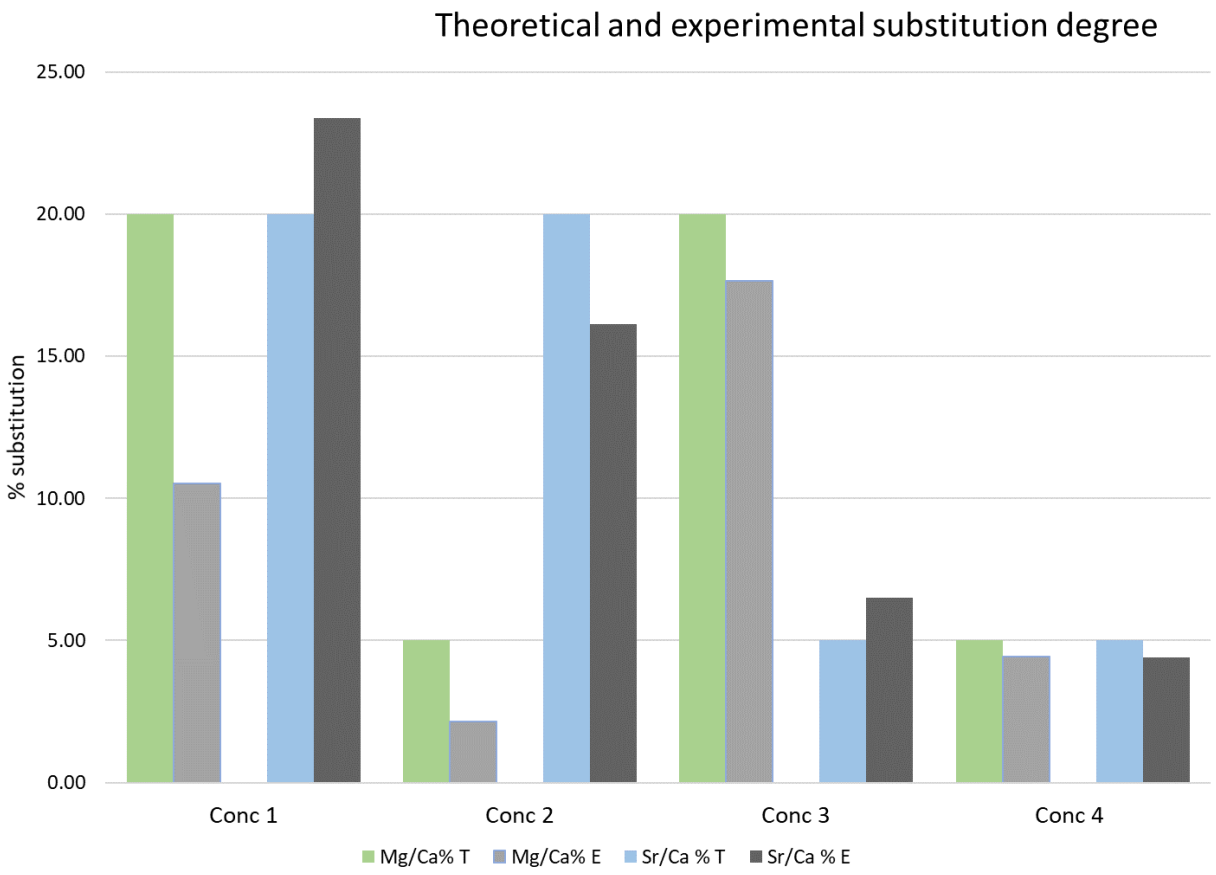
Results: Ratios with ICP Analysis



Conc	Mg	Sr
Conc1	20	20
Conc2	5	20
Conc3	20	5
Conc4	5	5

Single sample analysis

Results: Element Incorporation with ICP Analysis



Conc	Mg	Sr
Conc1	20	20
Conc2	5	20
Conc3	20	5
Conc4	5	5

Results: XRD Results

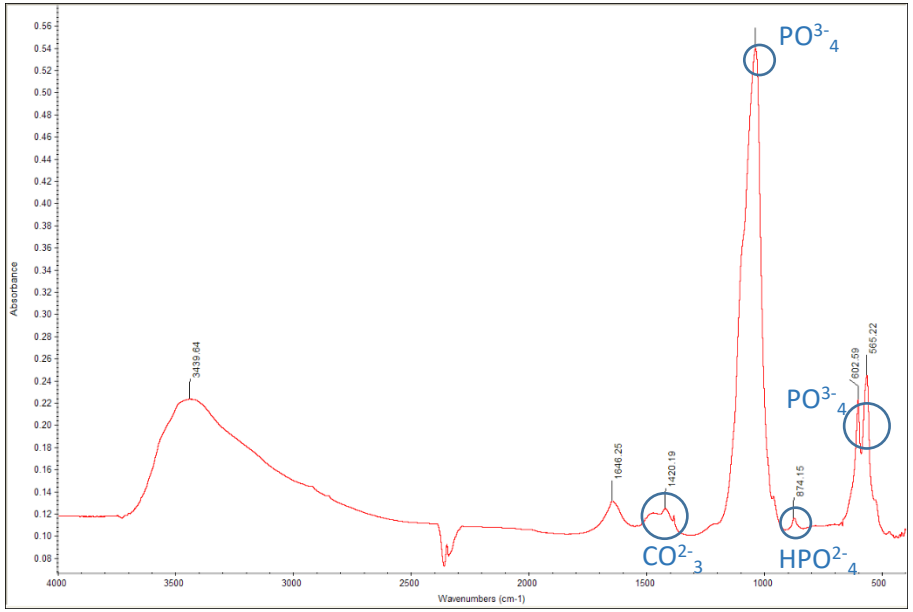
