Colloque ADEBIOTECH

Stabilité et formulation des protéines et peptides

23-24 October 2015, Romainville

Protein Encapsulation using Pressurized CO_2 based Processes: Challenges and Perspectives

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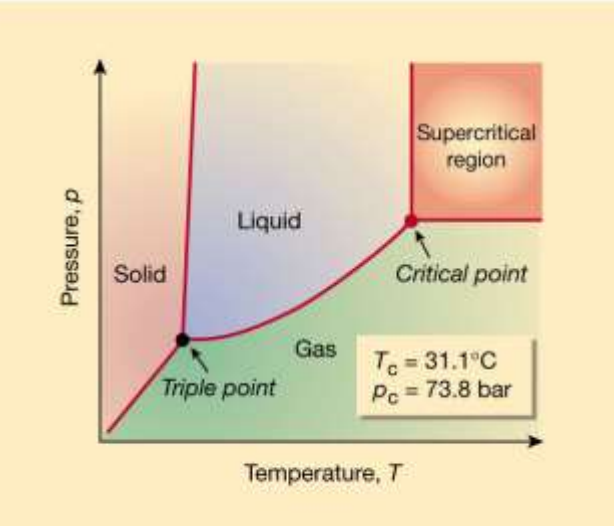
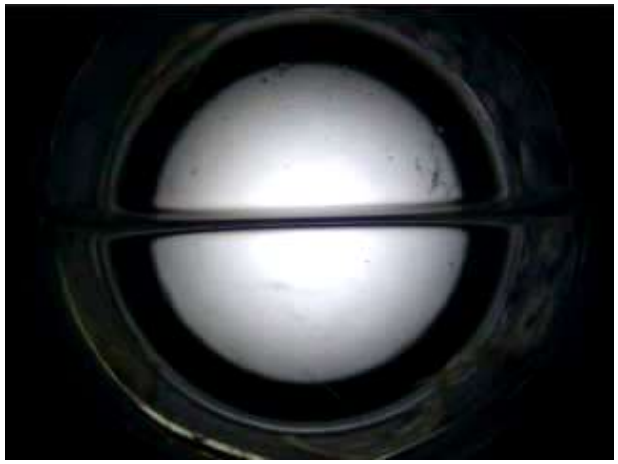
SUMMARY

- What is a supercritical CO_2 and its advantages ?
- The main issues in protein encapsulation
- $ScCO_2$ as a W/C emulsion continuous phase and reactant
  - Protein encapsulation into PLGA microspheres
  - Protein encapsulation into calcium carbonate microparticles
  - Preliminary evaluation for application in tissue engineering (cartilage)
- Perspectives and conclusions

What is a Supercritical Fluid?

Définition: The supercritical region refers to the state of compound which is above its critical pressure and critical temperature



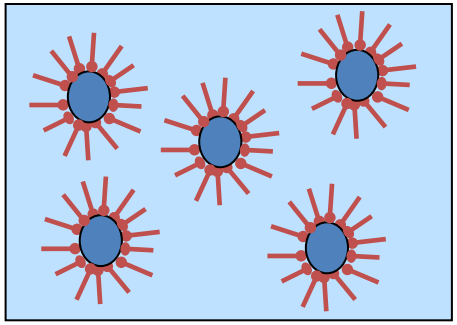


- ✓ Liquid-like properties (high density)
- ✓ Gas-like properties (high diffusivity, low viscosity)

Properties	Gas	Supercritical fluid	Liquid
ρ (kg m^{-3})	1	100–800	1000
η (Pa s)	0.001	0.005–0.01	0.05–0.1
$\mathcal{D}$ (m^2 s^{-1})	$1 \cdot 10^{-5}$	$1 \cdot 10^{-7}$	$1 \cdot 10^{-9}$

- ✓ Tunable solvent strength (density) by adjusting both the pressure and temperature

Applications of CO_2



CO_2 as chemical

Polymerization

Polymers modification

Polymers processing

CO_2 Technology

Polymers foaming

Anti-solvent processes

Extraction

CO_2 as monomer

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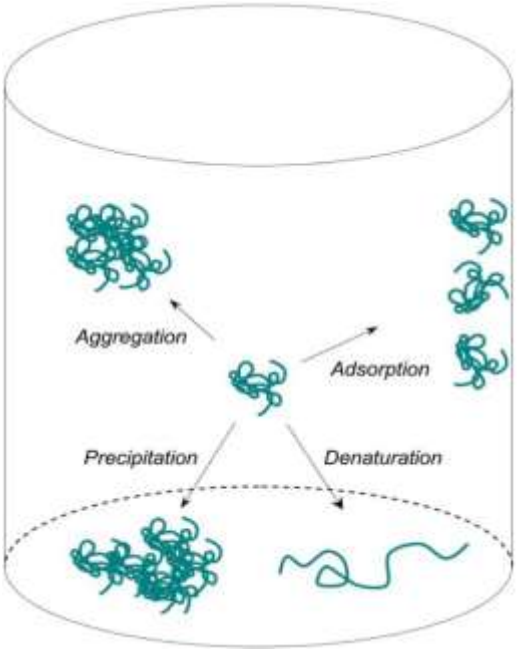
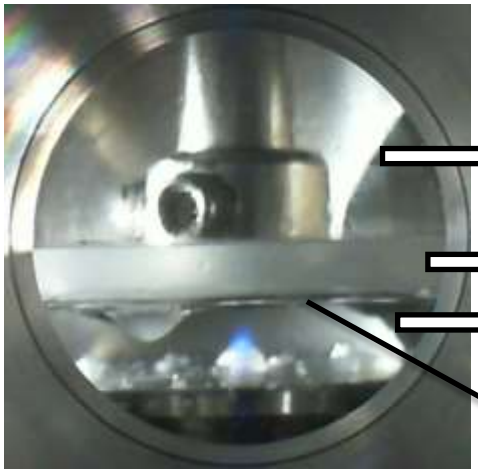
To take into protein unstabilities and solvent toxicity in formulation processes



Solvant	Limit concentration(ppm)	LD50 (mg/kg) oral route, rat	LD 50 (mg/kg)
Acetonitrile	410	2460	1680 (iv)
Methylene chlorhide	600	1600	
Acetone		5800	5500 (iv)
Ethyle acétate	5000	5620	
Tetrahydrofural		1650	2900 (ip)



GREEN PROCESSES

⟹ CO_2

⟹ Polymer solution

⟹ Aqueous solution

Interface → emulsion

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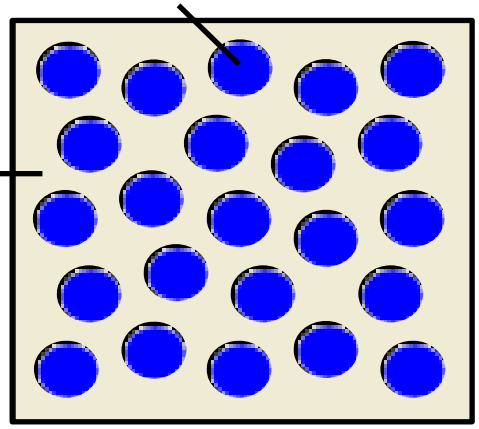
Particles formed from an emulsion with ScCO2 as the continuous phase

○ **Emulsion polar liquid-in-scCO$_2$**

Solvent+polymer+ API

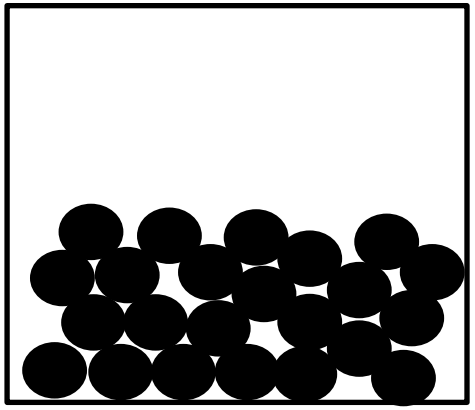
scCO$_2$



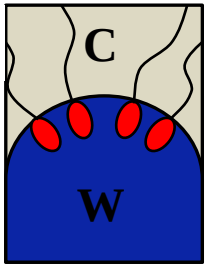
1) extraction

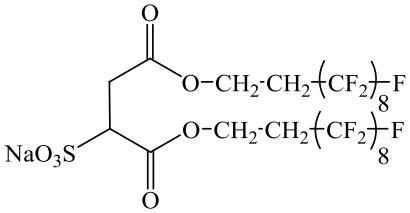
2) Dépressurisation

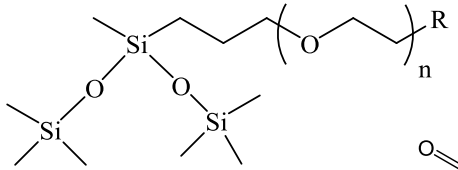
Solvent solubility in CO_2 (P,T)
Extraction media (water, ethanol…)

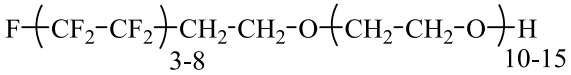


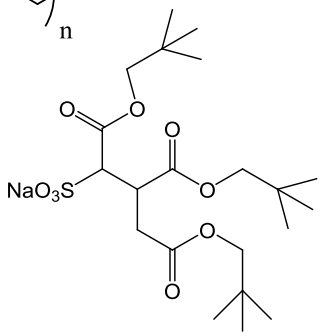
May involve stabilizers











But what about toxicity?

Eastoe *et al.*, Microemulsions with CO_2 as a solvent, *Curr. Opin. Colloid Interface Sci.*, **2012**, 17, 266–273

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Use of non toxic solvent

Solvent	LD50 (ml/kg)	Hemolytic activity IV route	Cardiovascular toxicity IV route	Angiotoxiciy in comparaison with DMSO Vasosapme (min)
Diméthyle sulfoxide	6.9 (iv./m), 12.6 (ip./m), 15(po./m)	Very high	Moderate	19.1
N-méthyl pyrrolidone	4.4 (ip./m)	Moderate	Toxicity +	5 (*)
Glycofurol	3.5 (iv./m)	Moderate	Toxicity --	10.5
Diméthyle isosorbide	8.54 (iv./r)	Low	Toxicity --	5.9 (*)
Polyéthylène glycol 200	7.6 (iv./m), 12.9 (ip./m), 26 (po./m)	Low	Toxicity -	1010

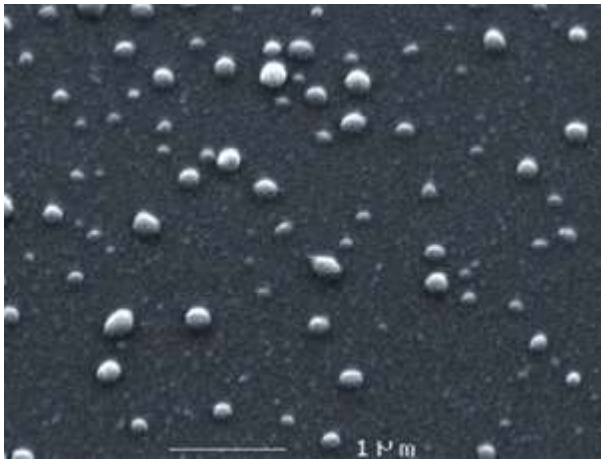
(*)Signicant difference between DMI / NMP et DMSO ($P<0.05$)
Iv, ip, po : Intrvenous, Intraperitoneal, oral ; m, r : mouse, rat

➔ DMI : less toxic solvent, miscible with water ($1x10^3$ g/L ;25°C), non flammable, non volatile (boiling T° : 234-242°C)

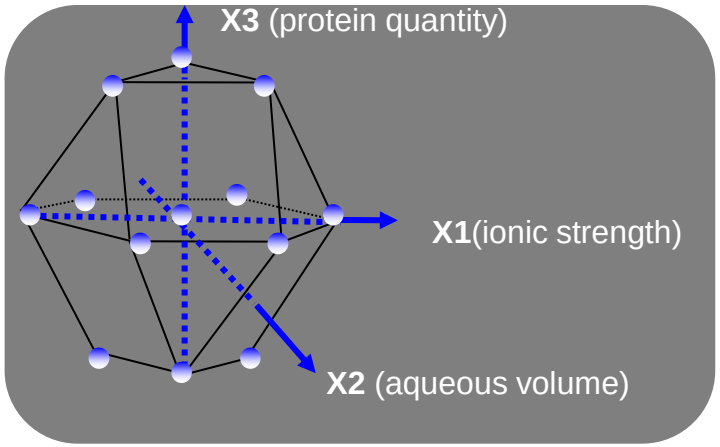
Protein encapsulation in PLGA microparticles

