In Vitro and *in Vivo* Characterization of a Photocrosslinkable Hyaluronan Hydrogel for Skeletal Muscle Repair

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Skeletal Muscle Diseases

- Skeletal muscle can self-repair through the activation of resident muscle satellite cells as a consequence of injury.
- When volumetric muscle losses (VMLs) occur due to trauma or surgical resection, lesions are so extensive that the regenerative potential of the muscle is overwhelmed.
- VLMs represent a challenging clinical problem in both military and civilan medicine
- Current approaches to treat VMLs include autologous tissue transfer and skeletal muscle tissue engineering, but both approaches have been hampered by several drawbacks.

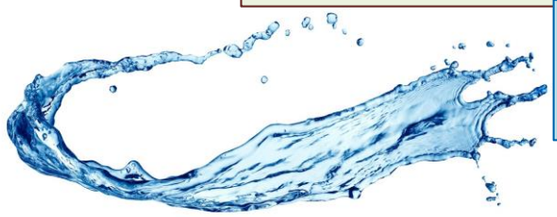
★ Clinical need for scaffolds that can be applied to bridge the gap of the lesion as contact guidance for muscle satellite cells growth and to act as a vehicle to deliver grow factors and nutrients in order to modify the microenvironment.

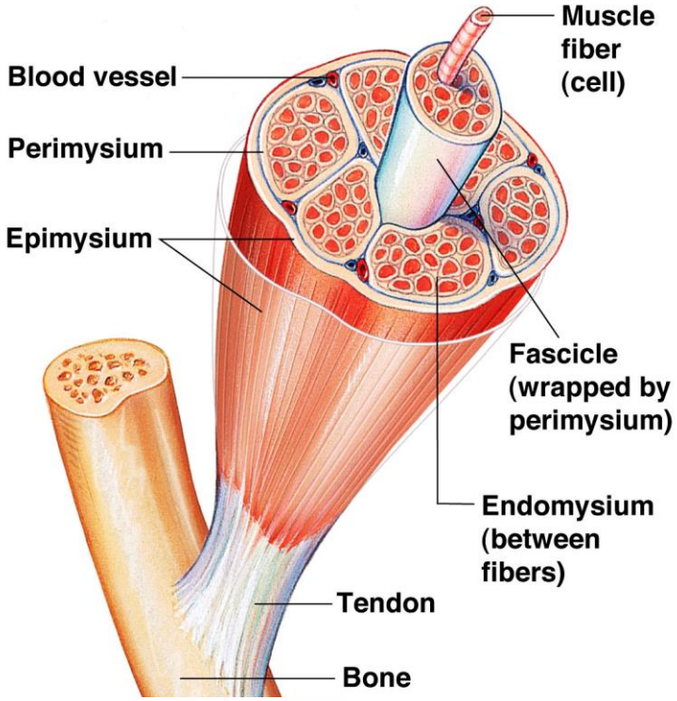
Hyaluronan hydrogels: increasingly attractive choice in the fields of regenerative medicine, wound care and tissue engineering as scaffolds.

High water content

Good biocompatibility

Good biodegradability

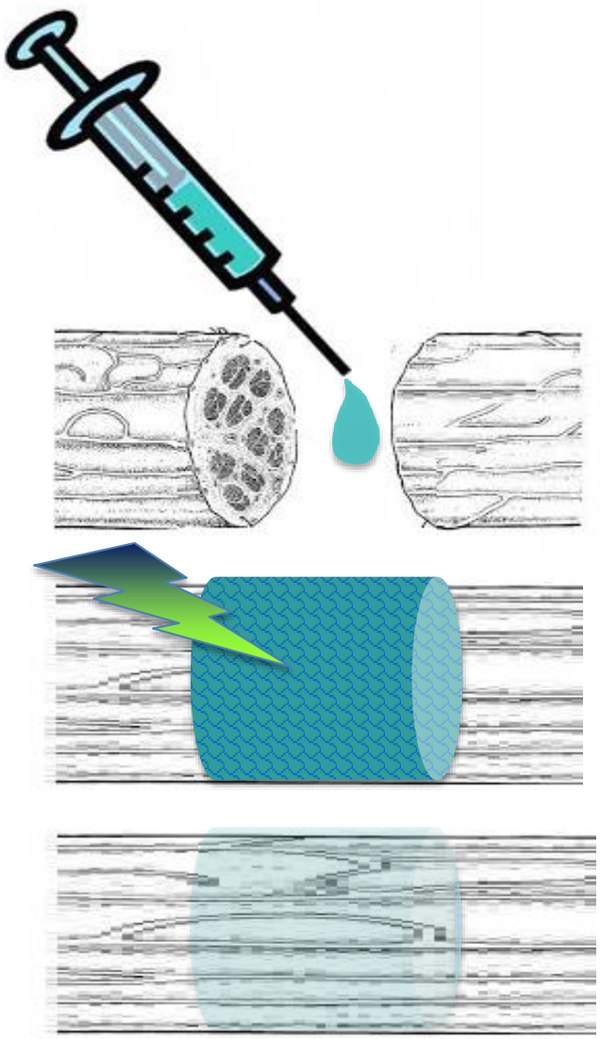




GOAL: In situ gel forming hyaluronan hydrogel

Injectable hyaluronan solution that fill the gap of the lesions and became a wall-to-wall hydrogel after an external stimulus

Ability to mimic the environment of the extracellular matrix



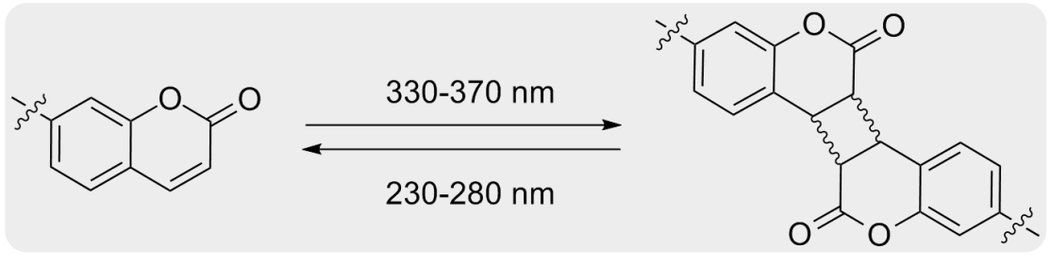
Hyaluronan solution

Cross-linked HA

Muscle regeneration

FID119: A NEW HYALURONAN DERIVATIVE

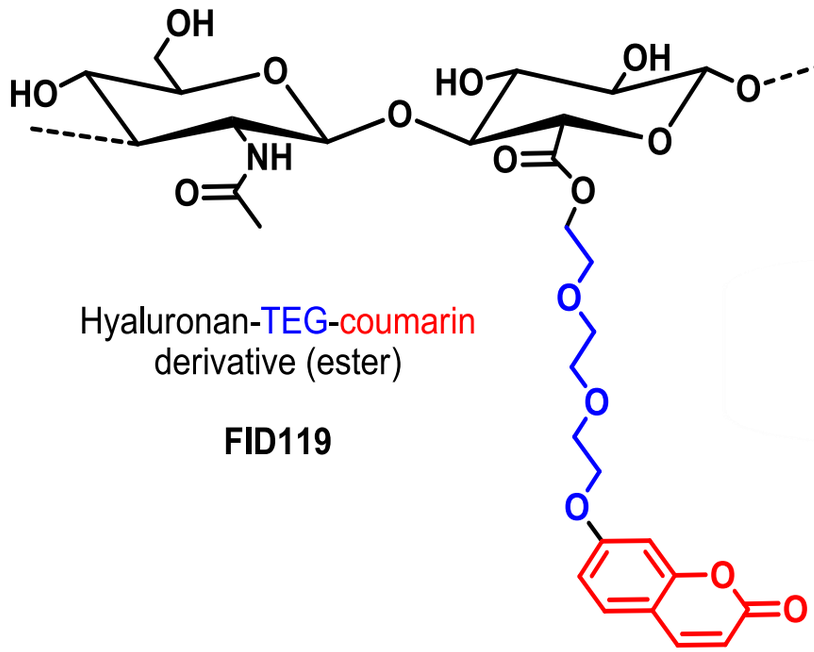
- *Strategy*



Near-UV irradiation induces coumarin [2+2] cycloaddition.

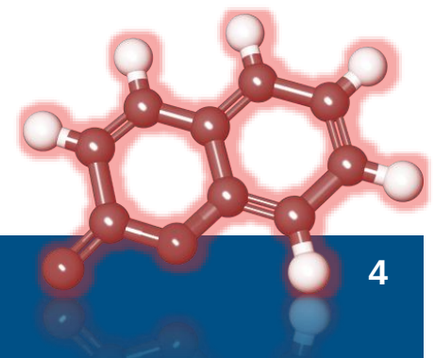
Ref. Wolff, T.; Gorner, H. Chem. Phys. 2004, 6, 368-376
Kehrlosser, D.; Trager, J.; Kim, H-C.; Hampp, N. Langmuir 2010, 26(6), 3878–3882.

- *Product*

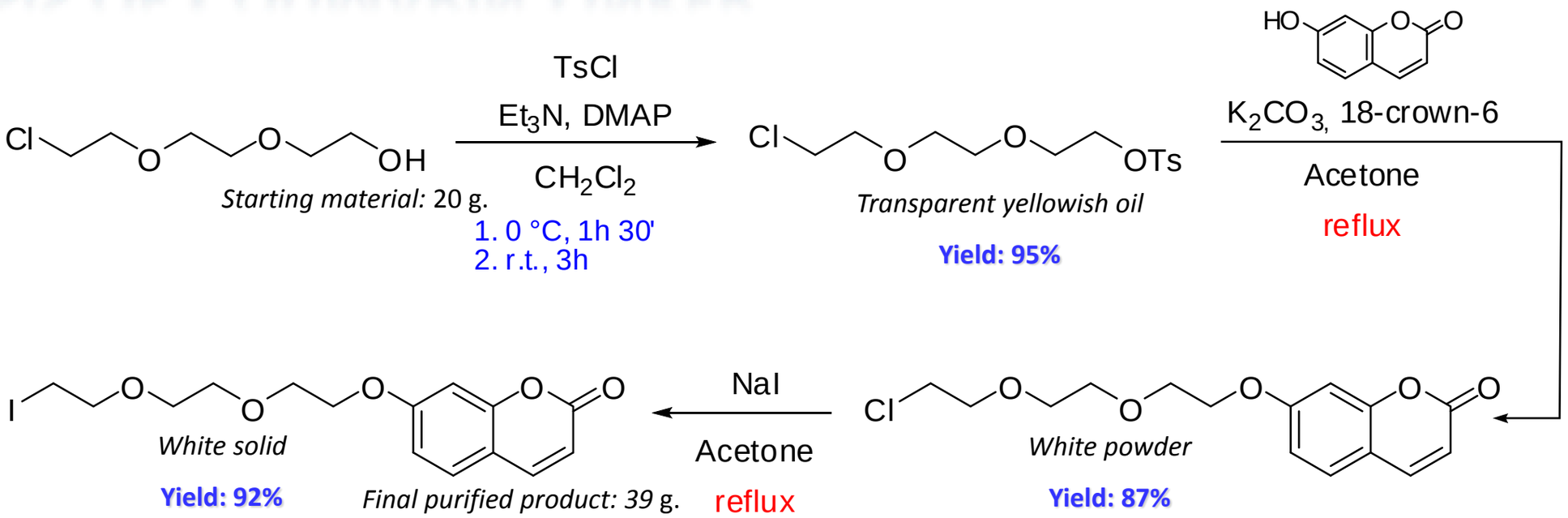


The product at a concentration from 10 to 40 mg/mL can be formulated as a solution can be sterilized by filtration (at 0,2 µm)

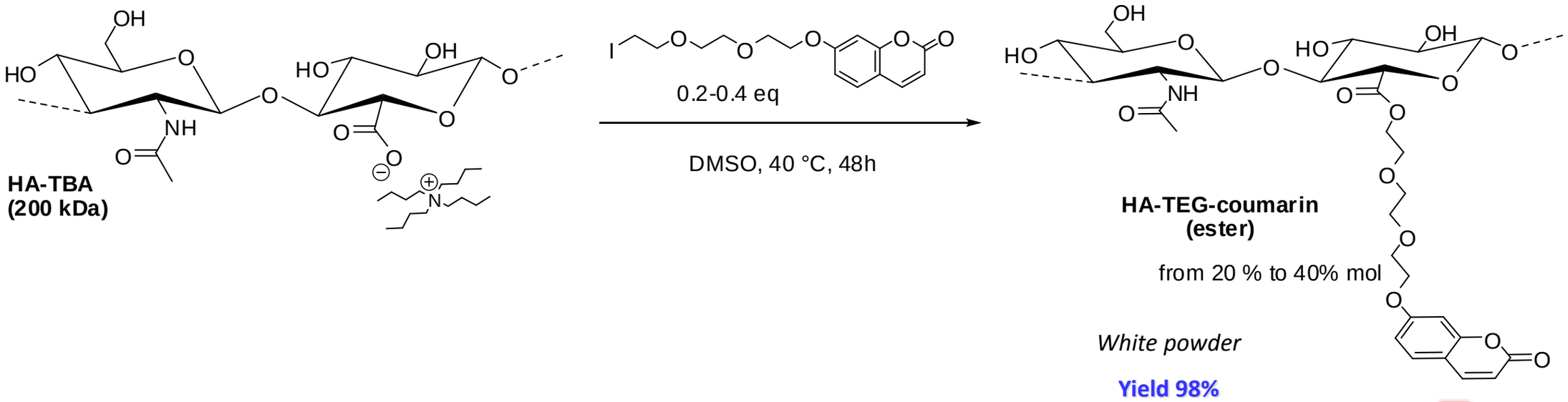
200 kDa hyaluronic acid (HA) was modified with coumarin moieties, using a polyethilenglycol spacer