Concentration	1	2	3	4
Brushite	72.4%	-	19.4%	23.2%
Amorphous phase	27.6%	-	71.2%	
HAP	-	100%	9.4%	76.8%

Brushite = Calcium phosphate crystal: $CaHPO_4 \cdot 2H_2O$, Ca/P ratio = 1

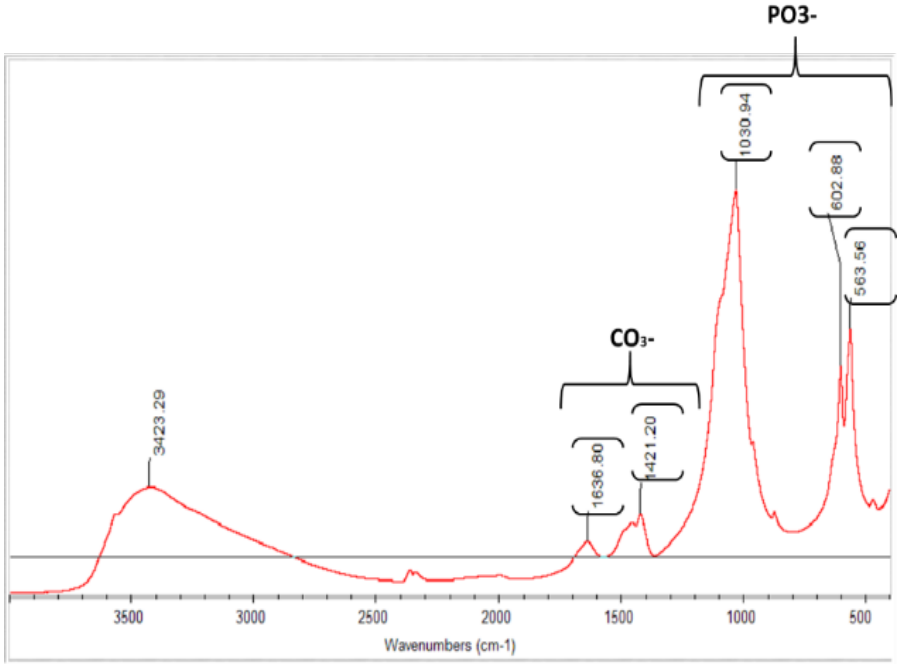
Amorphous phase = Calcium phosphate with lacking characteristic of a crystal: $Ca_9(PO_4),_6xH_20$, Ca/P ratio = 1.5