"solid dispersion is more stable than molecular state"

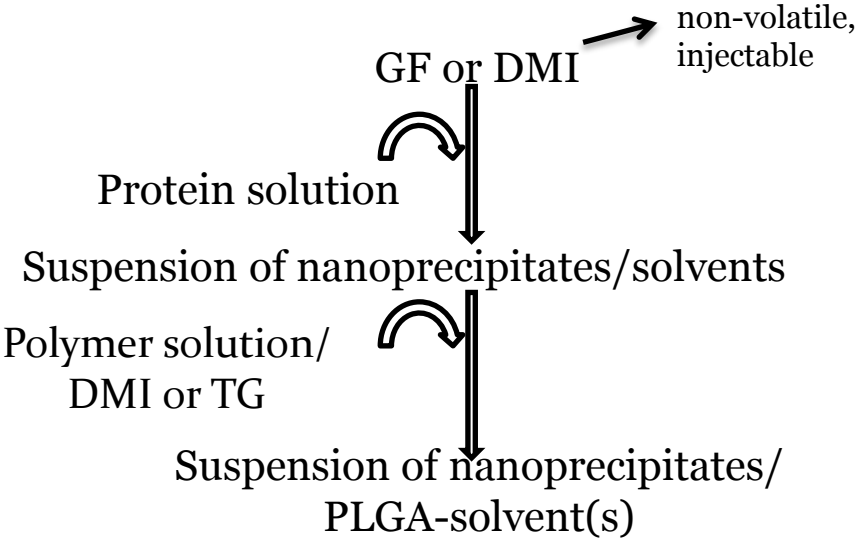
1/Protein nanoprecipitation :

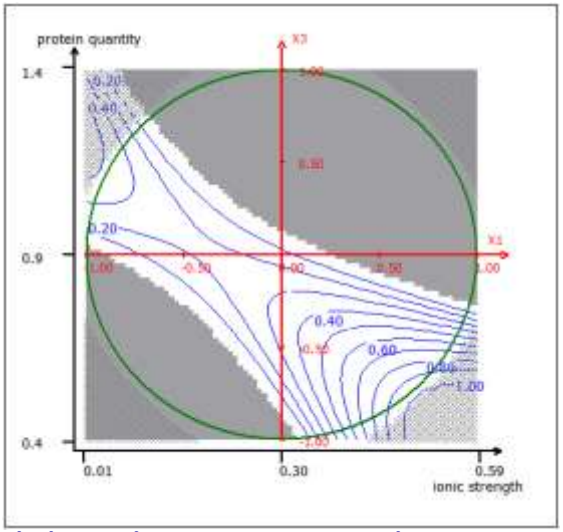


SEM image of lysozyme-nanoprecipitates



Doehlert matrix



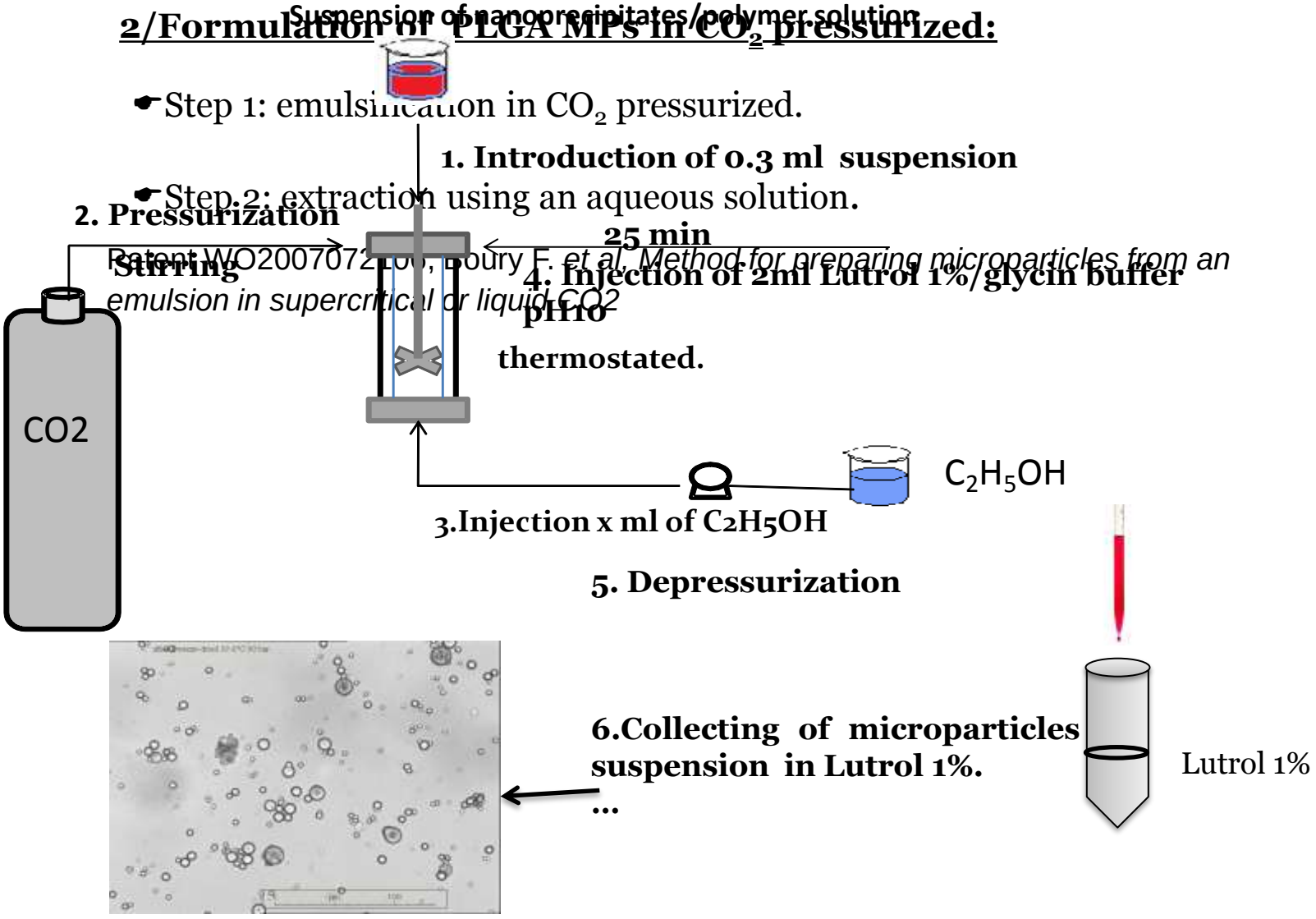


Giteau, A., et al., Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres. European Journal of Pharmaceutics and Biopharmaceutics, 2008. 70(1): p. 127-136.

Protein encapsulation in PLGA microparticles

2/Formulation of PLGA MPs in CO_2 pressurized:

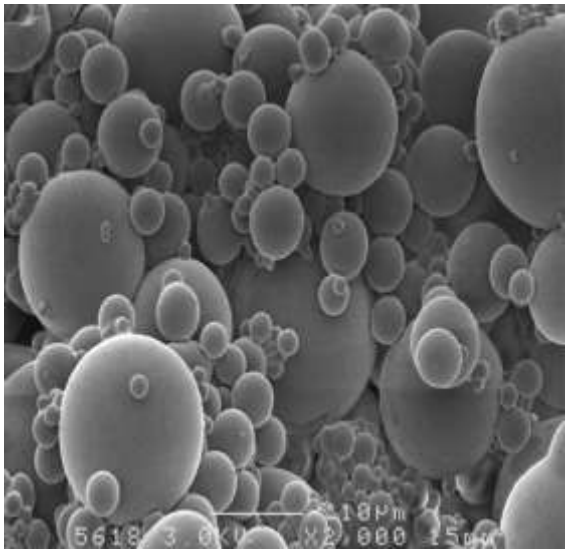
- Step 1: emulsification in CO_2 pressurized.
- Step 2: extraction using an aqueous solution.



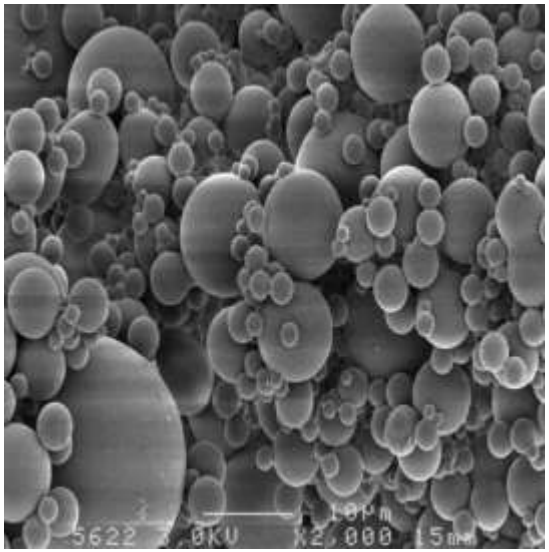
Patent WO2007072106, Boury F. *et al.* *Method for preparing microparticles from an emulsion in supercritical or liquid CO2*

Tran MK, Swed A, Boury, Preparation of polymeric particles in CO2 medium using non-toxic solvents: Formulation and comparisons with a phase separation method , F., Eur J Pharm Biopharm. 2012

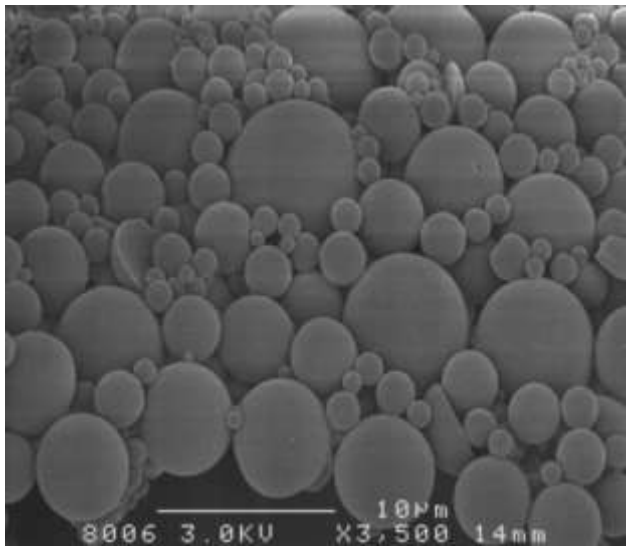
Microparticle observation with SEM



25°C, 63bar, 0,3 ml C_2H_5OH
100% GF



20°C, 58 bar, 0,15 ml C_2H_5OH
100% GF



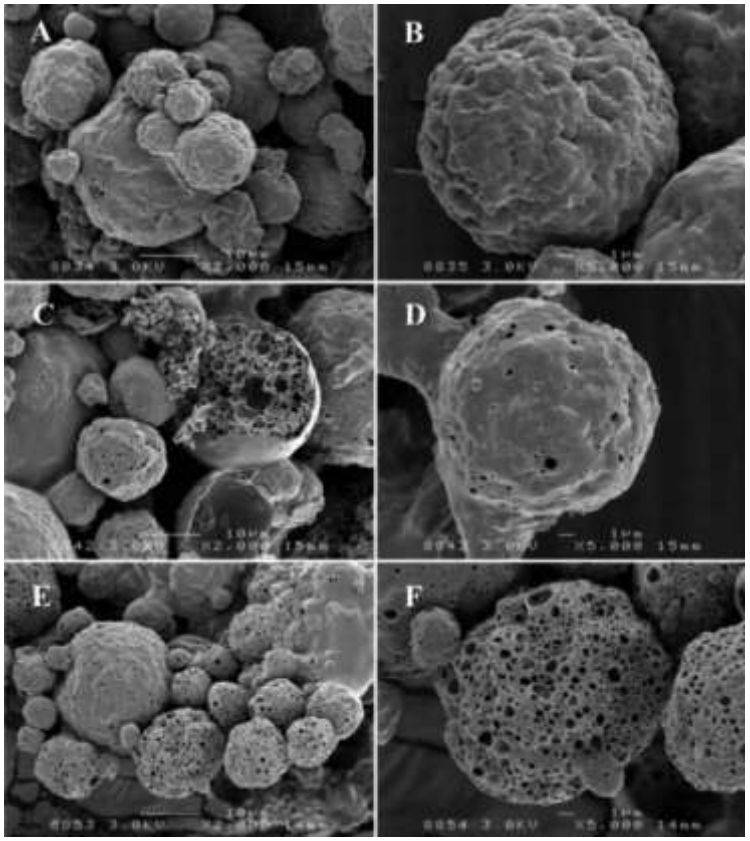
39.5°C, 82bar, 0,15 ml C_2H_5OH
GF-DMI (1:2/v:v)