SYNTHESIS OF COUMARIN LINKER

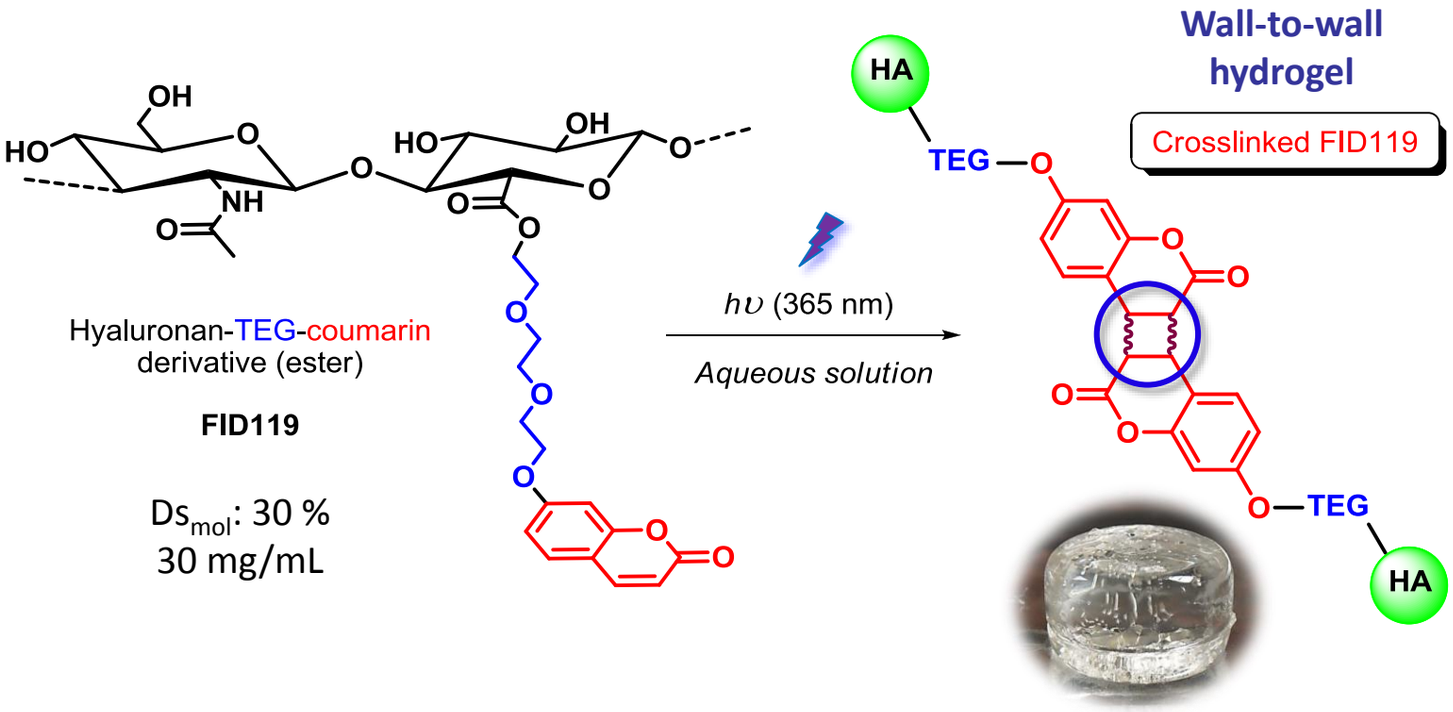


FID119 SYNTHESIS CONDITIONS



(a) Tomohiro, T.; Avval, P. A.; Okuno, H. *Synthesis* **1992**, *7*, 639-640. (b) Christensen, C.A.; Bryce, M.R.; Becher, J. *Synthesis* **2000**, *12*, 1695-1704. (c) Holmes, B.T., Snow, A.W. *Tetrahedron Letters* **2007**, *48(28)*, 4813-4815. (d) Motoyanagi, J.; Fukushima, T.; Ishii, N.; Aida, T. *J. Am. Chem. Soc.* **2006**, *128* (13), 4220-4221. (e) Ishow, E.; Credi, A.; Balzani, V.; Spadola, F.; Mandolini, L. *Chem. Eur. J.* **1999**, *5*(3), 984-989. (f) Koizumi, M.; Dietrich-Buchecker, C.; Sauvage, J-P. *Eur. J. Org. Chem.* **2004**, 770-775.

FID119: A NEW HYALURONAN DERIVATIVE



Photopolymerized into networks using a near-UV irradiation, that induces coumarin [2+2] cycloaddition.

- *In situ gel forming*:

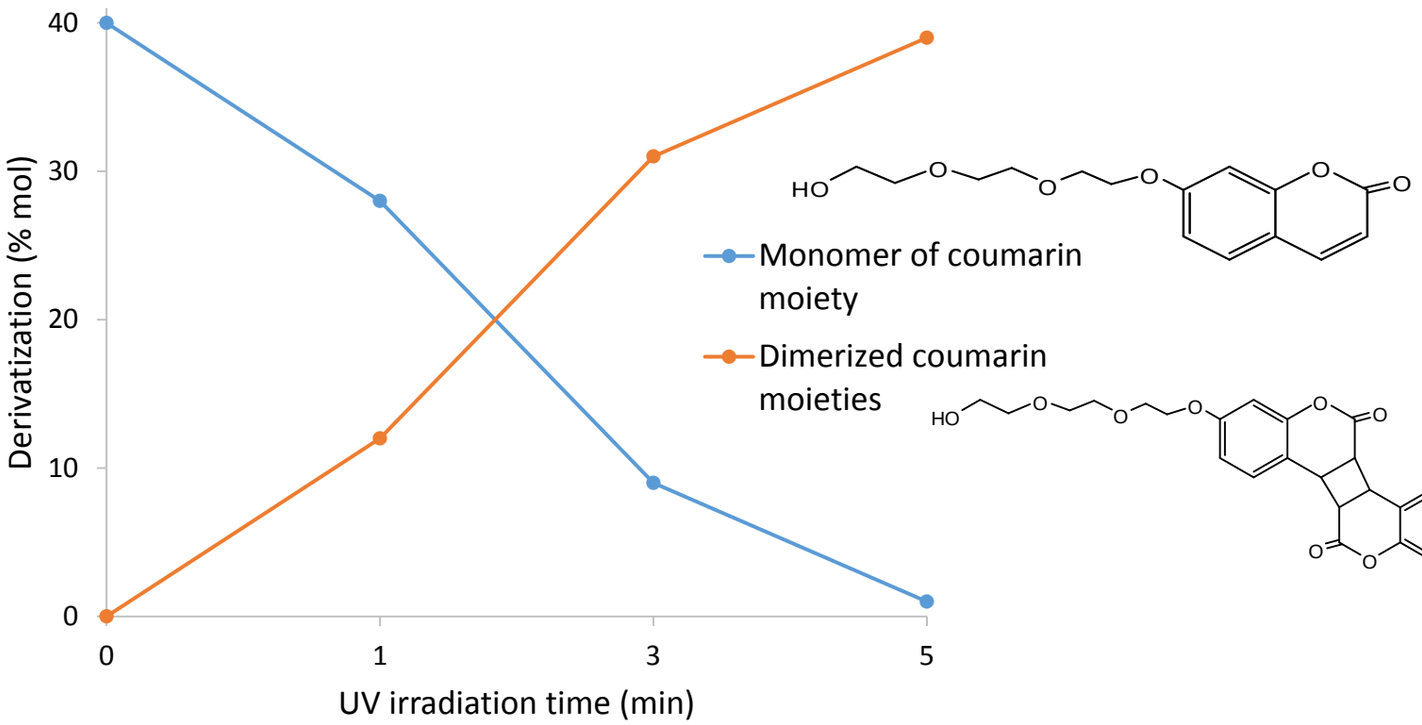
  solution → physical stimulus → gel.
- Irradiation time: 3-5 minutes.
- Novel UV quartz-led lamp (prototype of BTC *Medical Europe S.r.l.*) – emission max.: 365 nm

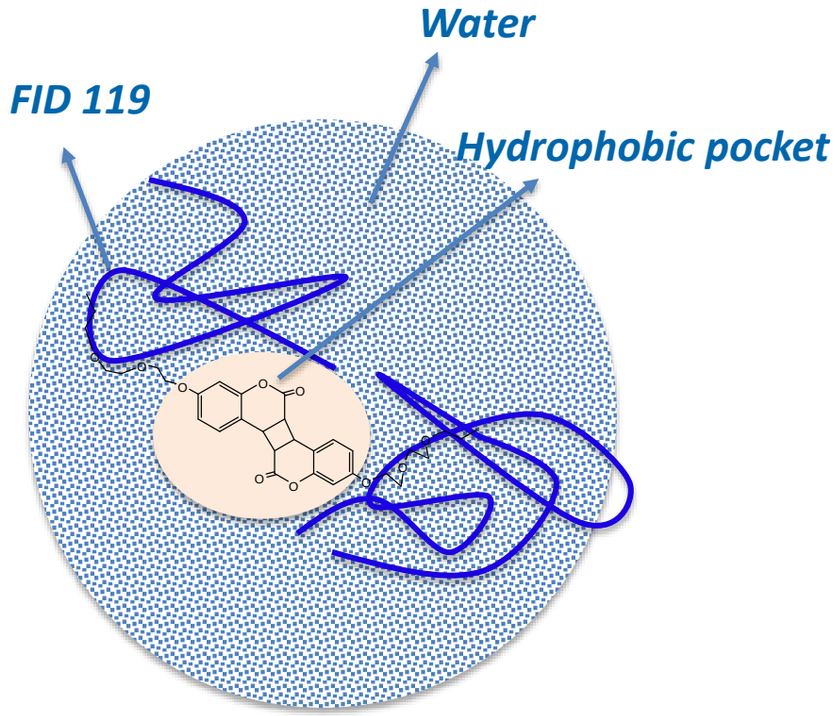
The resulting hydrogels have been tested in vitro for biocompatibility and in vivo in a mouse model of muscle loss.