Results: FTIR

- Shows similar results as Finceramicas product Sintlife

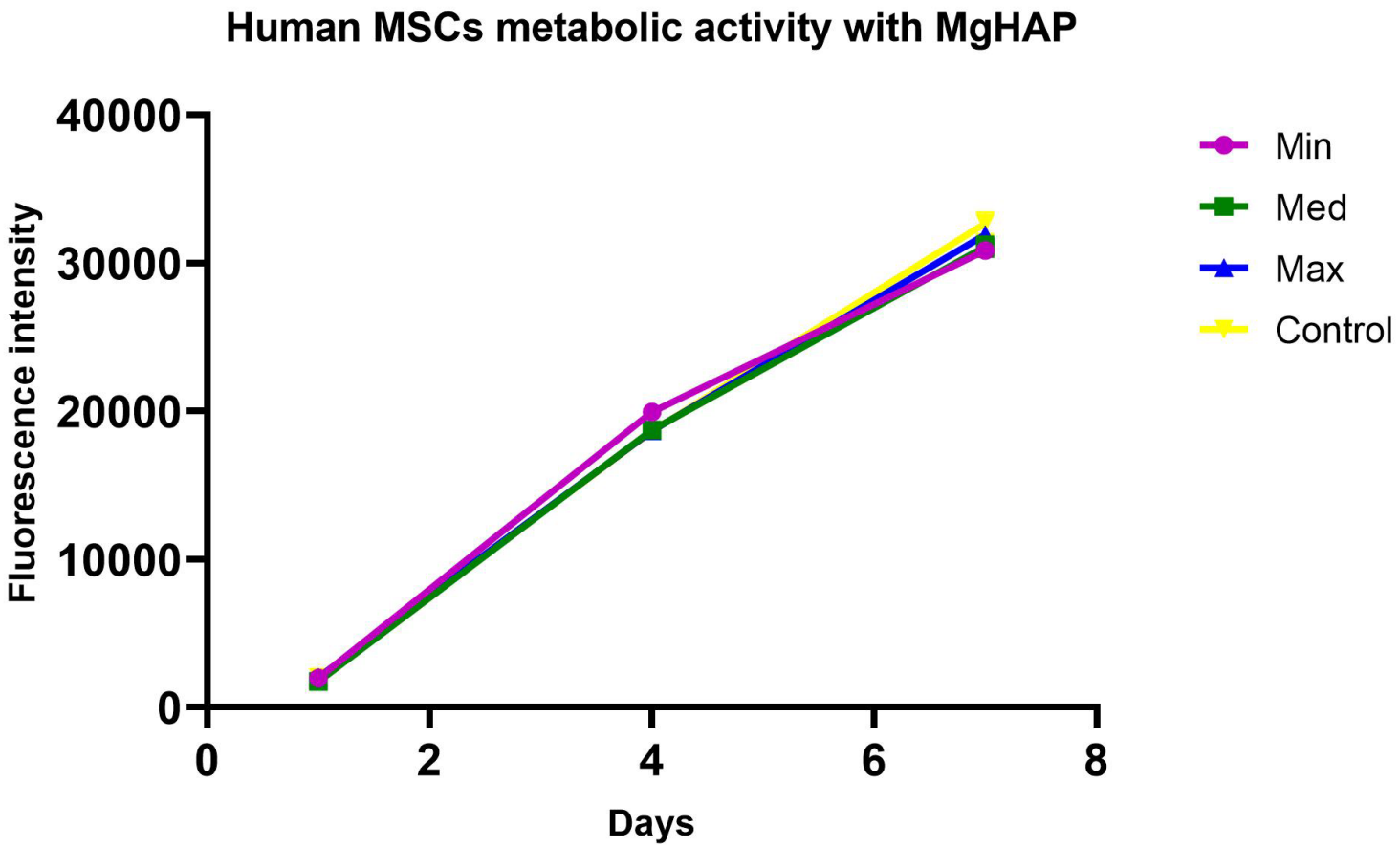


FTIR of Conc 4



FTIR of Sintlife

Results: Powders



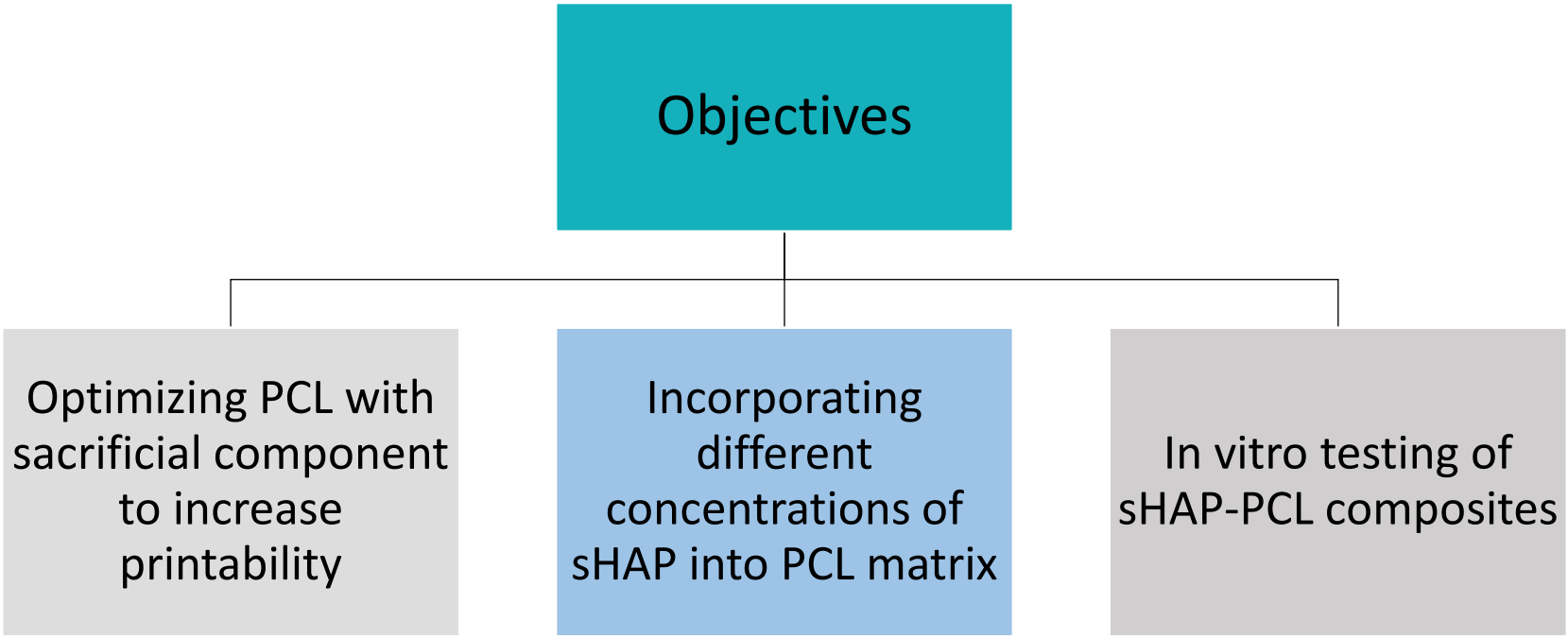
Conclusion

- Our continuous method is suitable for the synthesis for substituted HAP
- Increasing substitution degree leads to lower incorporation of Ca and substituents
- Substituted hydroxyapatite seems to not be toxic to human MSCs in the used concentrations

Incorporating sHAP in Polycaprolactone (PCL) matrix as carrier system for spinal fusion cages

Chapter#3

Objectives

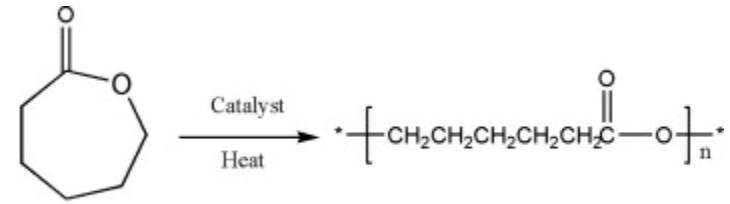

Objectives
Optimizing PCL with sacrificial component to increase printability
Incorporating different concentrations of sHAP into PCL matrix
In vitro testing of sHAP-PCL composites