« Fingerprint » of an emulsification step

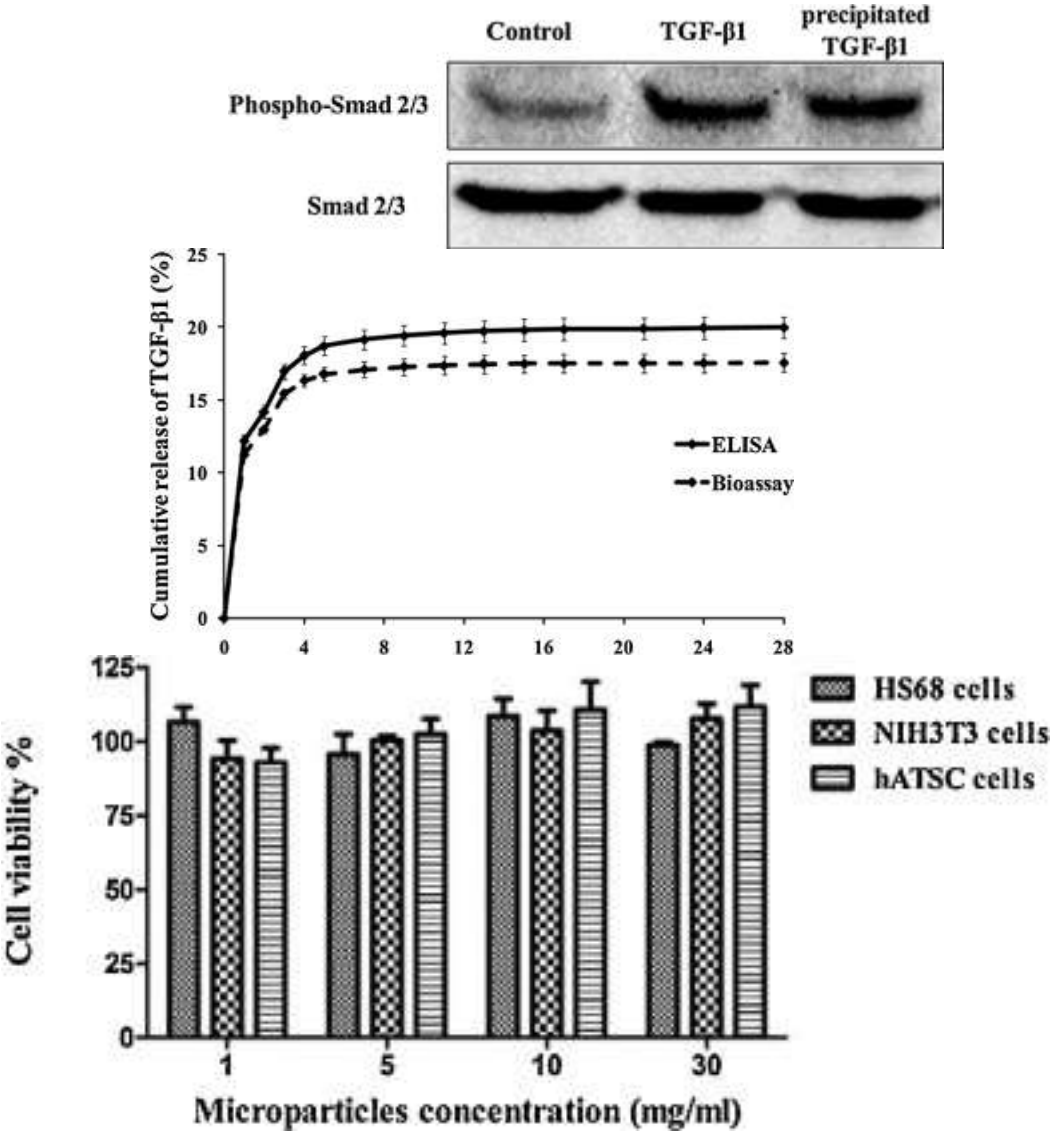
EncapsulationYield (max) ≈85%

Tran MK, Swed A, Boury, Preparation of polymeric particles in CO2 medium using non-toxic solvents: Formulation and comparisons with a phase separation method , F., Eur J Pharm Biopharm. 2012

Application to therapeutic proteins



SEM images of PLGA microparticles during the release study; after 1 week (A, B); after 2 weeks (C, D) and after 4 weeks (E, F).



Effect of unloaded PLGA microparticles on percentage viability of HS68, NIH3T3 and hATSC cells after 48 h of incubation as evaluated by a PicoGreen® assay.

A. Swed et al./International Journal of Pharmaceutics 493 (2015) 357 –365

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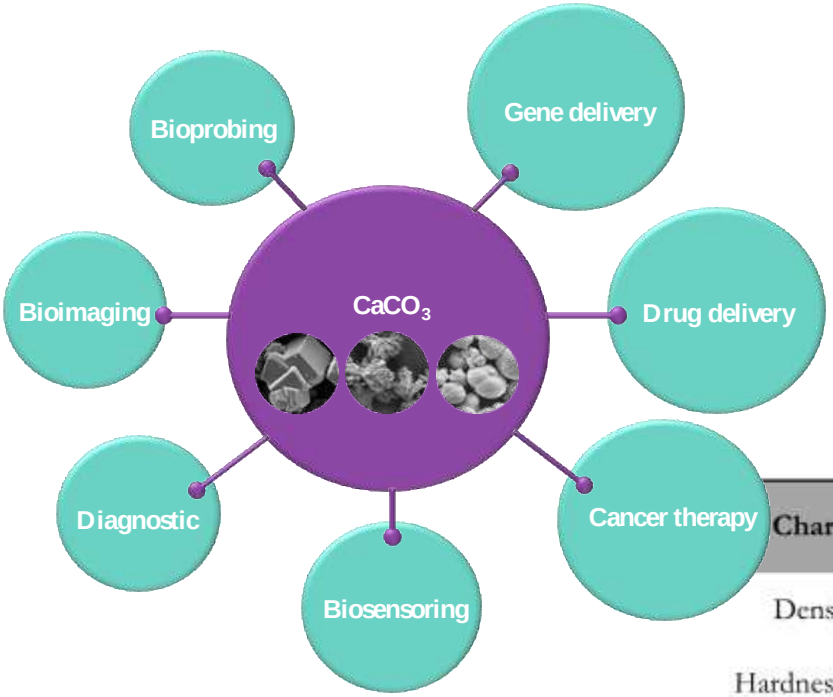
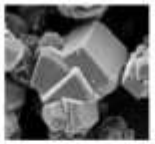
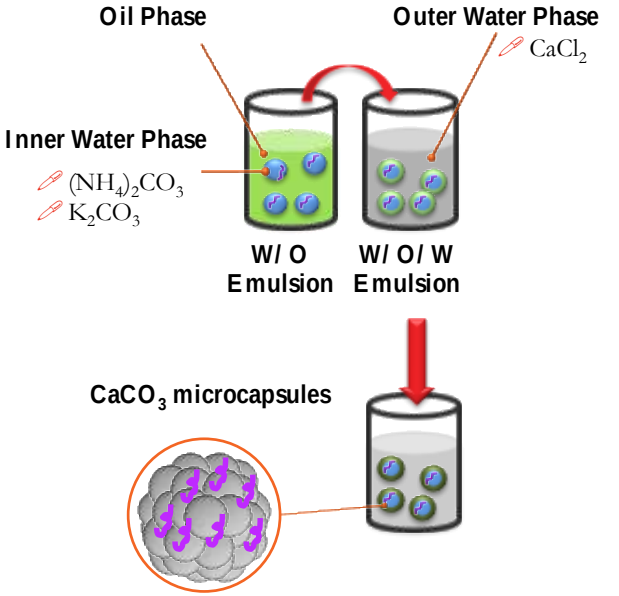
Differrent axes of $CaCO_3$ applications as drug delivery system



Characteristics	$CaCO_3$ polymorph		
	Calcite	Aragonite	Vaterite
Density (g/cm^3)	2.71	2.93	2.54
Hardness (Mosh scale)	3	3.5-4	3
Crystal structure			
	Rhomboedral	Orthorhombic	Hexagonal
Aqueous solubility at 25°C (K_{sp})	$10^{-8.48}$	$10^{-8.34}$	$10^{-7.91}$
Aqueous stability at 25°C	stable	stable	metastable

Different loading modes of drugs within $CaCO_3$ particles

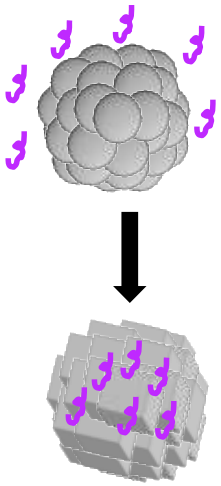
Interfacial reaction



FUJIWARA M., *et al.*, *Chemical Engineering Journal,* **2008**, 137, 14-22.

FUJIWARA M., *et al.*, *Crystal Growth & Design,* **2010**, 10, 4030-4037.

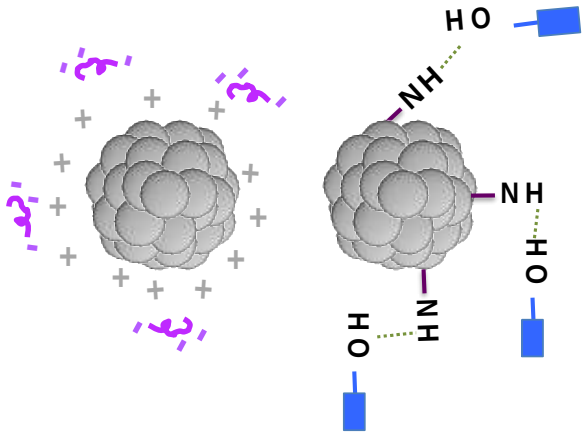
Phase transition



Adsorption-Impregnation

Physical

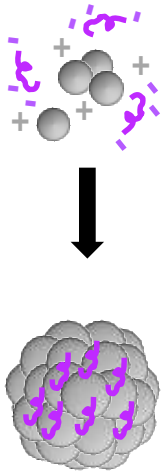
Chemical



VOLODKIN D. V., *et al.*, *Biomacromolecules,* **2004**, 5, 1962-1972.

LOPEZ-MARZO A., *et al.*, *Journal of Materials Chemistry,* **2012**, 22, 15326-15335.

Co-precipitation

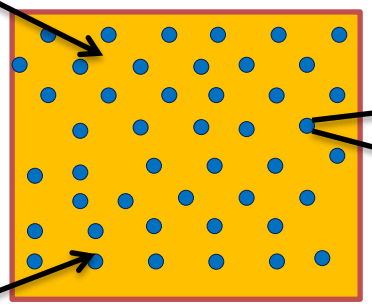


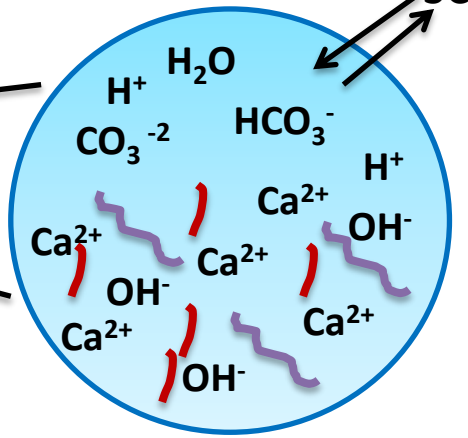
DE TEMMERMAN M.-L., *et al.*, *Biomacromolecules* , **2011**, 12, 1283-1289