FID119: CROSS-LINKING REACTION

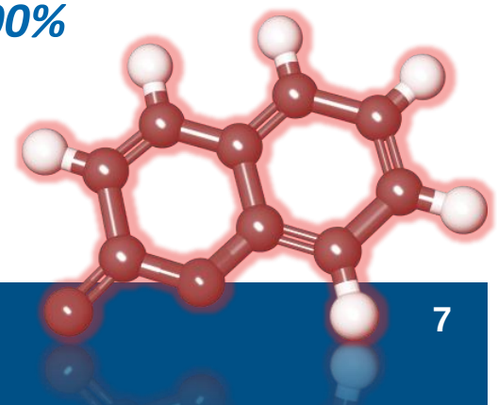
FID 119 (30% mol; 30 mg/mL)

- Four samples of the same batch were irradiated at 365 nm, each samples for a different time (0 or 1, 3 and 5')
- At each time point the sample was taken out, hydrolysed in NaOH 0,5M 40°C, and analysed by HPLC-MS





After 5' of irradiation efficiency of the cross-linking reaction is near to 100%

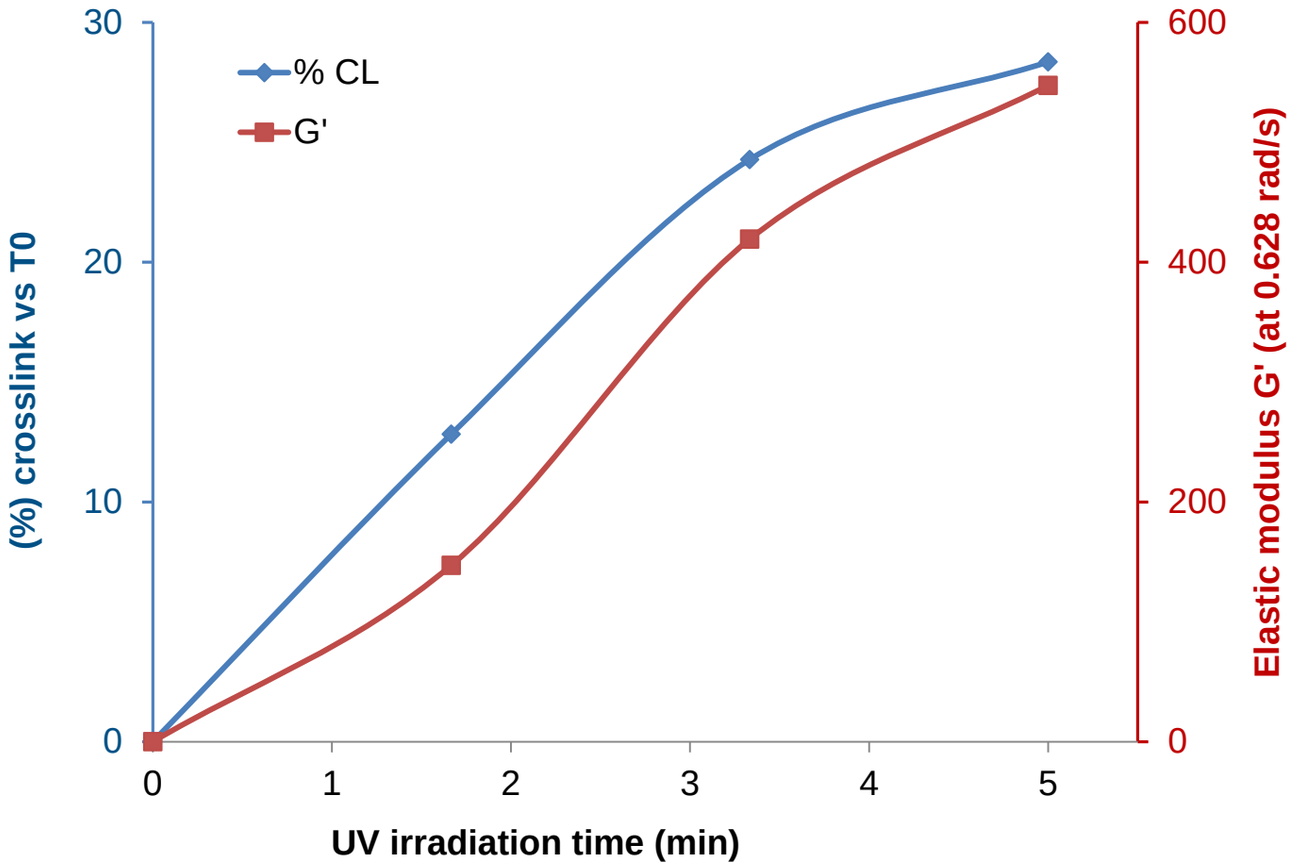


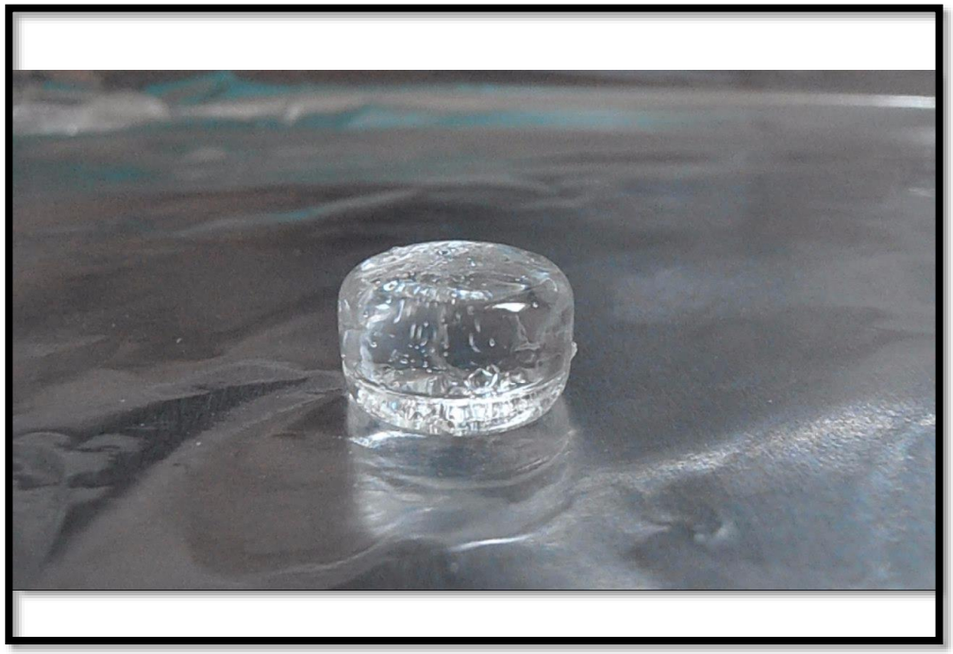
FID119: RHEOLOGICAL MEASUREMENTS

Viscoelastic moduli of irradiated FID 119 (30% mol; 30 mg/mL)

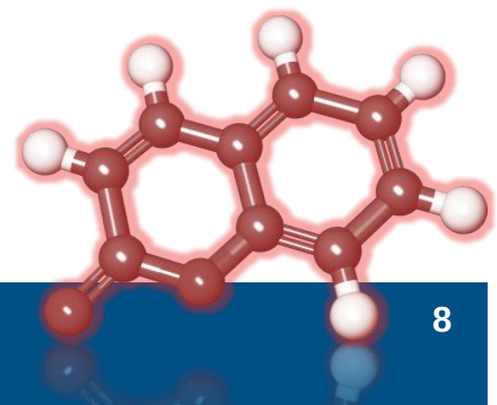
Cross-linking (%) vs. G'

- G' is proportional to the cross-linking degree: long-time irradiated solutions are more reticulated thereby displaying better viscoelastic properties.





- ✓ FID119 keeps its shape after cutting.
- ✓ Typical wall-to-wall hydrogel behavior.



POLYMERIZATION ABILITY AT DIFFERENT IRRADIATION TIMES

Molar Derivatization	Concentration (mg/ml)	Irradiation (3 min)	Irradiation (5 min)	
20%	10	✗	✗	
	20	✓	✓	
	30	✓	✓	
	40	✓	✓	
30%	10	✗	✓	
	20	✓	✓	
	30	✓	✓	p30
	40	✓	✓	
40%	10	✗	✓	
	20	✓	✓	p40
	30	✓	✓	

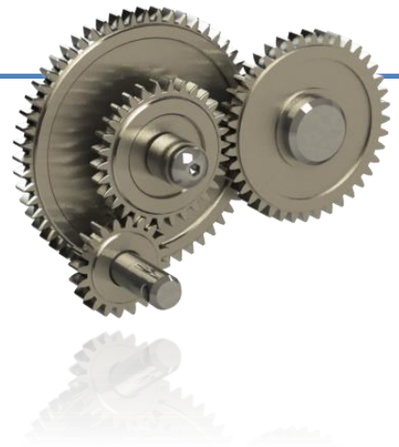
- Hydrogels at a concentration of 10 mg/mL do not polymerize to any of the degrees of derivatization tested (20%, 30% e 40%) after 3' of irradiation.
- Two hydrogel prototypes with different percentage (p) of derivatization (**p30** and **p40**) and concentration (30 and 20 mg/mL, respectively) were selected for the study.

- ✓ FID119 keeps its shape after cutting.
- ✓ Typical wall-to-wall hydrogel behavior.

PROJECT OVERVIEW

Mechanical properties

- Compression
- AFM
- FRAP

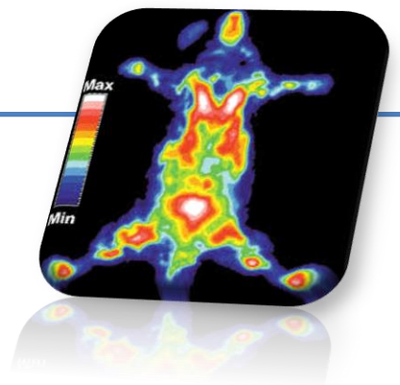


Biocompatibility

- Alamar Blue
- EdU
- Immunofluorescence (MyoD – Desmin)