Background

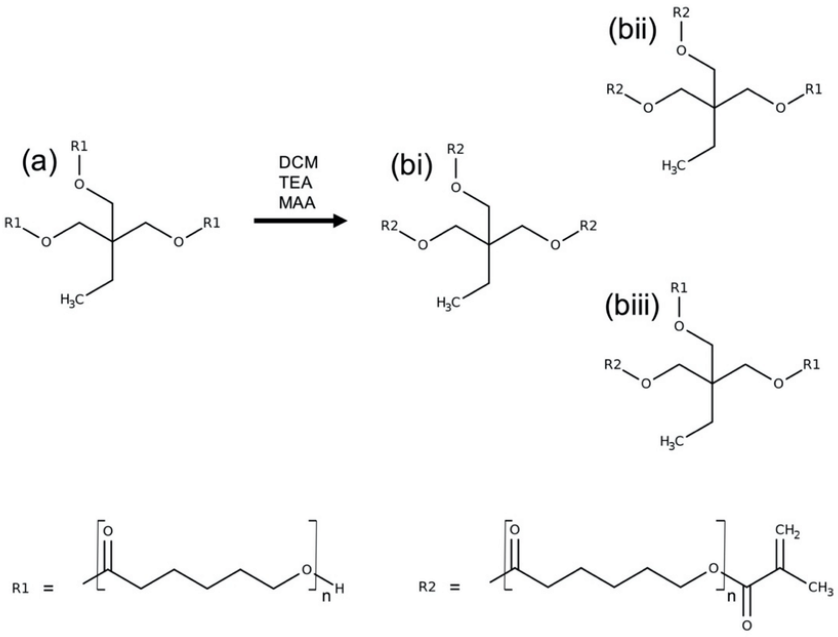
- PCL is a biodegradable polymer, it is non-toxic and tissue compatible
- The degradation time of PCL is 2–3 years and it is degraded by microorganisms or by hydrolysis
- Chemical and biological properties, physicochemical state, degradability and mechanical strength can be adjusted

Methods: Synthesis of PCL

1. Synthesis of PCL from monomers



2. Methacrylation of PCL
   - ❖To make it photo curable



Methods: Synthesis of PCL

› Molecules. 2021 Feb 24;26(5):1199. doi: 10.3390/molecules26051199.

A Tuneable, Photocurable, Poly(Caprolactone)-Based Resin for Tissue Engineering-Synthesis, Characterisation and Use in Stereolithography

Jonathan Field [1], John W Haycock [2 3], Fiona M Boissonade [1 3], Frederik Claeyssens [2 3]