The carbonation route

SC-CO_2 continous medium

Solution of Ca^{2+}



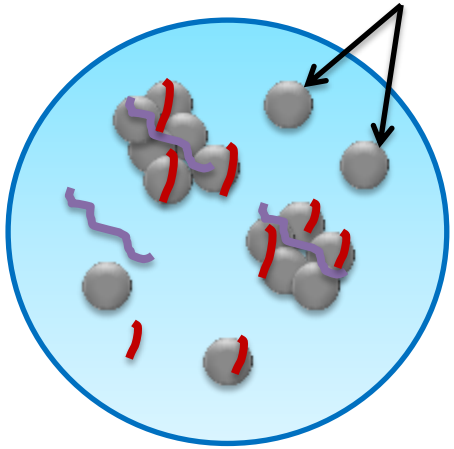


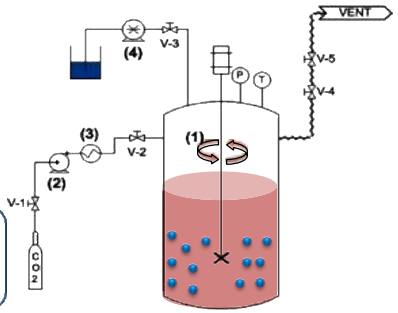
Emulsion H_2O/CO_2

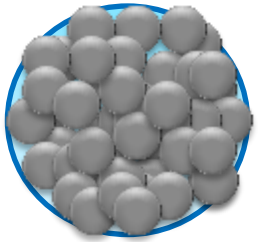
Hyaluronic acid:Template

Protein

Vaterite nanoaggregates



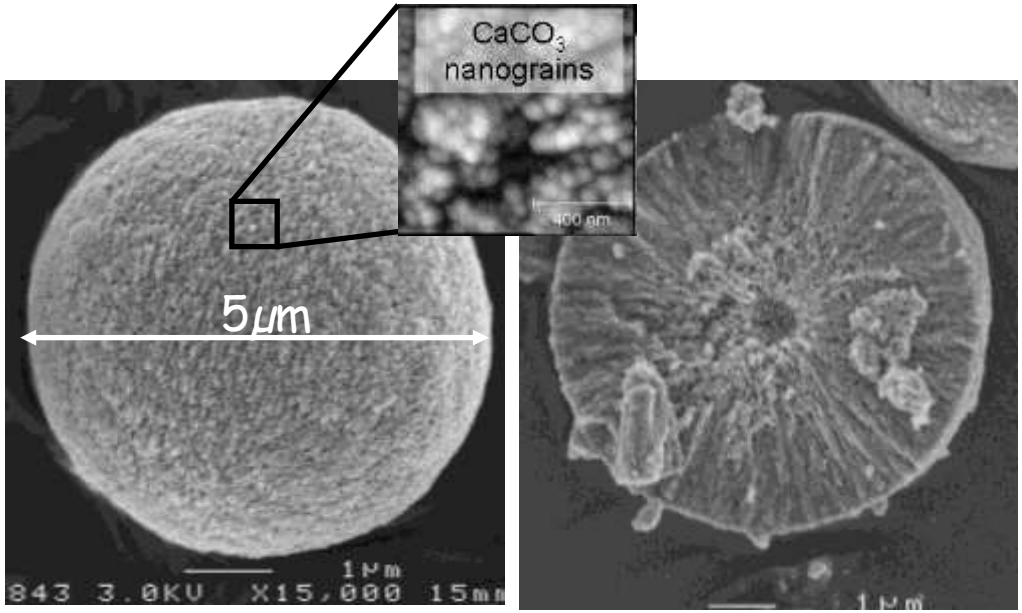




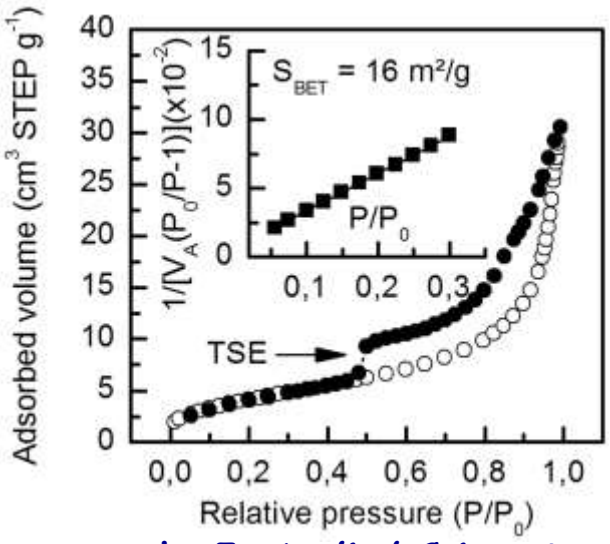
Beuvier *et al.* , *Journal of Materials Chemistry* 2011

Supercritical CO_2 process $Ca(OH)_{2\,(s)} + CO_{2\,(g)} \leftrightarrow CaCO_{3(s)} + H_2O_{\,(l/v)}$

$CaCO_3$ properties

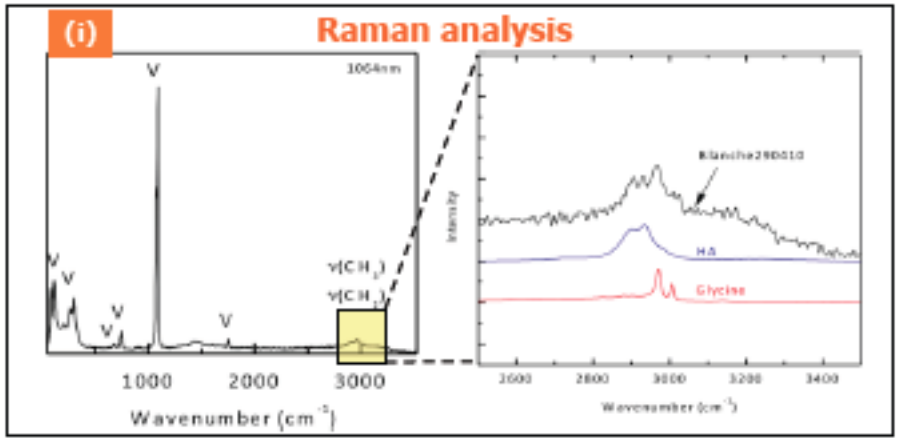


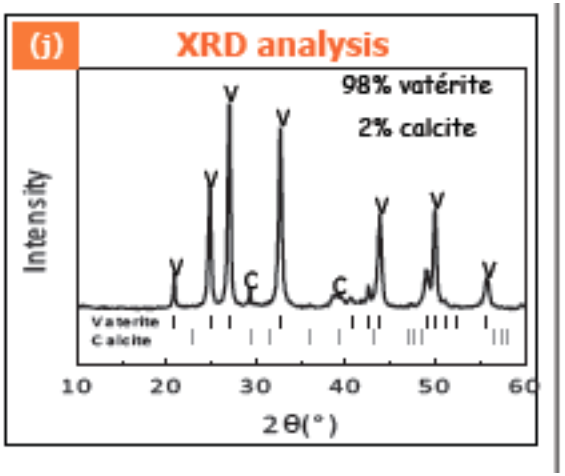
Mesoporosity



Polymorphism

E. Chavez et al, *J. Applied Crist.*, (2012). 45,881-889



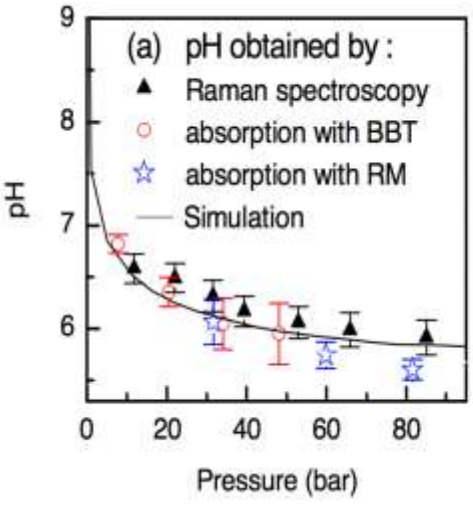


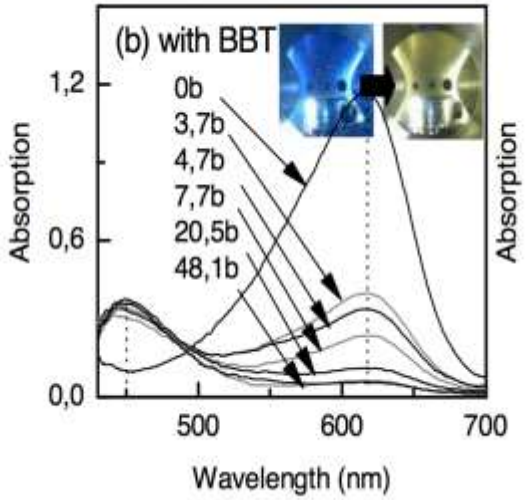
Beuvier et al J. Mater. Chem., 2011, 21, 9757

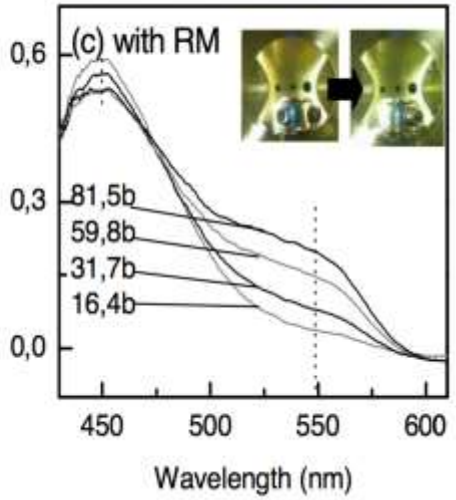
Formation of $CaCO_3$ microparticles
In situ analysis : effect of pH

Pressurization

Depressurization



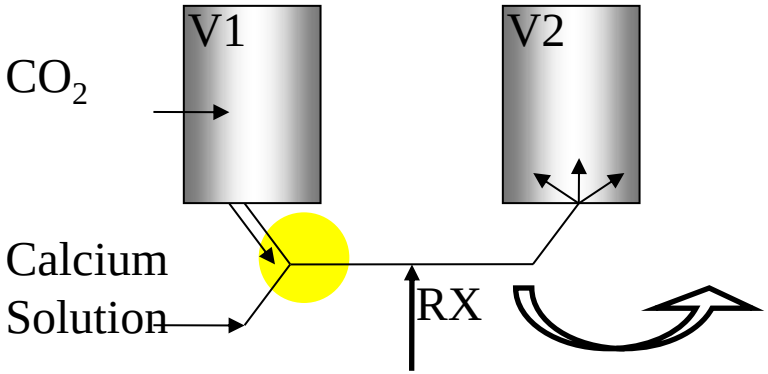




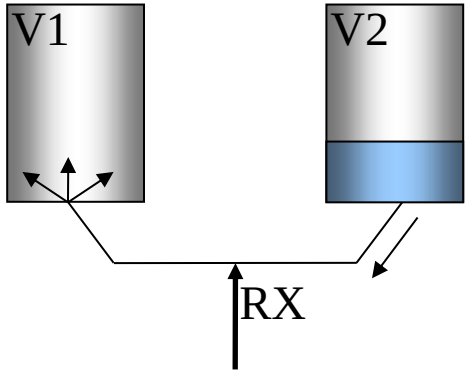
Beuvier et al, Anal.Chem. 2014, 86, 9895−9900

Formation of $CaCO_3$: *in situ* study of the cristallisation process (coll. A. GIBAUD, LPEC, Le Mans)