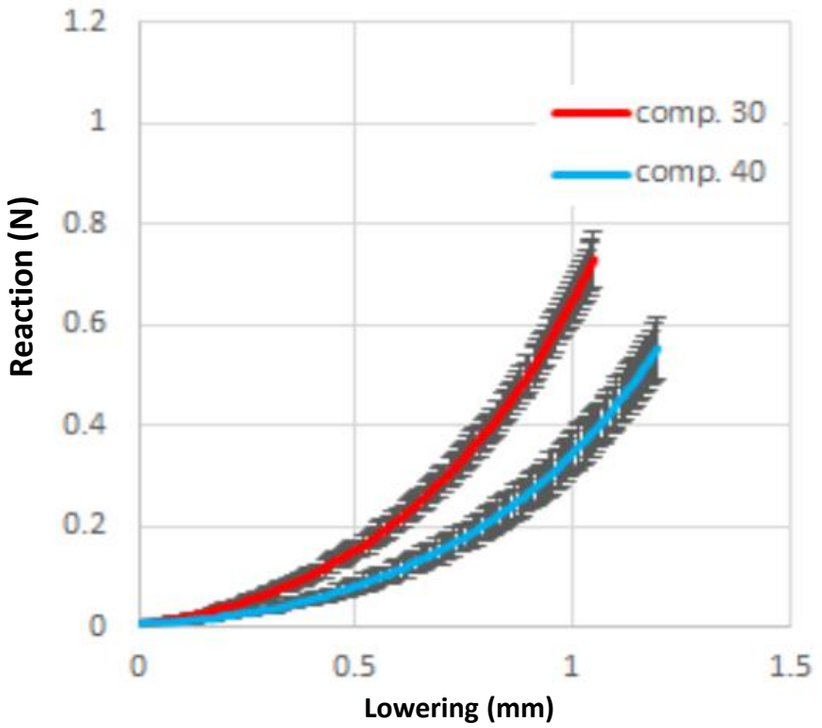
In vivo tests

- *In vivo* degradation

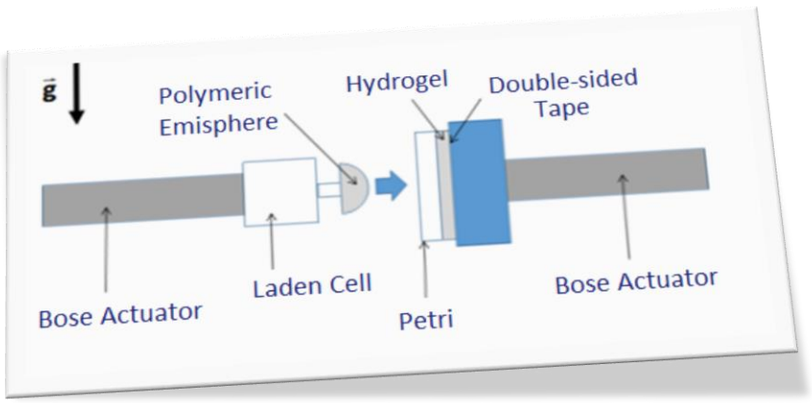


FID119: MECHANICAL PROPERTIES

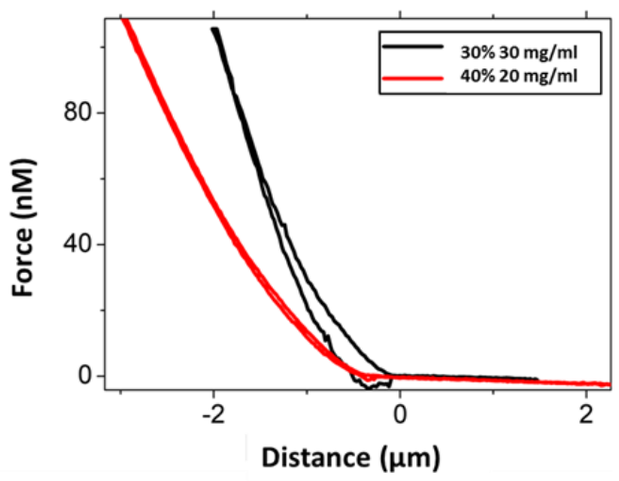
MECHANICAL COMPRESSION



MECHANICAL COMPRESSION	
p30	42.2 ± 3 kPa
p40	22.9 ± 2 kPa

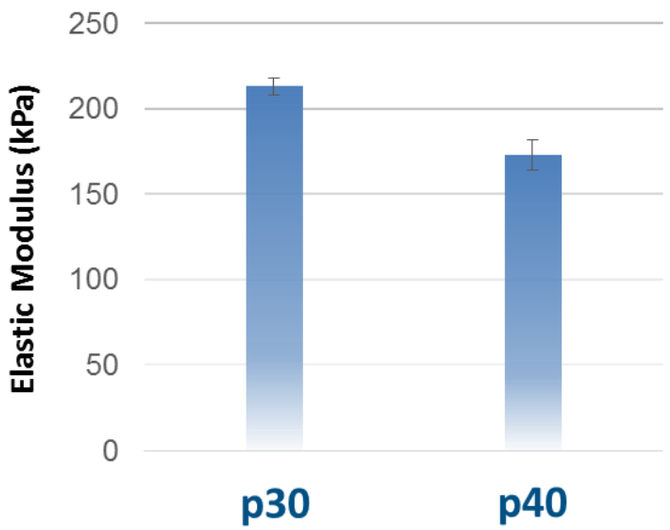


ATOMIC FORCE MICROSCOPY

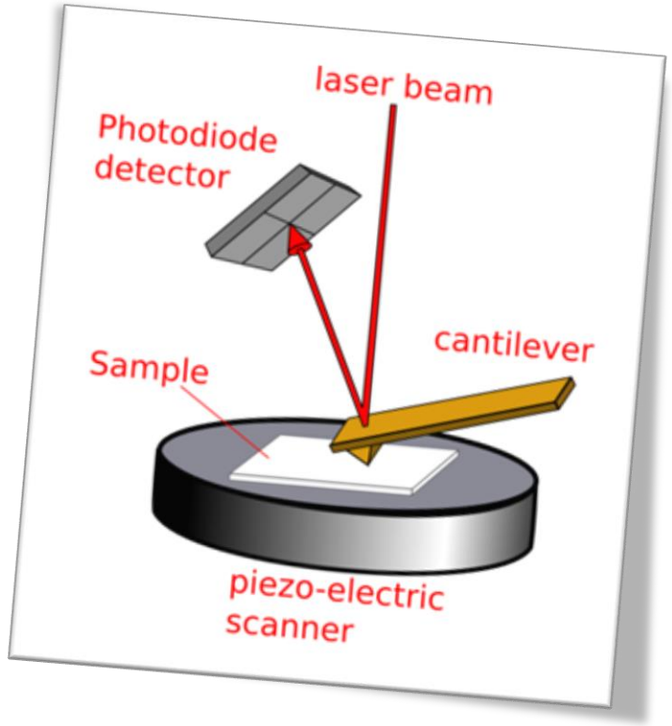


Force-distance curves for two hydrogels:
p30 (black lines)
p40 (red lines)

AFM	
p30	E_R = 213 ± 5 kPa
p40	E_R = 173 ± 9 kPa

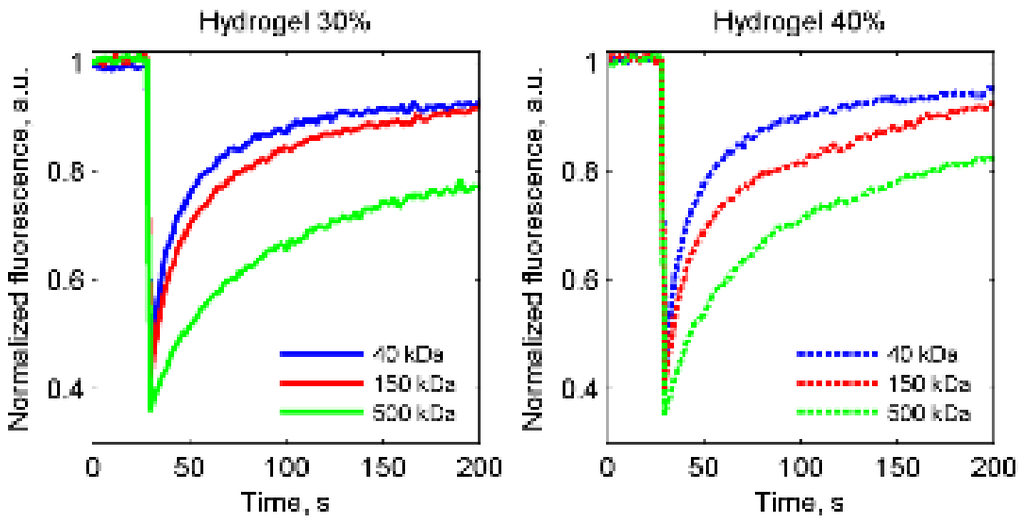


- The **p30** hydrogel is more adhesive than the **p40**, since trace and retrace do not track each other.
- Hydrogel's elastic modulus is 213 kPa for **p30** and 173 kPa for **p40**.



FID119: MECHANICAL PROPERTIES - NANOPOROSITY

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) ASSAY



Hydrogel	Dextran-FITC (kDa)	Diffusion coefficient (μm²/s)	Hydrodynamic radius (nm)
p30	40	57±3	4±1
	150	39±1	9±2
	500	NA	16±4
p40	40	55±3	4±1
	150	45±3	9±2
	500	NA	16±4

Cut-off for the diffusion of molecules with molecular weight above **150 kDa ~ 10 nm**

- The FRAP assay has been used to evaluate the hydrogels permeability.
- The hydrogels are porous: good permeation to nutrients and growth factors.
- In this field, no differences between **p30** and **p40**.

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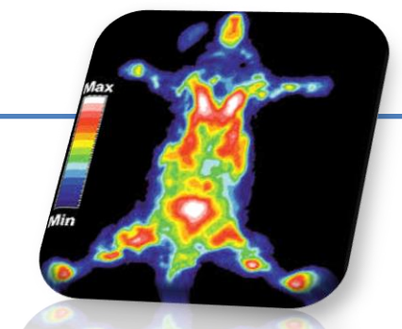
- Immunofluorescence (MyoD – Desmin)

In vivo tests

- *In vivo* degradation



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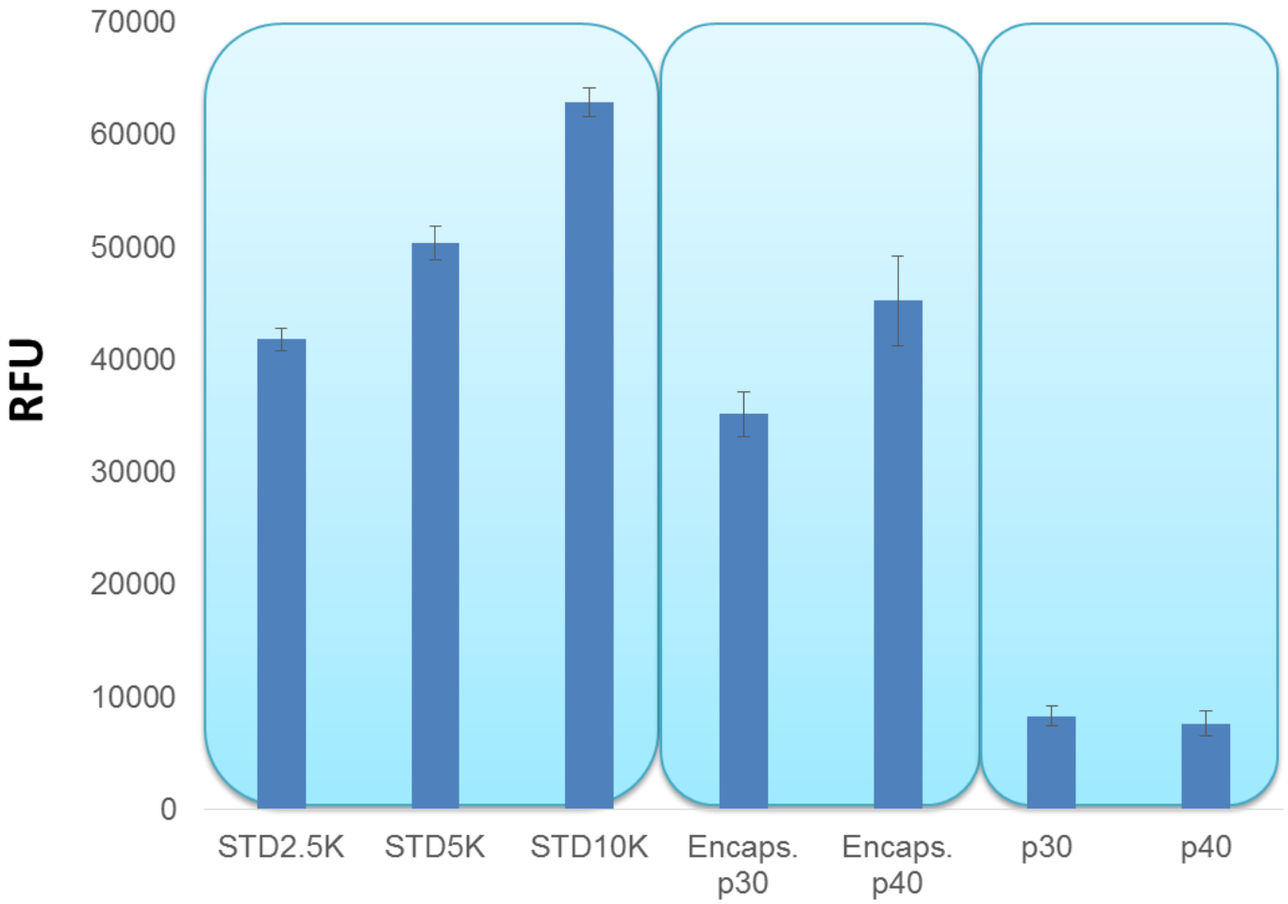
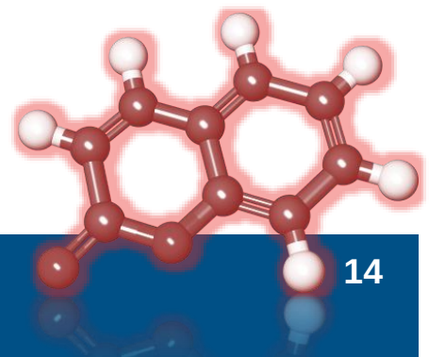
FID119: BIOCOMPATIBILITY - *Primary murine myogenic precursors (MPC)*



Alamar Blue metabolic assay

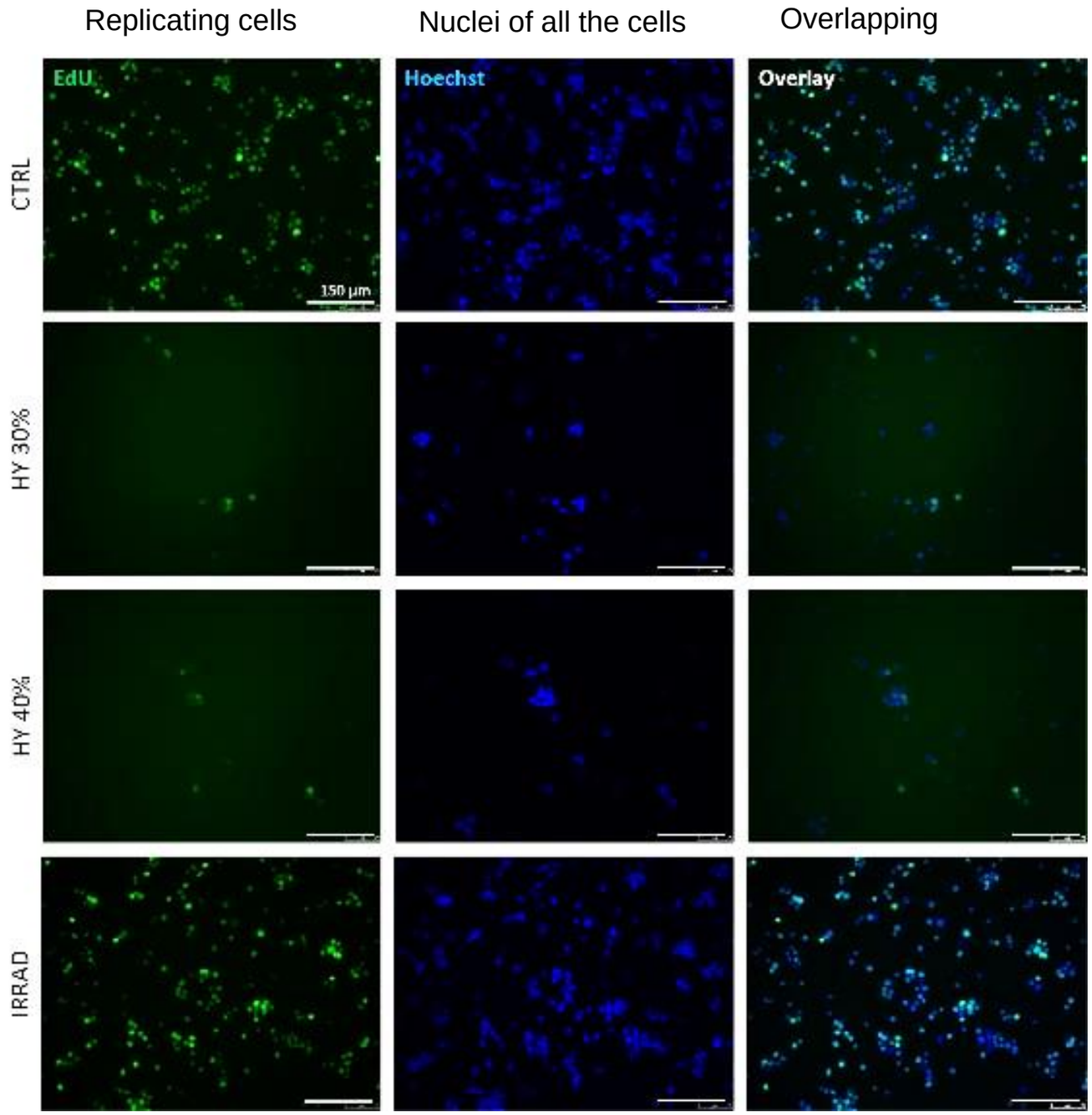
– Proliferation after 48 hours

- 10K MPC cells encapsulated within the hydrogels (5' of irradiation) showed metabolic activity values similar to the standard with 2500 cells.
- **p40** encapsulated cells showed a higher metabolic activity compared to **p30**.