Affiliations + expand

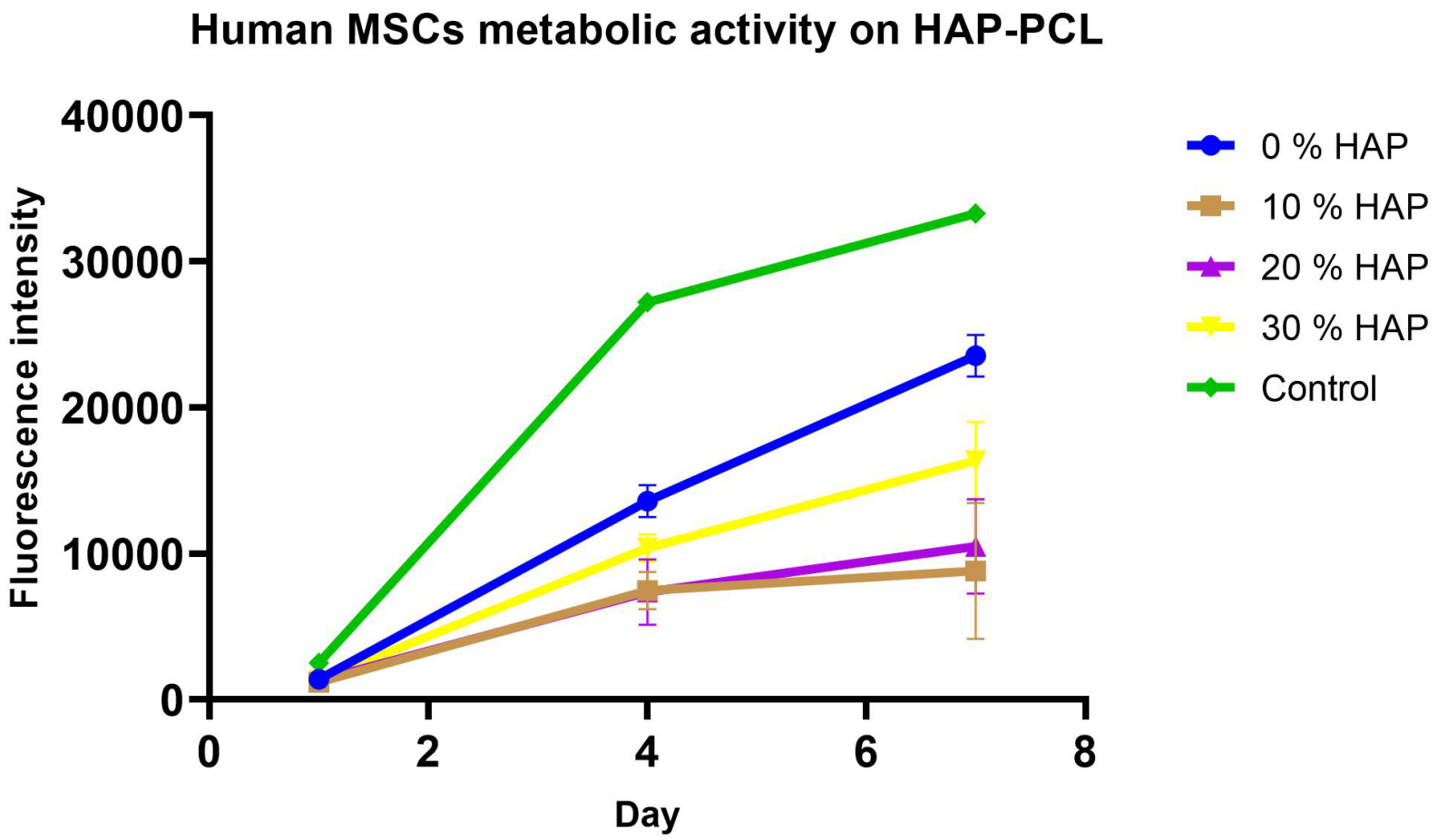
PMID: 33668087 PMCID: PMC7956195 DOI: 10.3390/molecules26051199

Free PMC article

Method: Incorporating sHAP in PCL

- Optimization with HAP only
- Concentrations: 0 %, 10%, 20%, 30% (w/w)
- Discs/films
  - Discs
    - r=4.5 mm
    - h=3 mm
    - A=2.12 cm^2
  - Films
    - r=6.5 mm

Results for Films



Conclusion

- HAP-PCL films are non toxic to human MSCs

- Further experiments necessary
  - To determine osteogenic capacity
  - Incorporate sHAP into PCL films
  - Increase printabilty of bioinks
  - Print fusion cage filler

Thesis summary

1. We have successfully synthetized a substituted HAP with Mg and Sr using a continuous method
2. An SOP was develop to test powders and composites *in vitro*
3. On going: composite as filler for spinal cages is currently investigated

Acknowledgments

- Supervisors
  - Professor Gwendolen Reilly
  - Dr Frederik Claeyssens
- Industrial Collaborators
  - Lucia Forte
  - Laura Grillini
  - Riccardo Bendoni
  - Claudio De Luca
- ESRs
  - Jose Rodrigues
  - Chloé Techens
  - Jennifer Fayad
  - Marco Sensale
  - Cameron James

Questions?

Further steps

- Testing of different powder concentrations in vitro
- Optimizing printing conditions and bioink composition
- Testing of sHAP-PCL composites