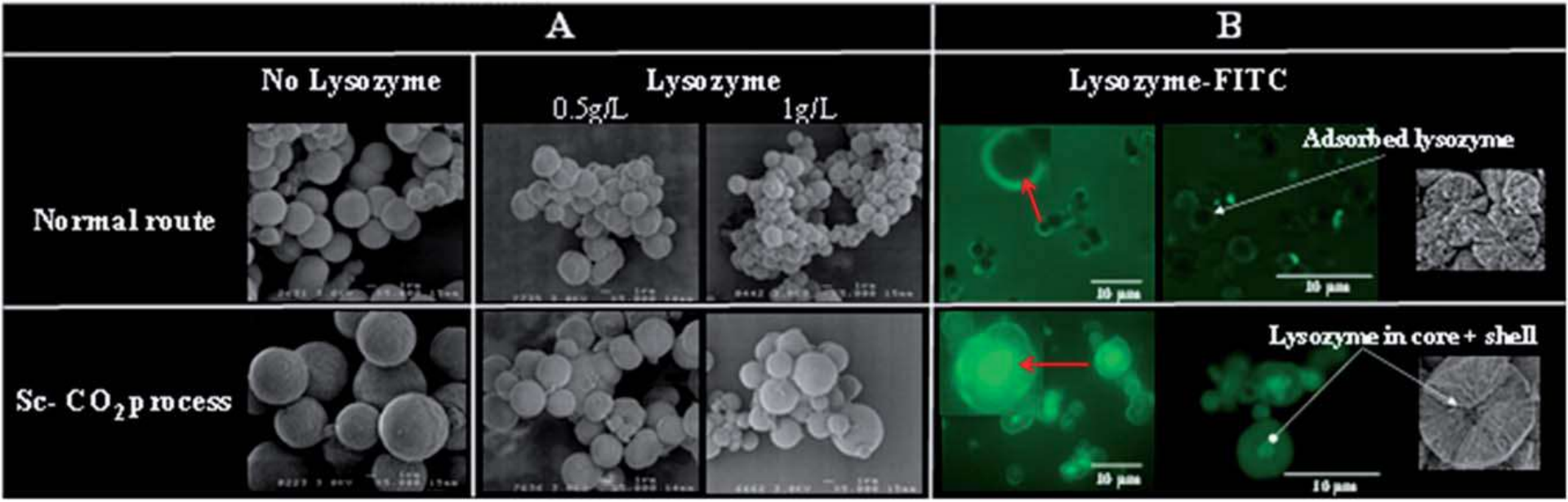
Pressure increase

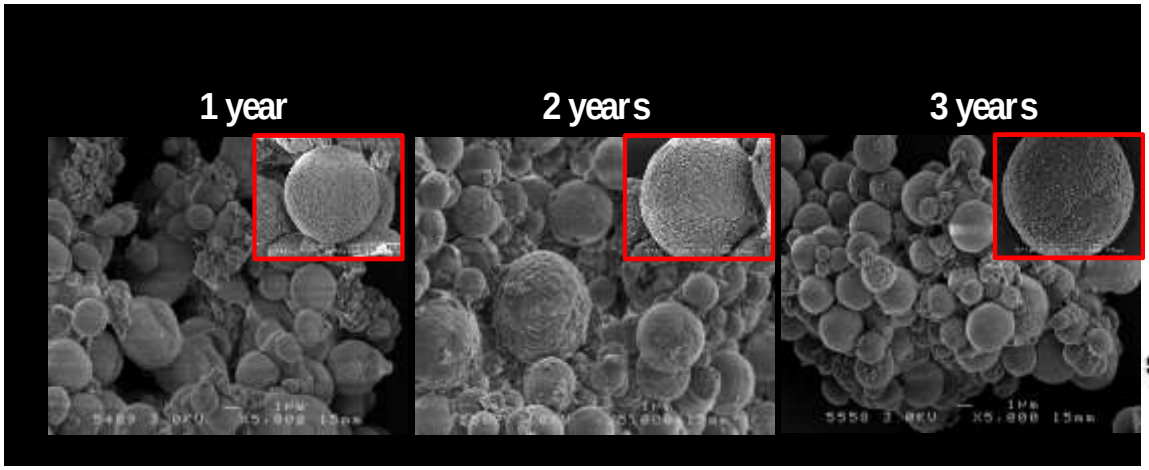


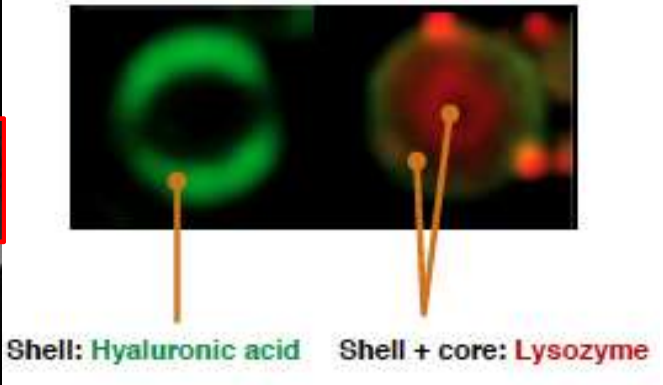
Pressure decrease



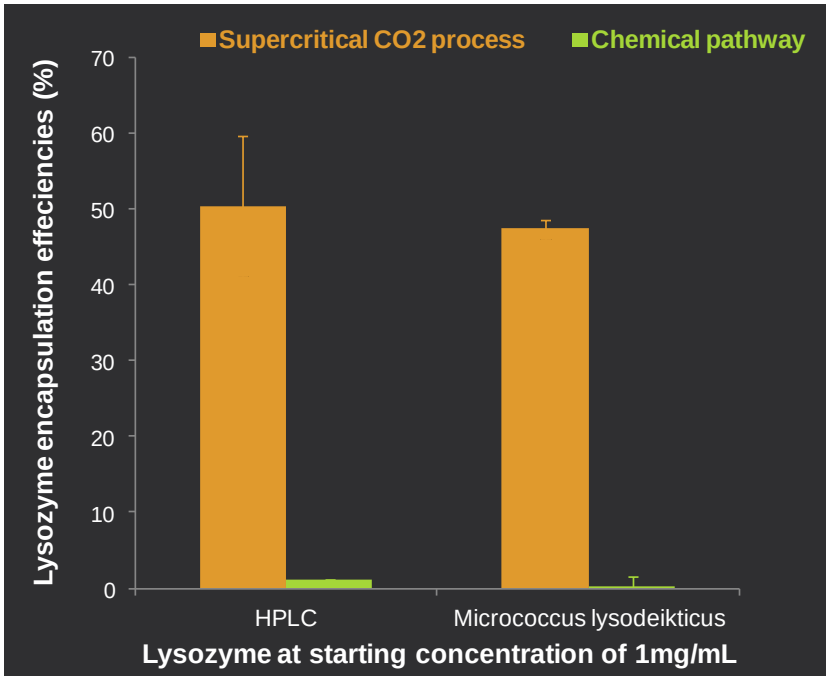
Lysozyme encapsulation in $CaCO_3$ microparticles
Influence of the formulation process

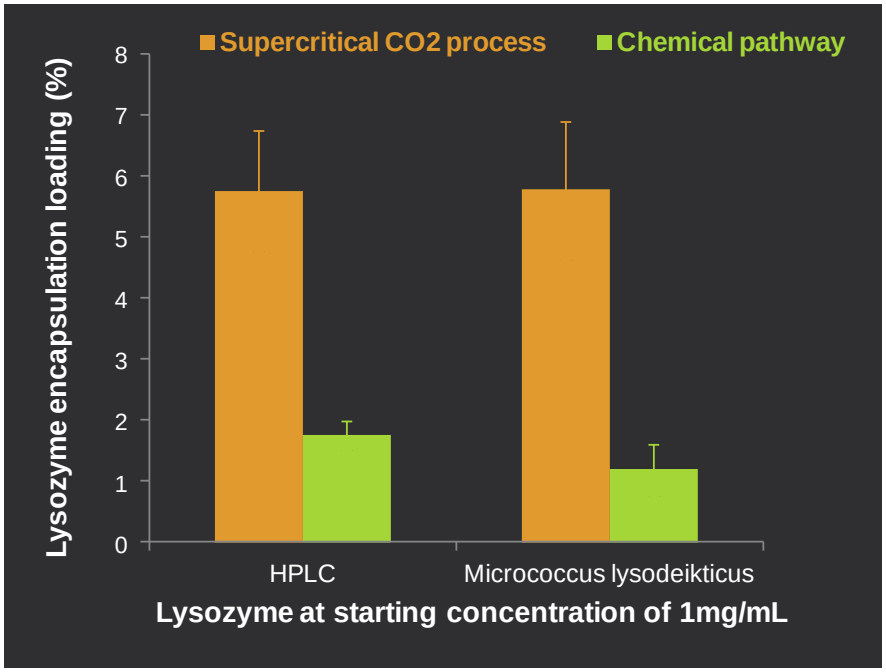






Lysozyme encapsulation in $CaCO_3$ microparticles Influence of the formulation process

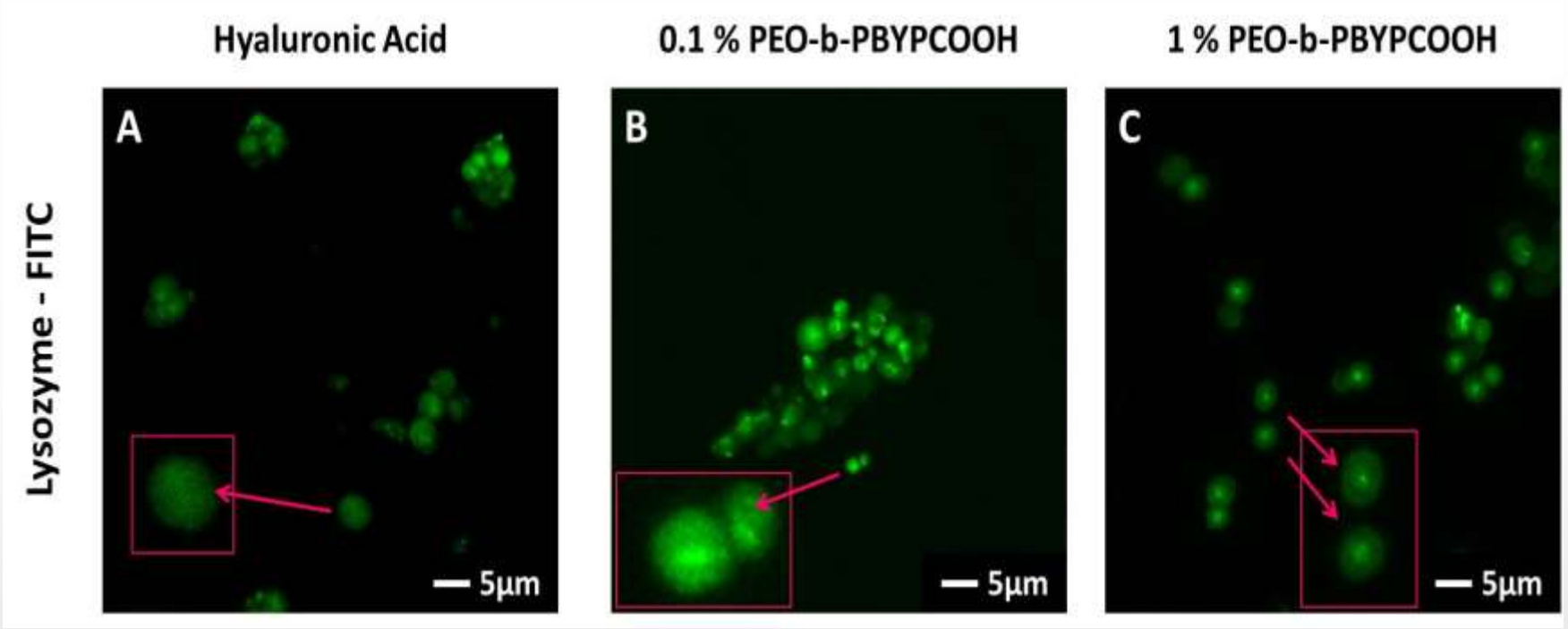


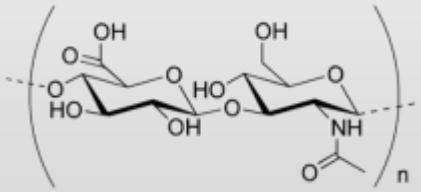


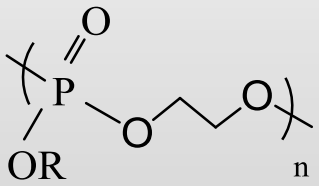
Hassani et al.J.Mater.Chem.B , 2013, 1, 4011-4019

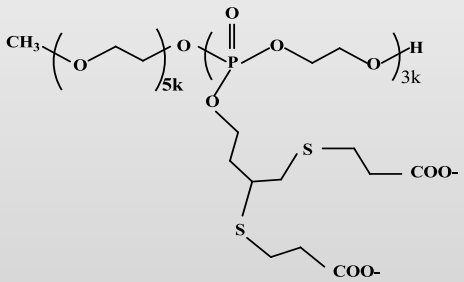
Formation of $CaCO_3$ microparticles Influence of templating macromolecules

(collaboration CERM, Liège, Prof. C. Jérôme)





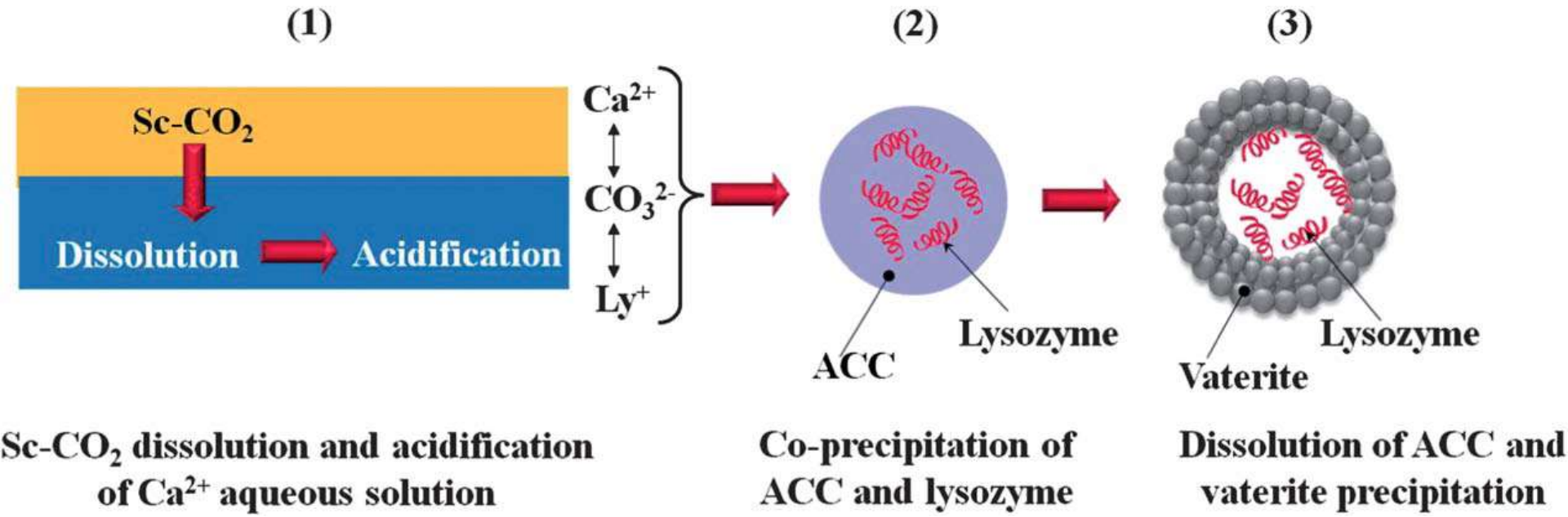




Co, H.; Antonietti, M. **1998**, *382*, 582–589.