FID119: BIOCOMPATIBILITY

EdU *(5-ethynyl-2'-deoxyuridine) Assay: DNA synthesis in proliferating cells*



SAMPLE	PROLIFERATING CELLS [%]
CTRL	90 ± 5
p30	25 ± 4
p40	35 ± 3
IRRADIATED	95 ± 6

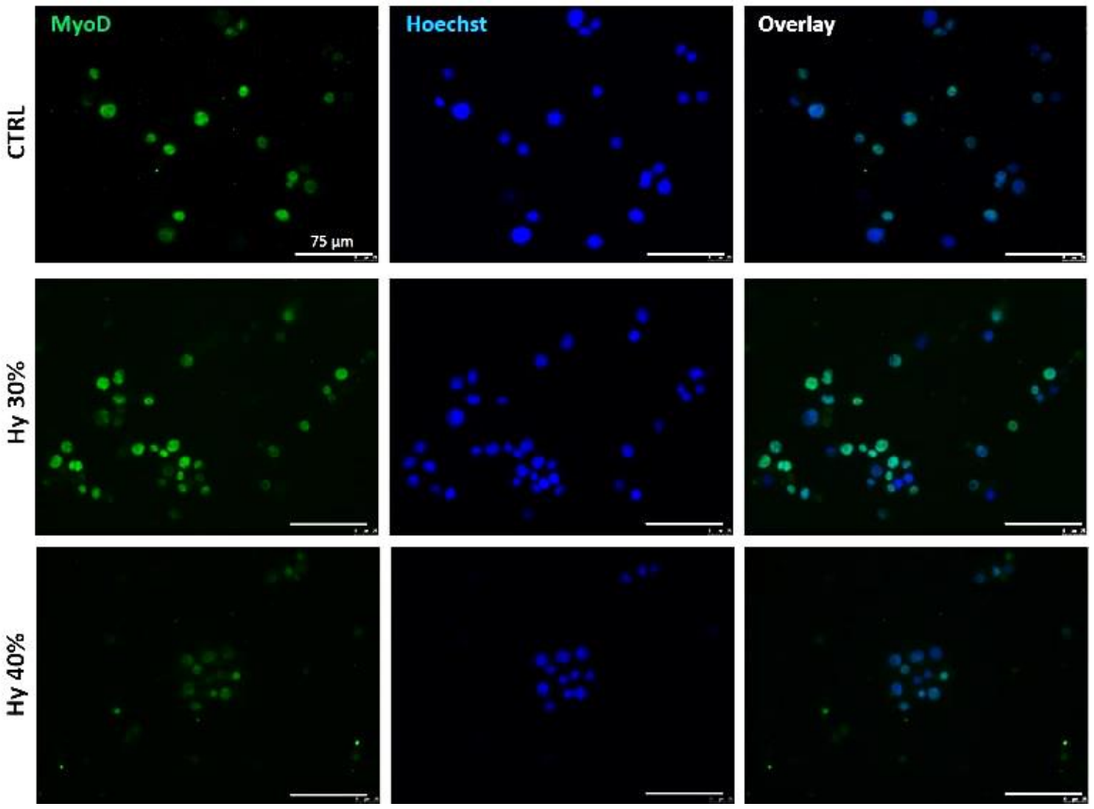
- In case of encapsulated cells, the hydrogel was degraded with hyaluronidase in 12 hours, then cells were transferred to microscope slide by means of Cytospin.
- Cells are proliferating for both the FID119 (**p30** and **p40**).
- UV irradiated cells (not encapsulated) showed the same proliferation level of the control. → *the UV irradiation isn't cytotoxic.*

FID119: BIOCOMPATIBILITY

Expression of muscular biomarkers

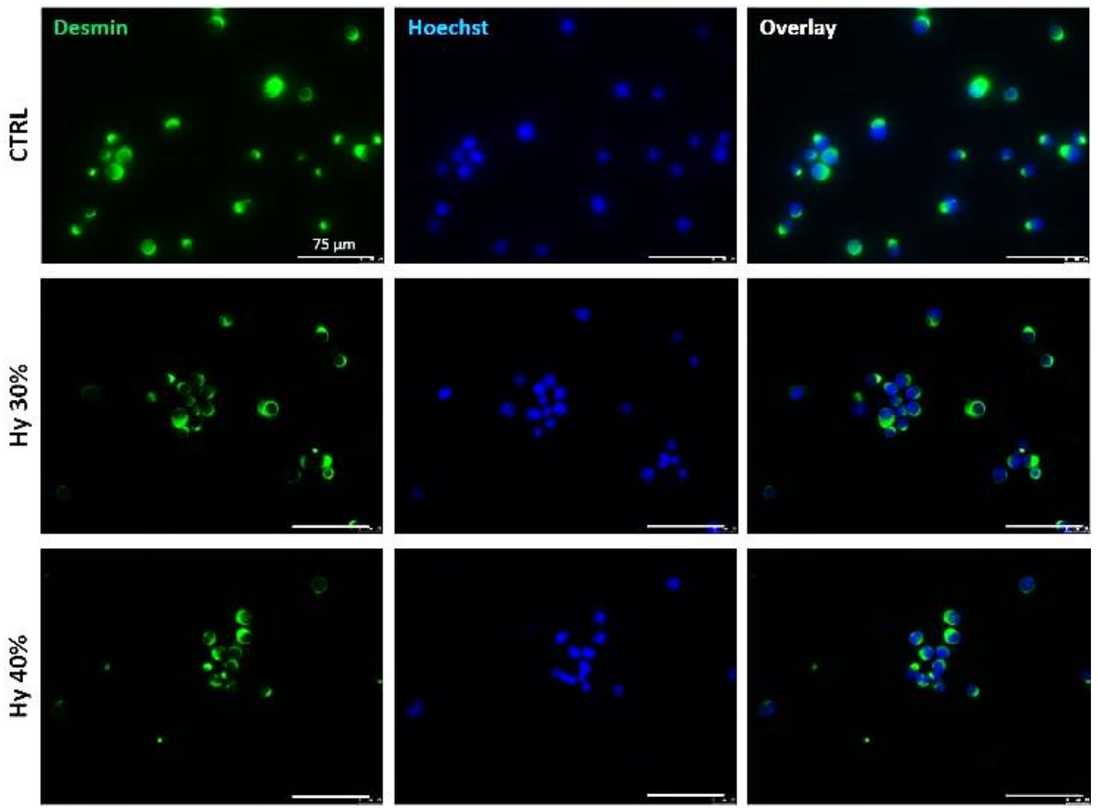
Cellular proliferation (EdU) and immunofluorescence were carried out to determine the ability of encapsulated cells to maintain the expression of two muscular markers: MyoD and Desmin, present in different stages of myogenesis.

MyoD – the main muscular transcription factor.



Sample	MyoD positive [%]
CTRL	86 ± 5
p30	72 ± 6
p40	60 ± 7

Desmin – intermediate fiber near Z line in sarcomeres.



Sample	Desmin positive [%]
CTRL	99 ± 3
p30	99 ± 2
p40	94 ± 4

Encapsulated samples were treated with hyaluronidase

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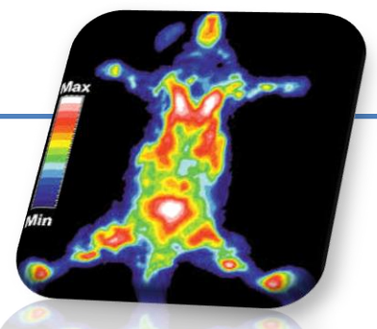

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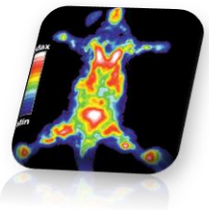
FID119: *IN VIVO* TESTS



1) *In vivo* degradation

Muscle ablation (Tibias anterior): 20%.

2 mice: right paw p40, left paw p30.
Sacrifice at 5 days.

2 mice: right paw p40, left paw p30.
Sacrifice at 2 weeks.

1 mouse: sham operated (no hydrogel).
Sacrifice at 2 weeks.

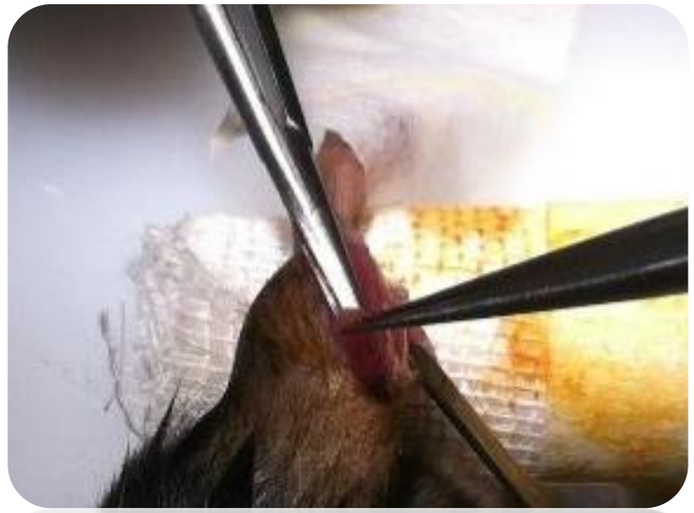
2) Degradation rate (p30 only)

Muscle ablation: 20% and 50%.

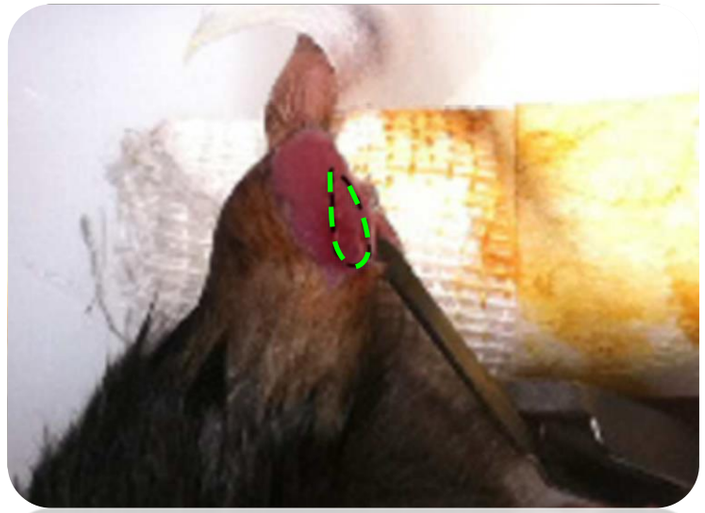
2 mice: right paw 20%, left paw 50%.
Sacrifice at 2 weeks.

2 mice: right paw 20%, left paw 50%.
Sacrifice at 6 weeks.

[A] Muscle exposure.

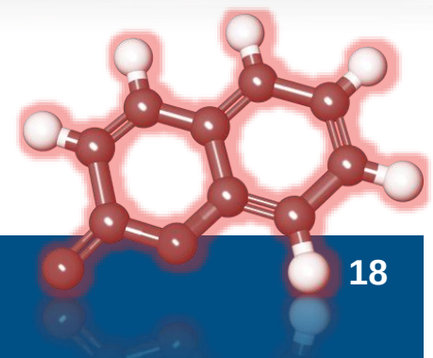


[B] Pocket creation.



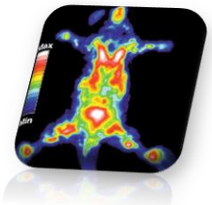
[C] Pocket area.

[D] Suture.



FID119: *IN VIVO* TESTS

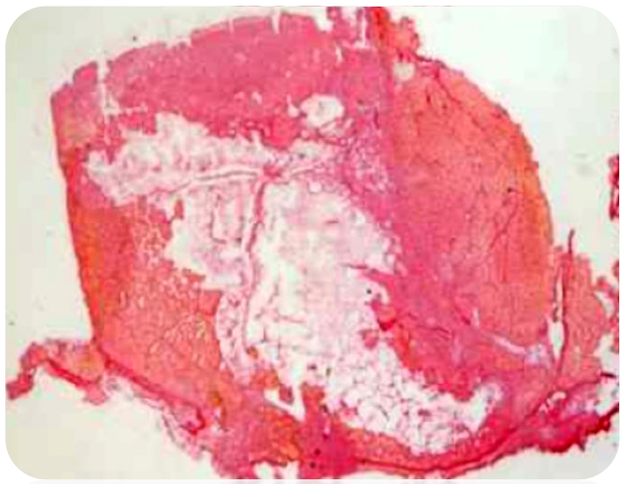
Degradability

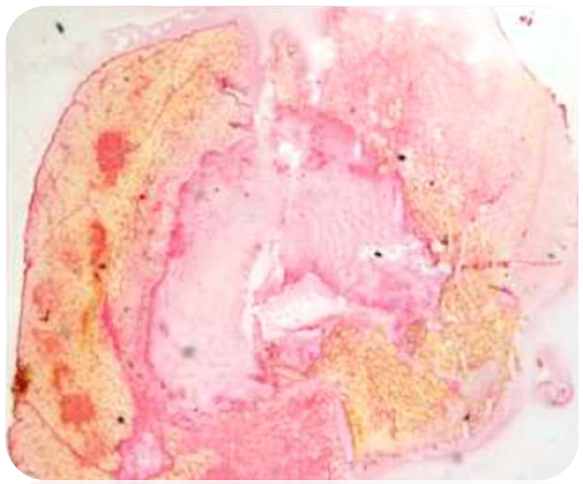


p30

p40

Sirius red





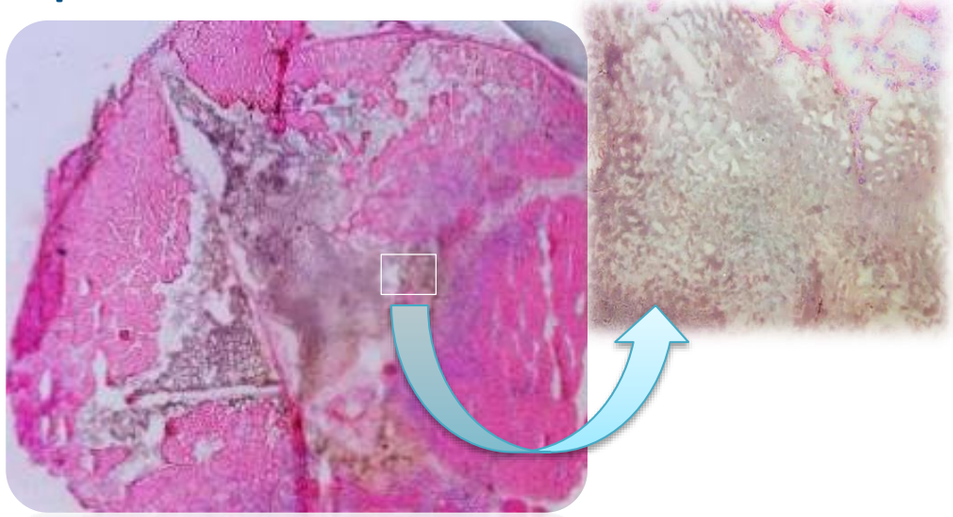
Muscle sections after 5 days

- ≈ 20% muscle removal.
- Hydrogels are still present: **p40** seems to be more compact.
- Evidence of cellular infiltrate at the edge of the hydrogels, not in the center.

p30

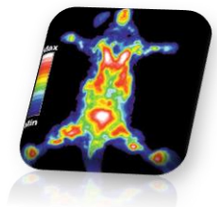
p40

Hematoxylin & Eosin



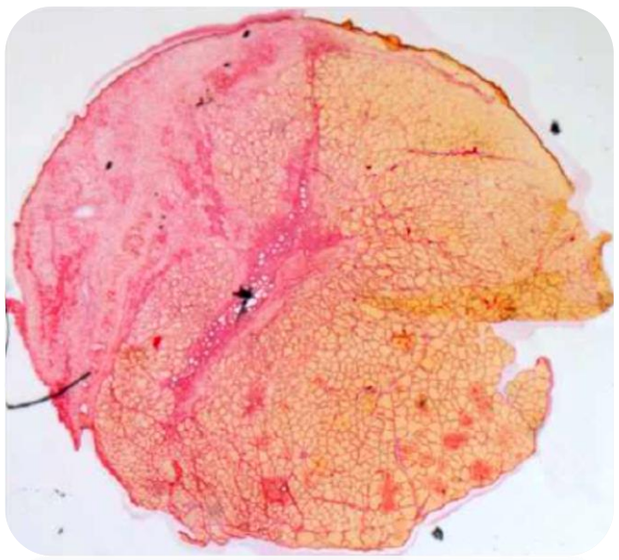
FID119: *IN VIVO* TESTS

Degradability

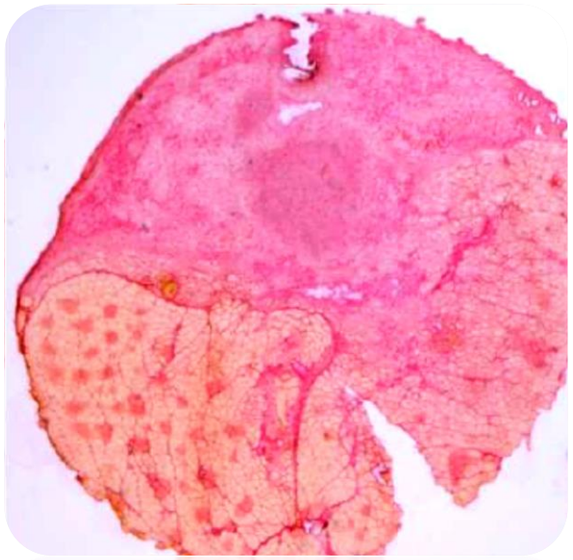


Sirius red

p30

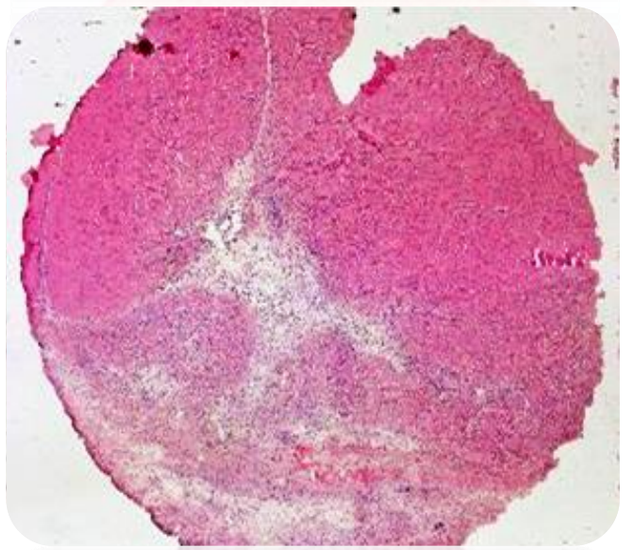


p40

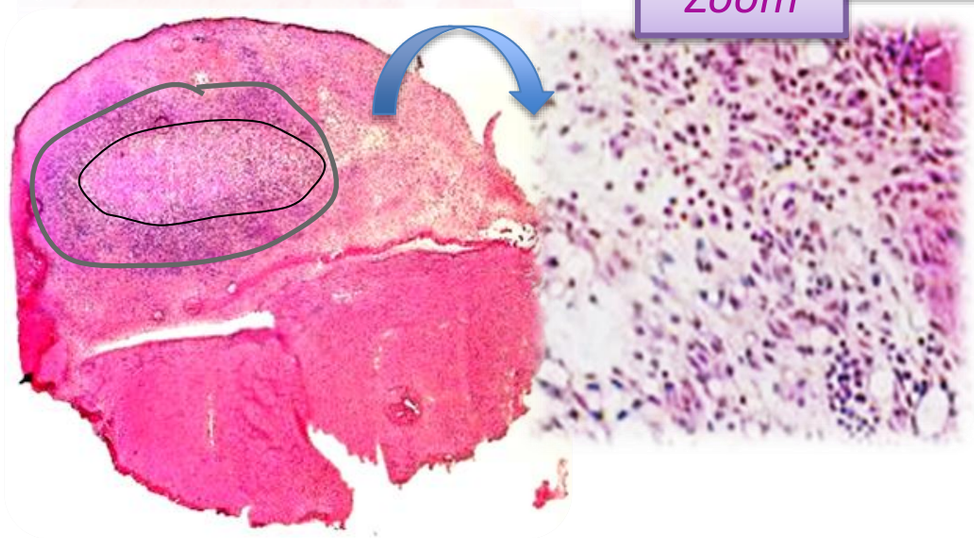


Hematoxylin & Eosin

p30



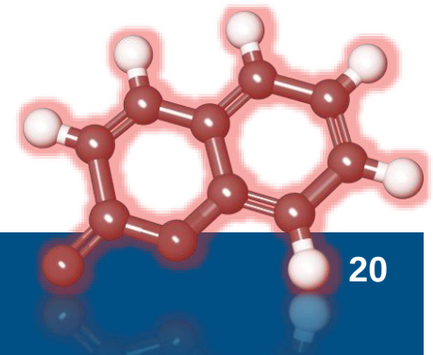
p40



Muscle sections after 2 weeks

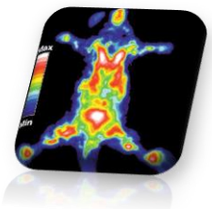
- Most of the hydrogels were degraded.
- Significant muscular regeneration.

- Small, center-nucleated myofibrils at the edge of the hydrogel.
- New muscle is infiltrating the hydrogel.



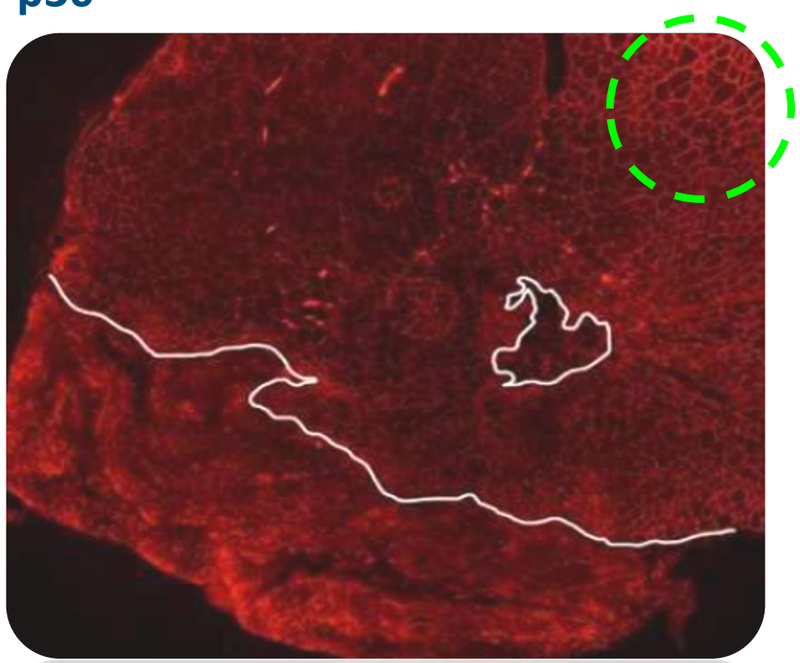
FID119: *IN VIVO* TESTS

Muscular regeneration at 2 weeks

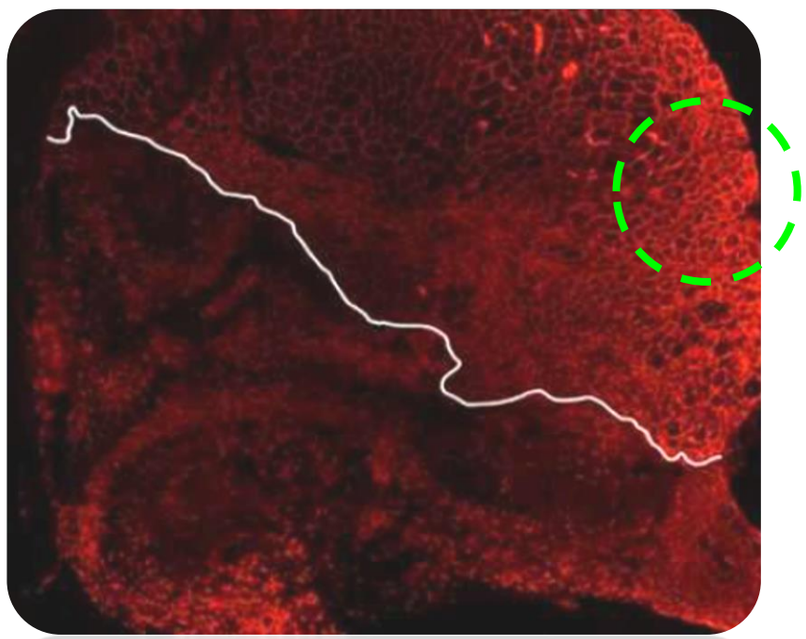


Dystrophin (immunohistochemistry)