Ergul Yilmaz, Z.; Debuigne, A.; Calvignac, B.; Boury, F. ; Jérome C. (J.Mater. Chem. B, 2015, 3, 7227).

Proposed mechanism for microparticle formation and lysozyme encapsulation

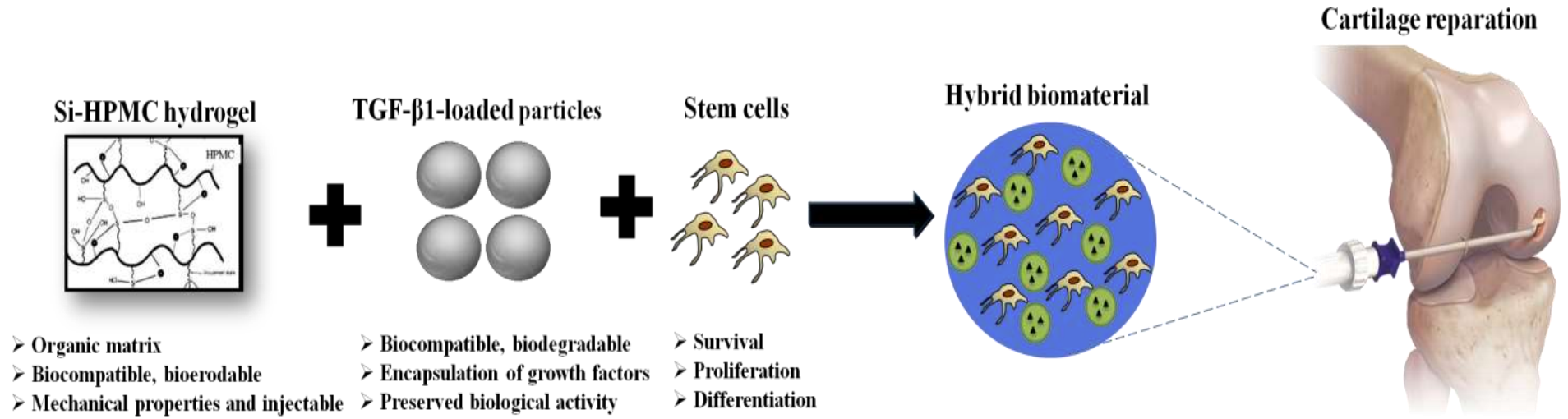


Hassani et al., J. Mater. Chem. B, 2013, 1, 4011–4019

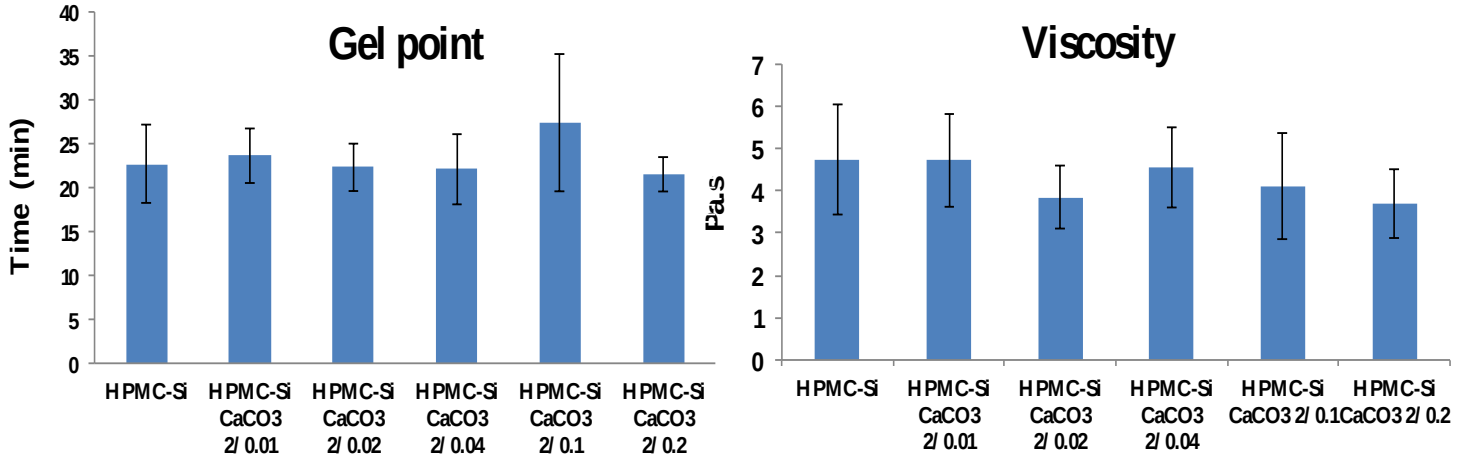
SUMMARY

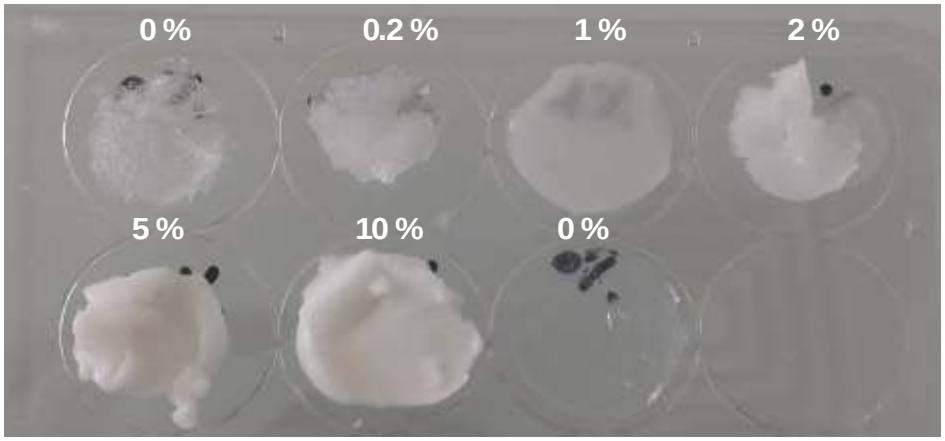
- What is a supercritical CO_2 and its advantages
- The main issues in protein encapsulation
- $ScCO_2$ as a W/C emulsion continuous phase and reactant
  - Protein encapsulation into PLGA microspheres
  - Protein encapsulation into calcium carbonate microparticles
  - **Preliminary evaluation for application in tissue engineering (cartilage)**
- Perspectives and conclusions

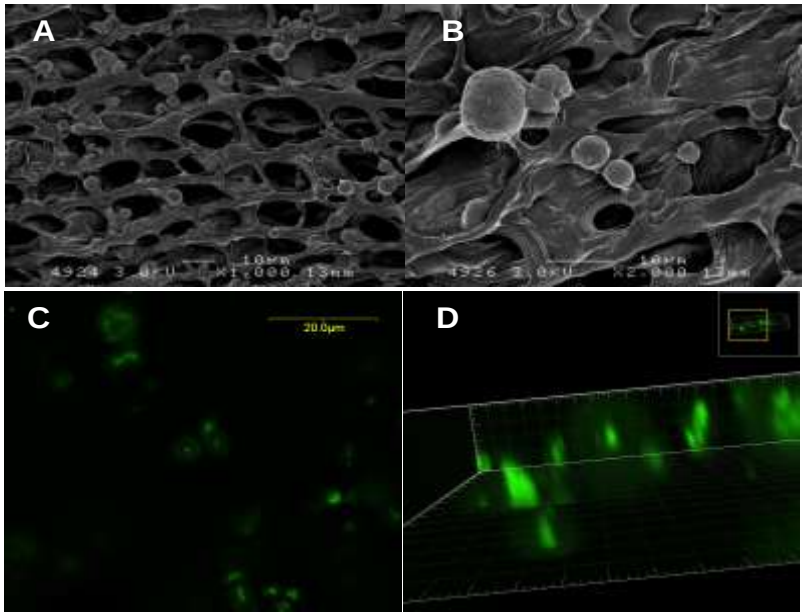
Principles of tissue engineering



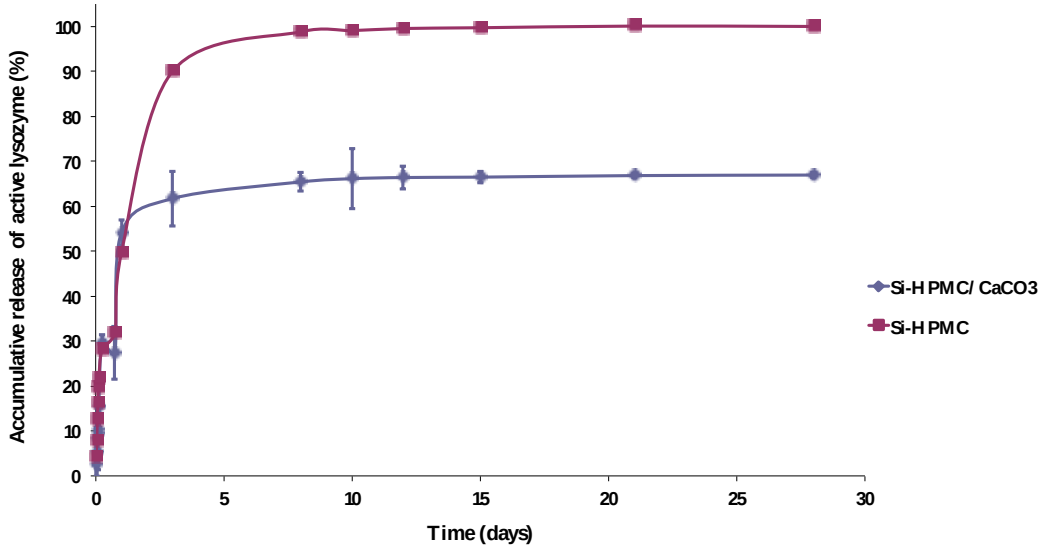
Distribution of $CaCO_3$ microspheres Inside the hydrogel (Coll. P. WEISS, LIOAD, Nantes)

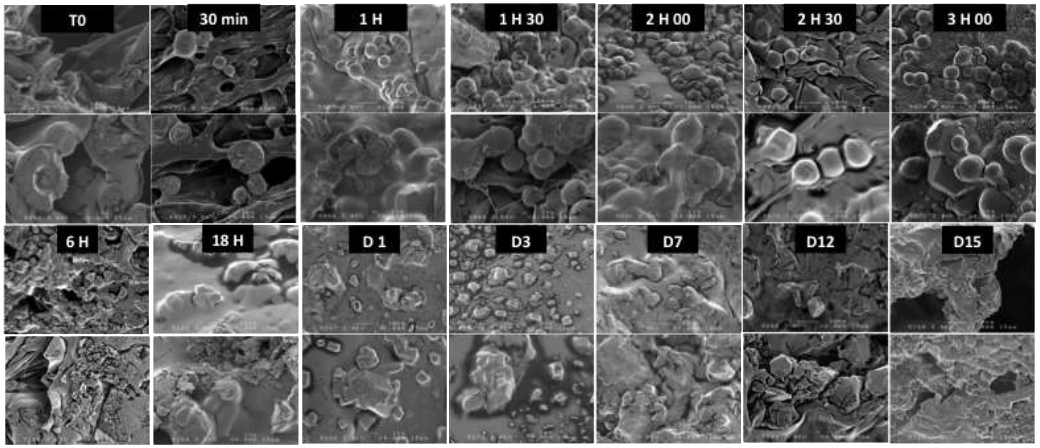




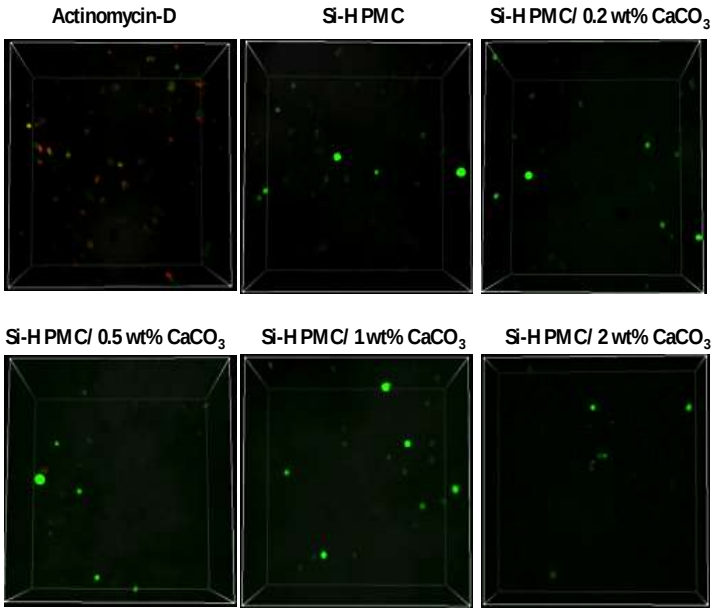


Lysozyme release study from Si-HPMC hydrogel and Si-HPMC/$CaCO_3$ combined hydrogel





SEM images of Si-HPMC/$CaCO_3$ 2 wt % hybrid hydrogel at different time point during lysozyme release experiment.