p30

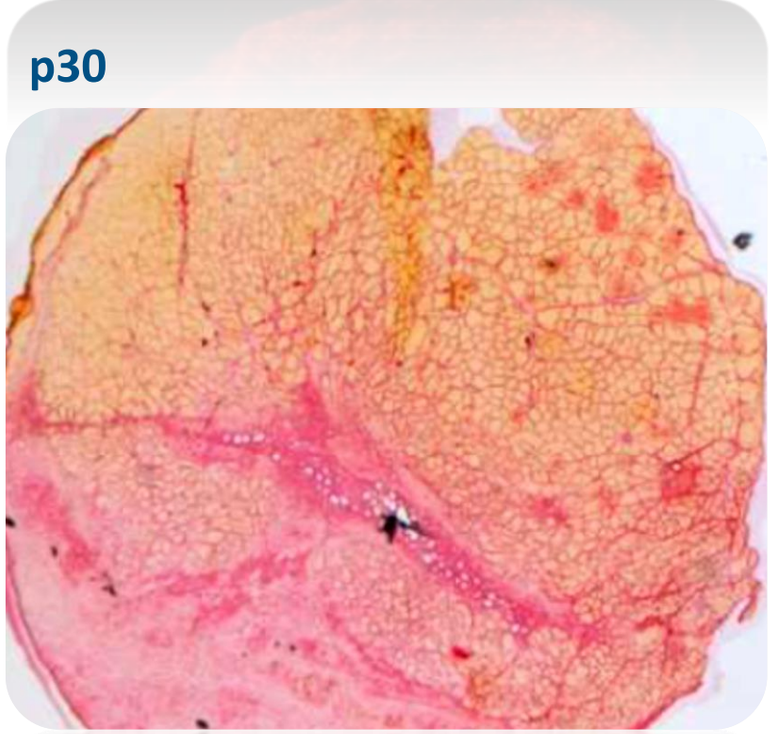


p40

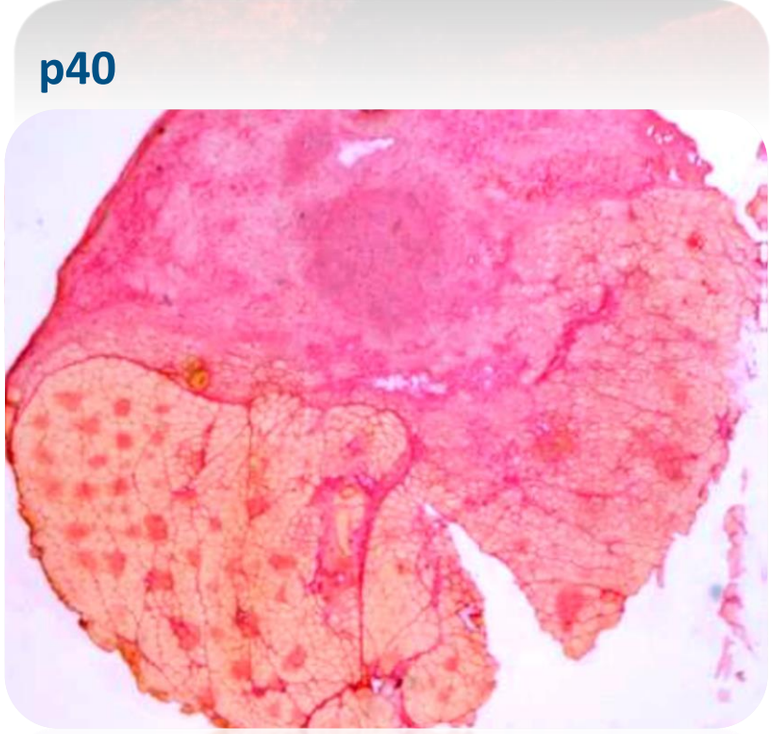


Collagen (Sirius Red)

p30



p40

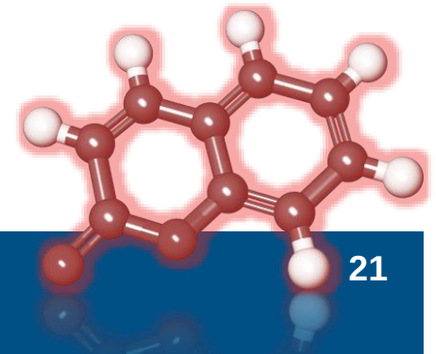


Dystrophin expression

- In evidence: regenerated muscle area, with small fibers.
- Muscle reconstitution area is larger in case of **p30**.
- Degree of functionalization affects the kinetic of process regeneration.

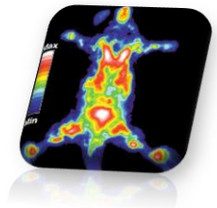
Collagen analysis

- Muscles treated with **p40** have a higher collagen deposition respect of **p30**.
- **p30** promotes a faster regenerative process.



FID119: *IN VIVO* TESTS

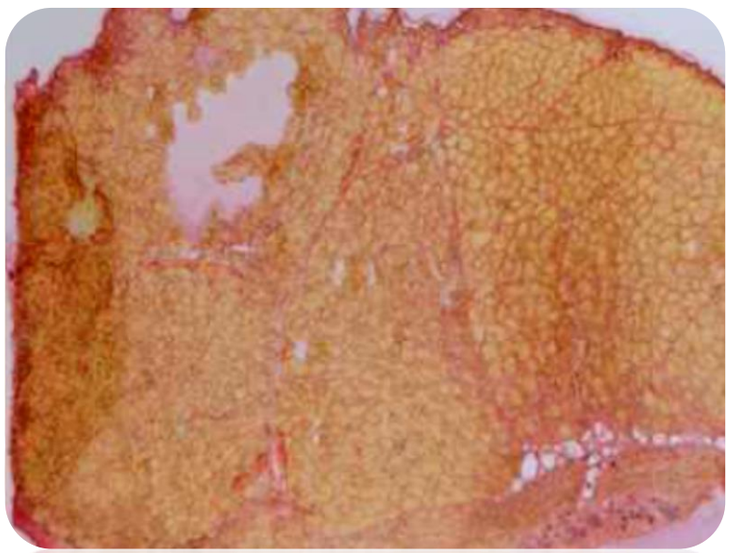
Muscular regeneration at 6 weeks

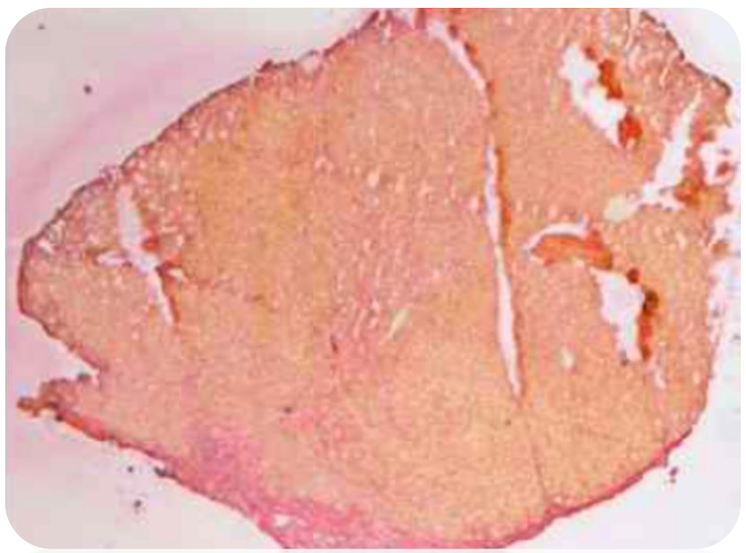


p30, 20% muscle ablation

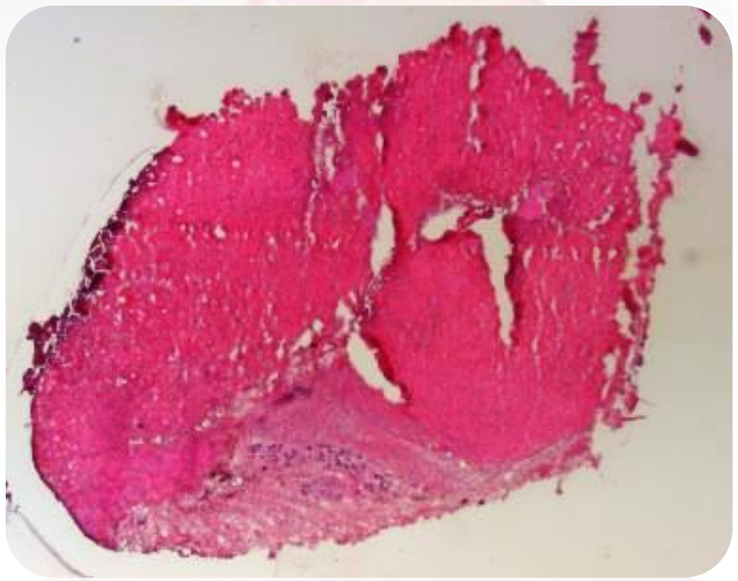
p30, 50% muscle ablation

Sirius red

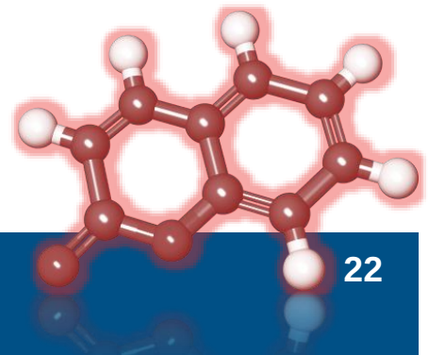




Hematoxylin & Eosin



- 20% muscle ablation (12 µL FID119 treatment): major regeneration.
- 50% muscle ablation (24 µL FID119 treatment): less regeneration.

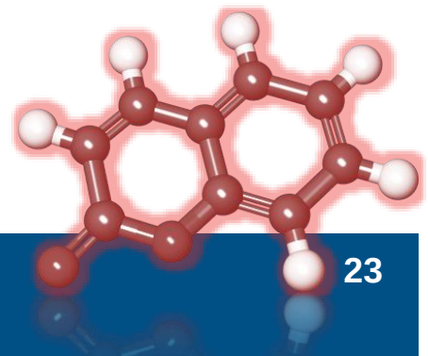


CONCLUSIONS

New hyaluronan hydrogels for skeletal muscle repair have been developed and characterized.

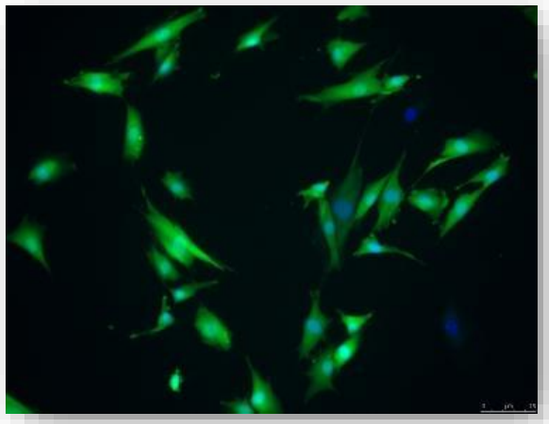
- Good mechanical properties (elastic moduli in line with muscular values).
- Permeable to nutrients and small soluble factors (diffusion cut-off larger than 150 kDa).
- Able to sustain cellular proliferation and maintain myogenicity after 48 hours culture.
- *In vivo* experiments highlighted the hydrogels efficacy.
- Good degradation profile (residence time: 2 weeks).

Muscle repair is a balance between new tissue formation and dissolution of engineered scaffolds.

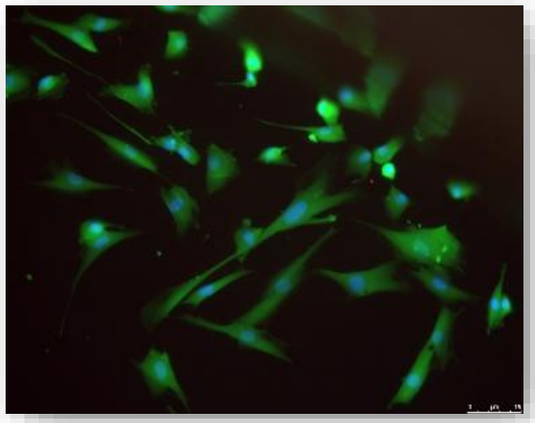


Thank you for Your attention!