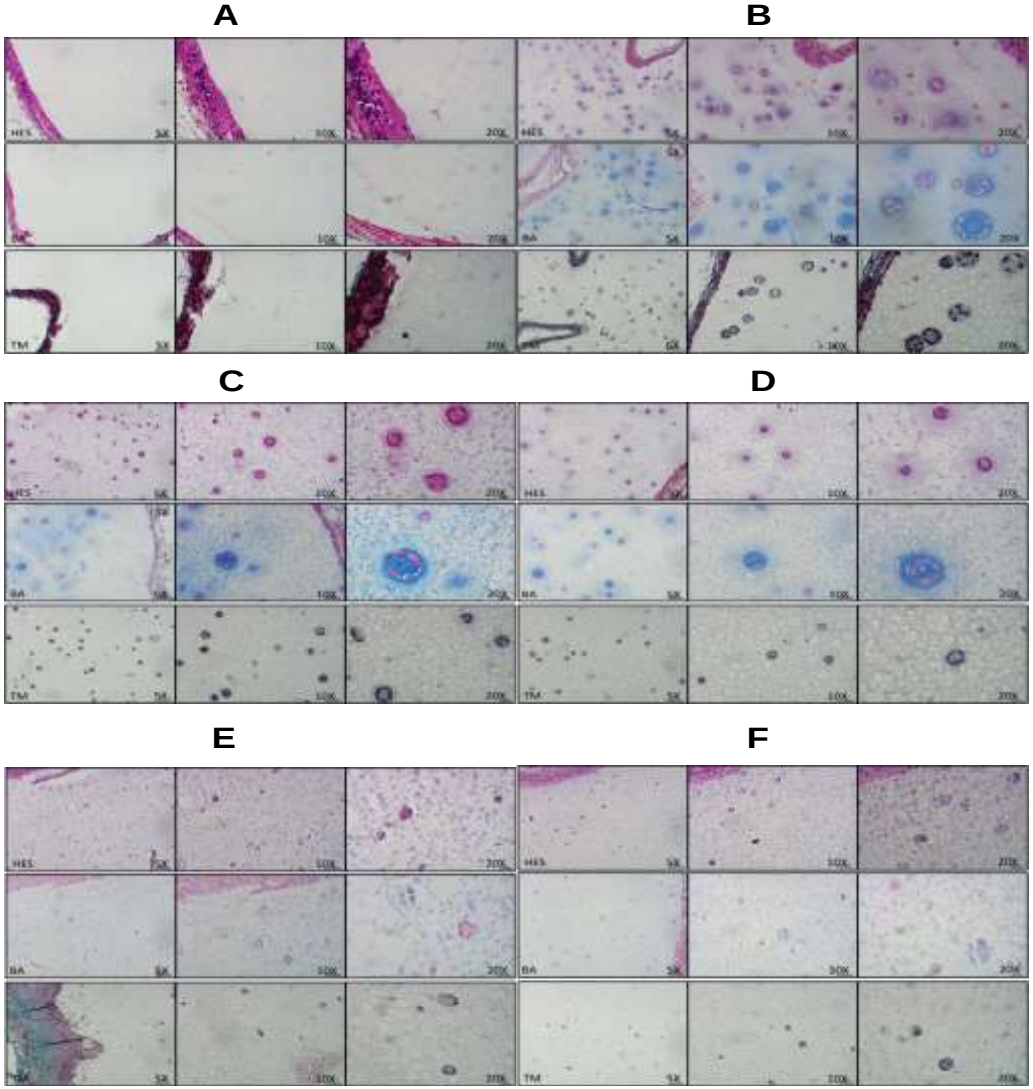


***In vitro* viability tests**

CLSM images of hASC cells seeded within pure Si-HPMC hydrogel and Si-HPMC/$CaCO_3$ hydrogels

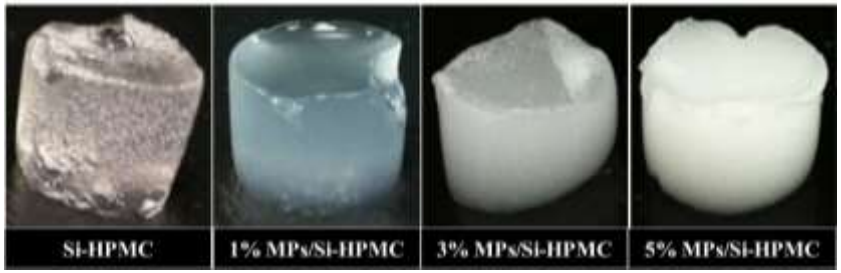
(Coll. P. WEISS, LIOAD, Nantes)

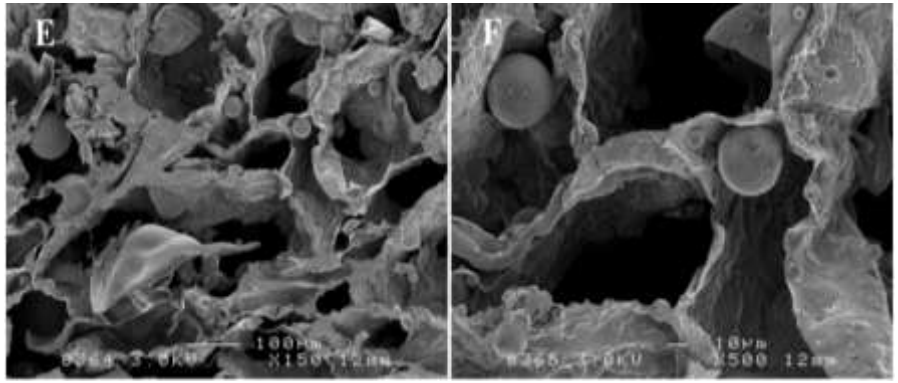
Histological analysis after *in vivo* implantation (Coll. P. WEISS, LIOAD, Nantes)

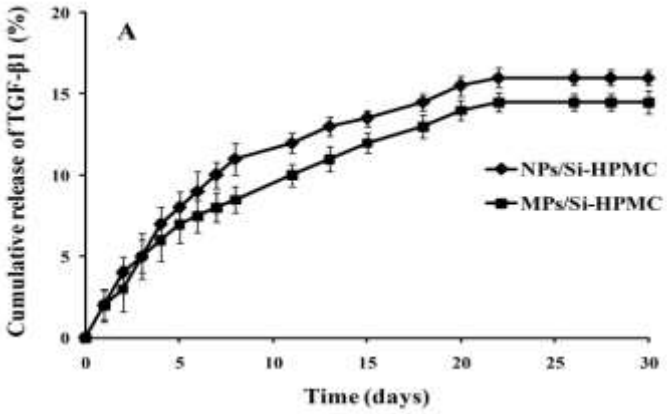


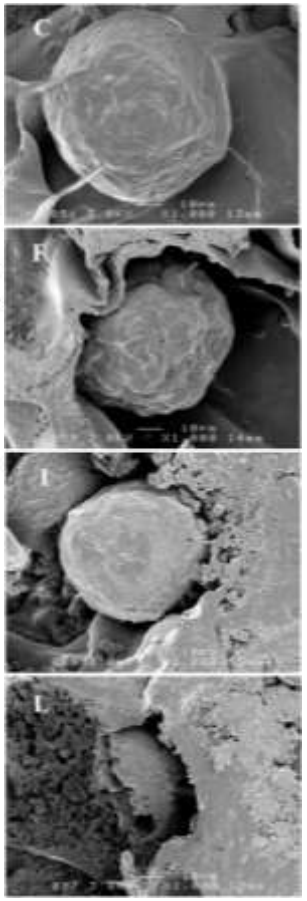
A) pure Si-HPMC hydrogels, B) Si-HPMC hydrogels combined to chondrocytes, C) Si-HPMC/ 1 wt%$CaCO_3$ hydrogels combined to hNC cells, D) Si-HPMC/ 2 wt%$CaCO_3$ hydrogels combined to hNC cells, E) Si-HPMC/ 1 wt%$CaCO_3$ hydrogels combined to hASC cells, F) D) Si-HPMC/ 2 wt%$CaCO_3$ hydrogels combined to hASC cells.

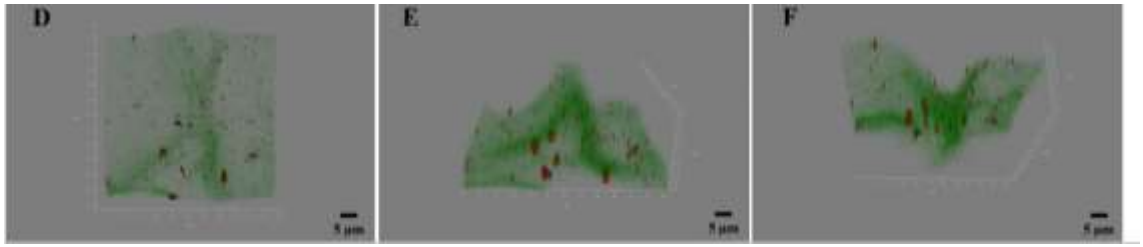
TGFβ1 release study : Si-HPMC/PLGA MP











SUMMARY

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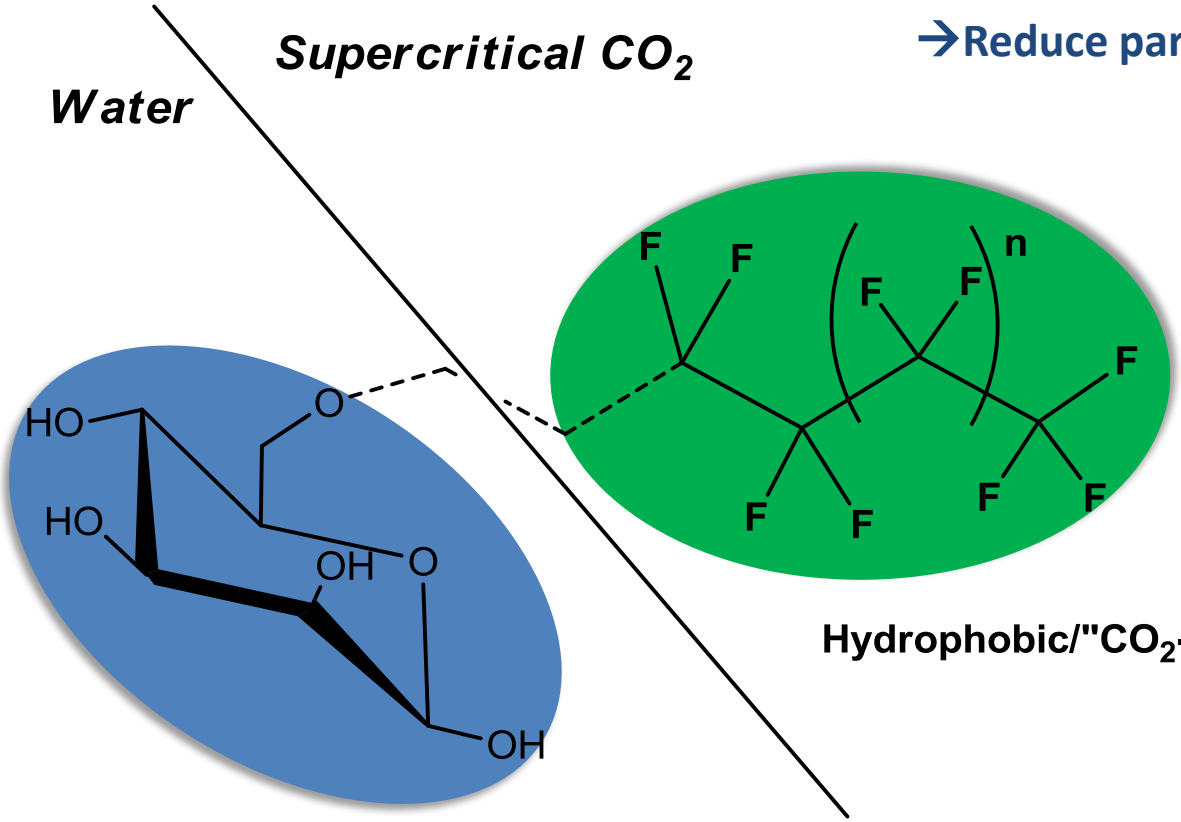
Synthesis and use of specific surfactants for emulsification in $scCO_2$

→Reduce/control the particle size

→Increase the emulsion stability

→Reduce particle agregation



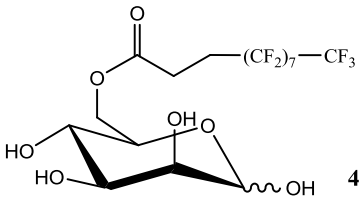
Hydrophobic/"CO_2-philic" moiety

Hydrophilic part

Center for Education and Research on Macromolecules

Cédric Boyère *et al.*, *Carbohydr. Res.* 2011, *346*, 2121-2125

Synthesis and use of fluorinated surfactants for emulsification in $scCO_2$



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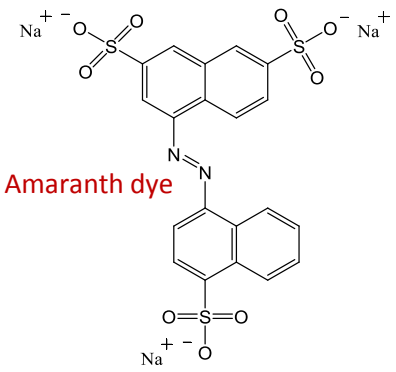
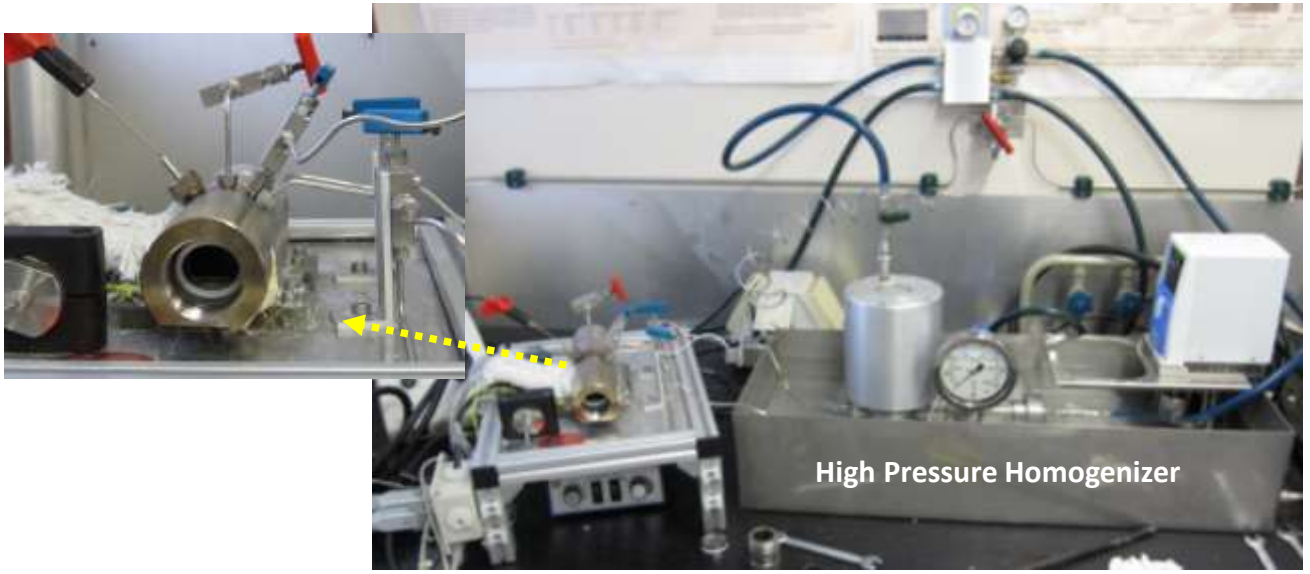
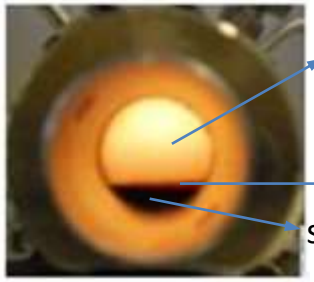




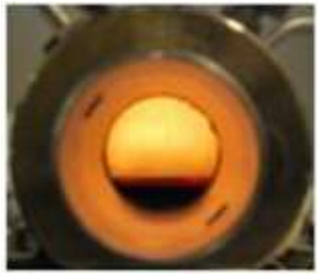
150 bar



without homogenization

150 bar, homogenization 15 min

homogenization



stop homogenization

250 bar, homogenization 15 min

t = 0

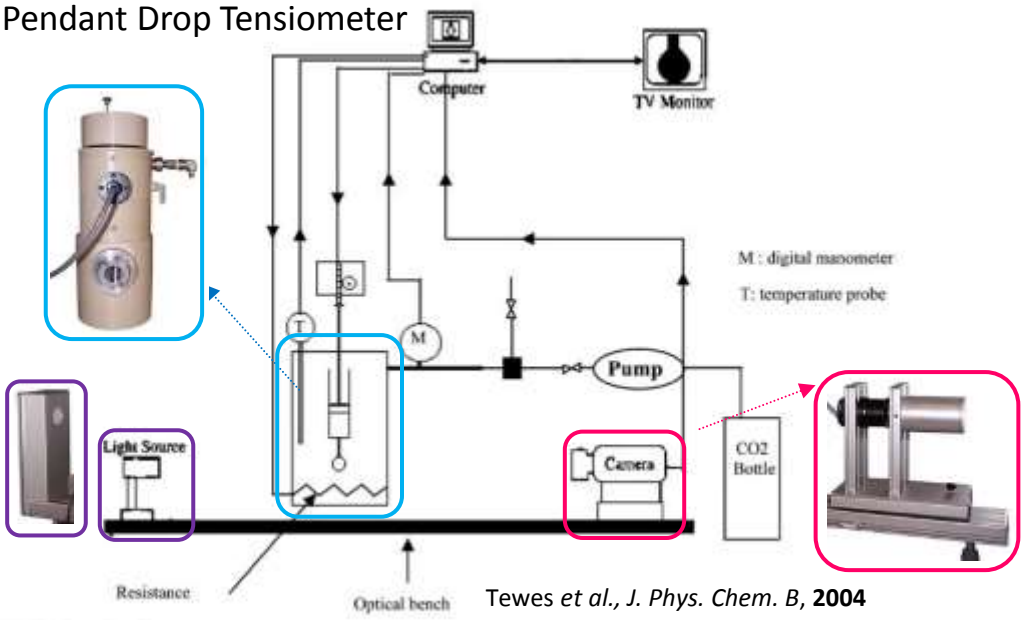
overnight

Assessment of the capacity of Man-C2F8 **4** (C = 90,3 mM) to stabilize (water+amarante)/$scCO_2$ (1:9 v/v) emulsion at high pressure and 45°C.

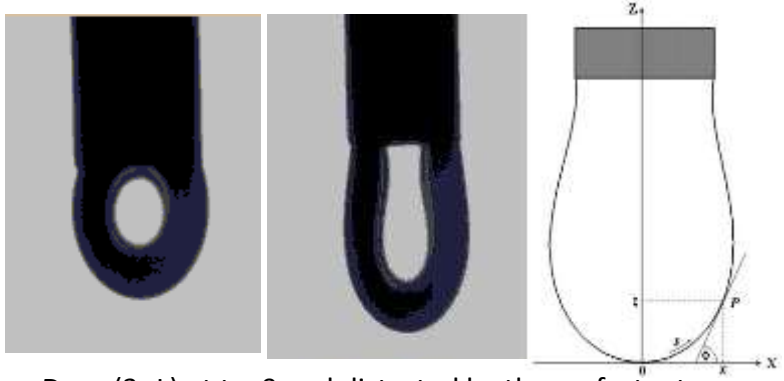
A. Favrelle, C. Boyère *et al.*, *J. Colloid Interface Sci.*, 398, 2013, 273-275

Tensiometry at the water/scCO2 interface

Pendant Drop Tensiometer

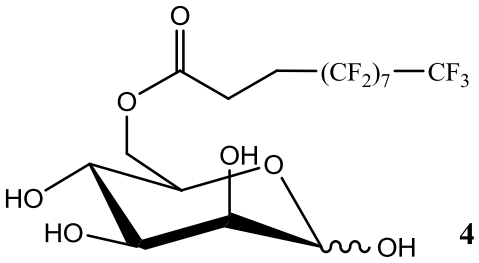


Tewes *et al., J. Phys. Chem. B*, **2004**



Drop (3µL) at t = 0 and distorted by the surfactant

$$\frac{1}{x}\frac{d}{dx}(x \sin\phi) = \frac{2}{S} - cz \qquad c = g\rho/\Upsilon$$



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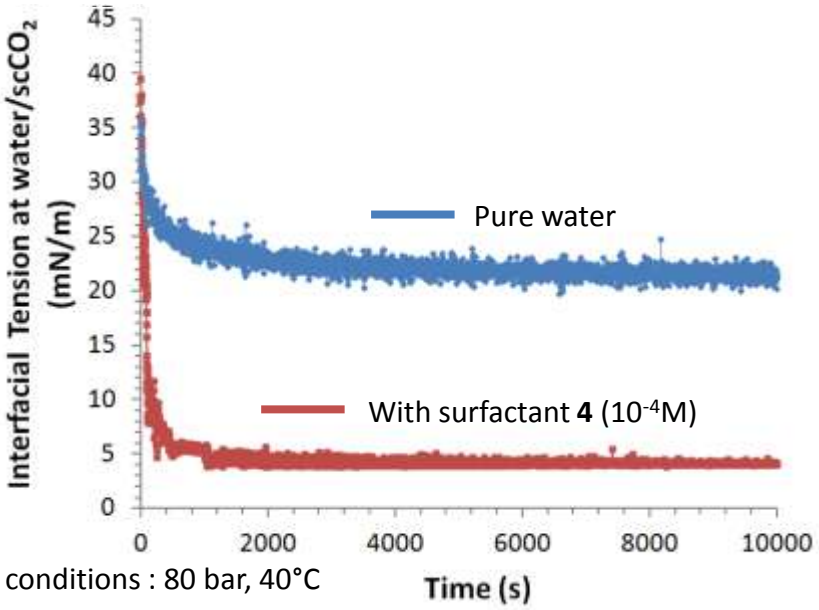

conditions : 80 bar, 40°C

A. Favrelle, C. Boyère *et al.*, *J. Colloid Interface Sci.*, 398, 2013, 273-275

Conclusions

- SCF technologies is a tool box:
  - For protein encapsulation avoiding toxic solvent
    - polymeric particles
    - mineral particles
  - To process preforming polymers
    - Foaming, polyHIPES
    - Impregnation of actives substances
  - Developing scaffolds and new materials
  - To scale-up and produce GMP batches

Conclusions

- BUT it needs further development to improve:
  - The knowledge of processes
  - In situ characterization
  - The development of GRAS additives adapted to this tunable medium
  - To convince the pharmaceutical companies of the interest of “green chemistry”
  - To overcome industrial bottleneck

Aknowlegments

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Pr. A. Debuigne
A. Farvelle
C. Boyère

SCIAM (“Service Commun d'Imagerie et d'Analyse Microscopique”) for electron microscope images.
“Région Pays de la Loire”(Bioregos 2 project),
ANR (“Agence Nationale pour la Recherche”, grant (ANR-09-PIRI-0004-01)
EACEA (EC)

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