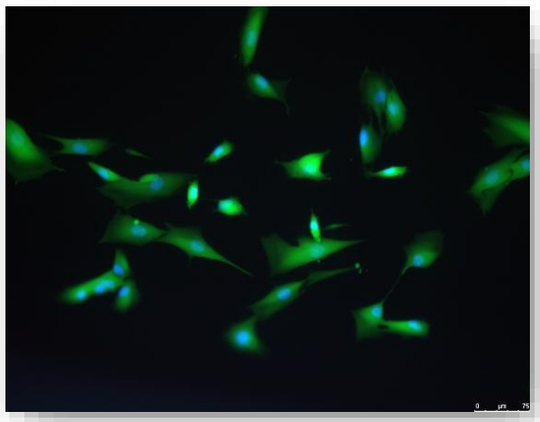
CELL VITALITY ASSAY AFTER UV EXPOSURE/ENCAPSULATION



Control (no UV)



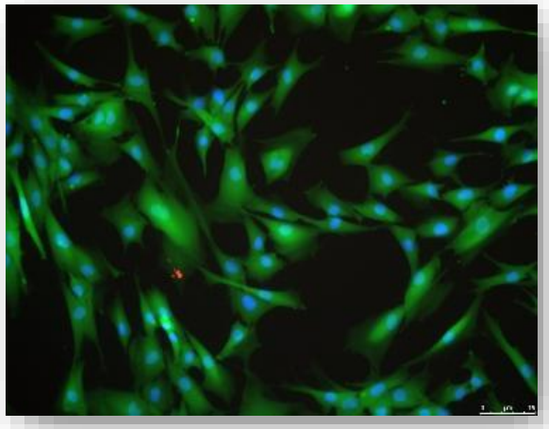
3’ irradiation



5’ irradiation

Fibroblasts on tissue culture plates

- All cells are viable 24 hours post-UV irradiation.
- No significant differences between 3 or 5 minutes UV irradiation.



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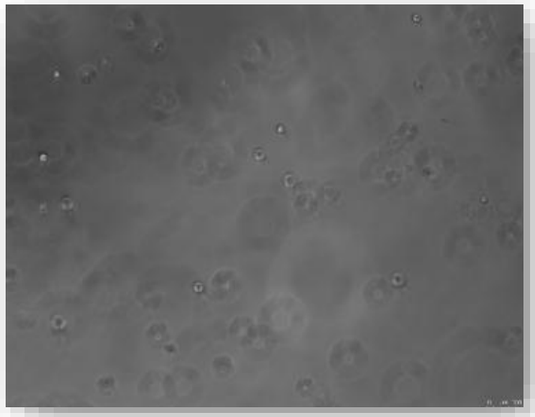
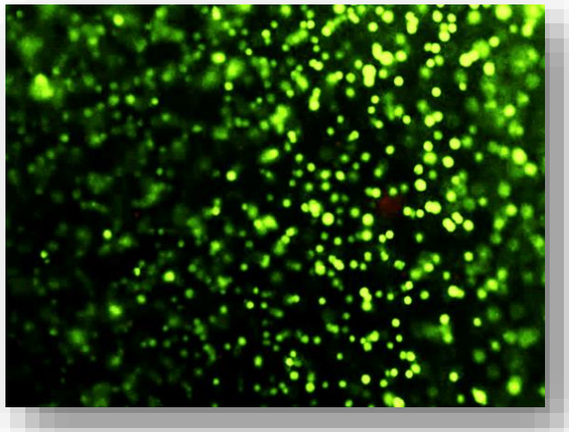


Encapsulated
(*phase contrast*)



Encapsulated
(*live & dead*)

Encapsulated cells

- Hydrogel-encapsulated cells are viable, show a spherical morphology and are present in different layers of the hydrogel.

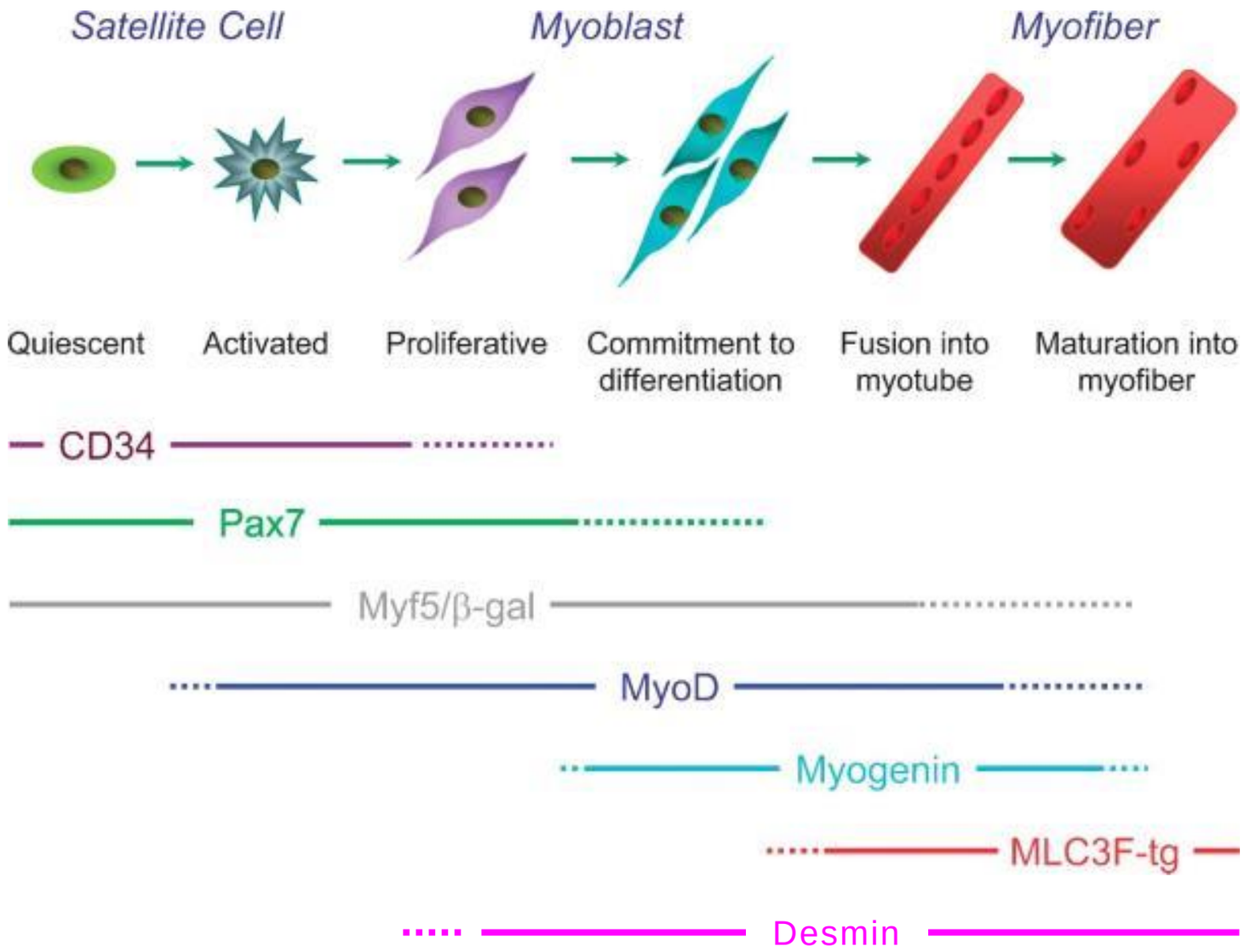
FID119: BIOCOMPATIBILITY

Expression of muscular biomarkers

- Two muscular biomarkers were analyzed:

1. **MyoD** – the main muscular transcription factor.
2. **Desmin** – intermediate fiber near Z line in sarcomeres.

- Encapsulated samples were treated with hyaluronidase.

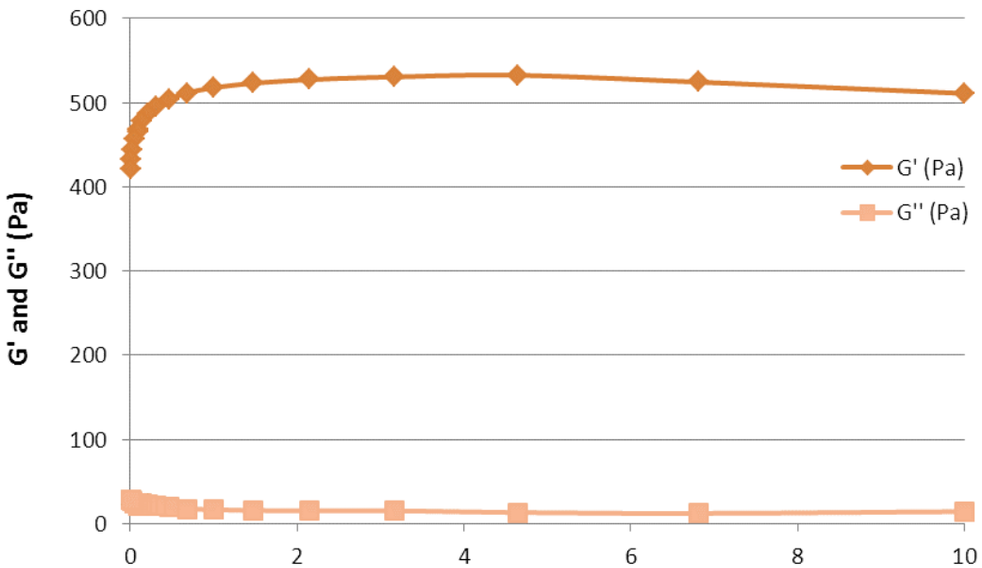


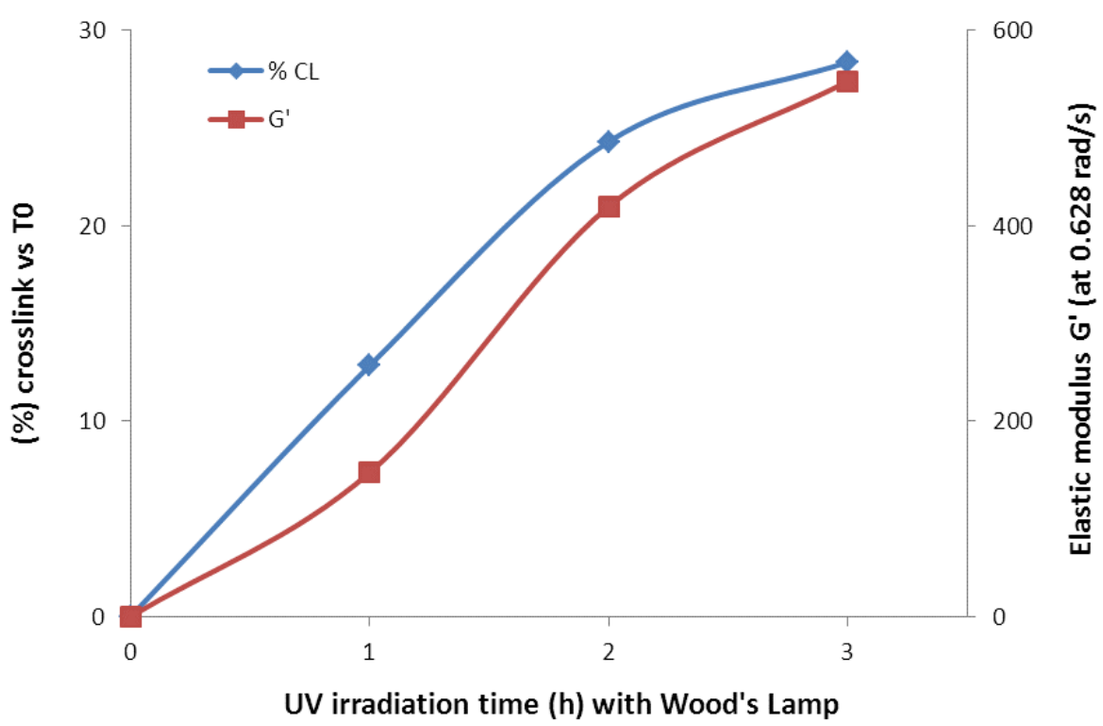
Zammit et al., J Histochem Cytochem 54:1177-1191, 2006.

FID119: RHEOLOGICAL MEASUREMENTS

Viscoelastic moduli of irradiated FID 119

- T=25 °C.
- G’ (elastic modulus) and G’’ (viscous modulus) are observed from 0.07 to 90.0 rad/s, with a strain value equal to 10%.
- G’ > G’’ in the complete frequencies range: FID 119 is a real wall-to-wall hydrogel.





Cross-linking (%) vs. G’

- G’ is proportional to the cross-linking degree: long-time irradiated solutions are more reticulated thereby displaying better viscoelastic properties.