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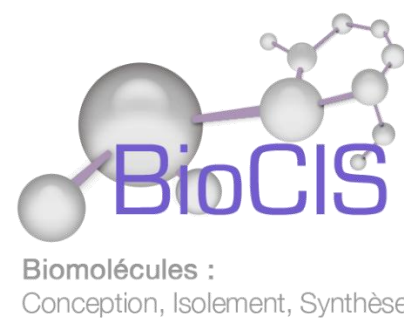


# Enhancing biomedical metal implants with synthetic peptides: improved bioactivity and in vitro safety

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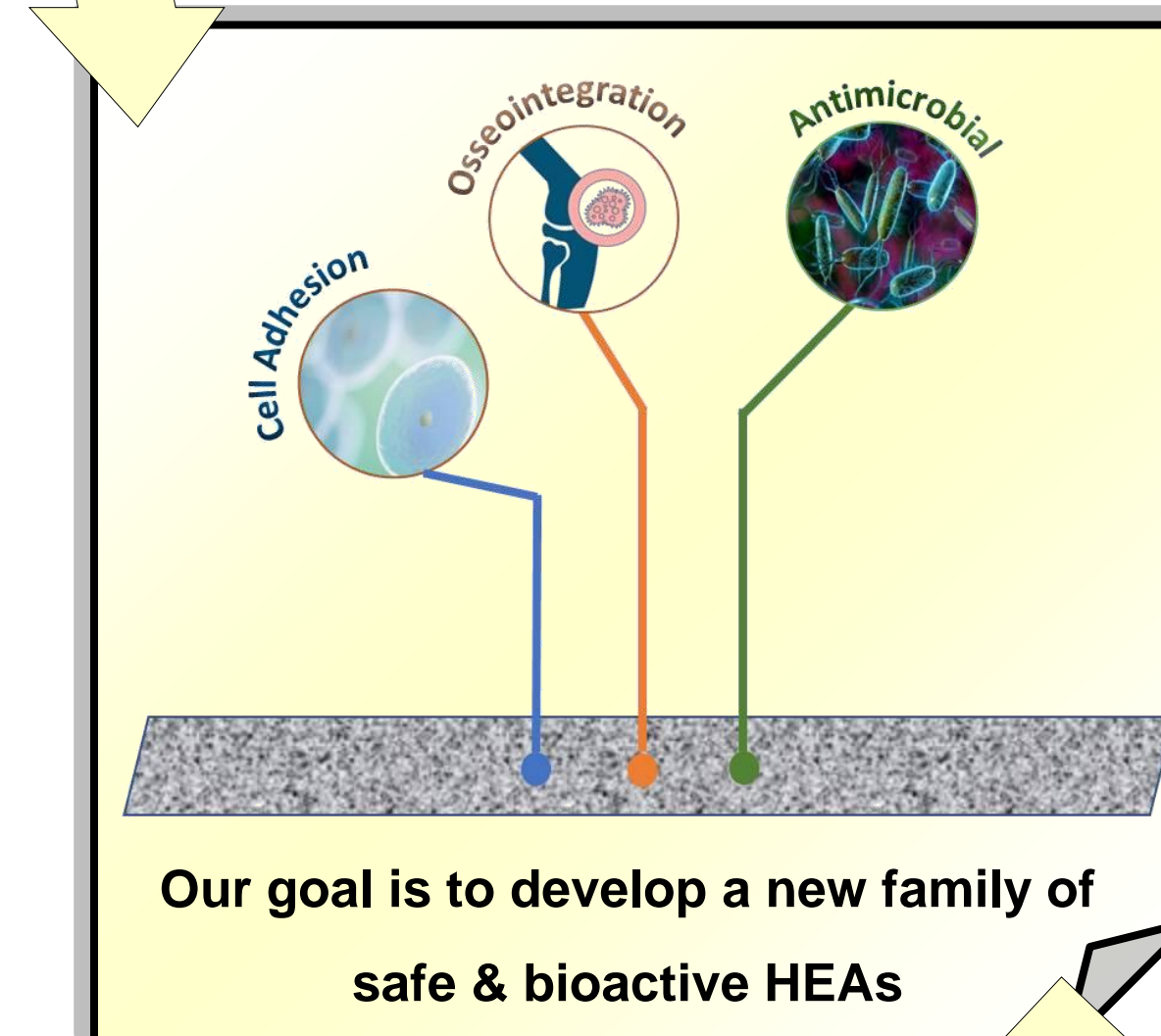
Modern solutions in biomedical engineering imply innovative strategies at the frontier between chemistry, material science, and biology to develop biofunctionalized substitutes able to restore, maintain or improve tissue integrity. These substitutes are materials that are designed to positively interact with a living biological system for a therapeutic purpose while introducing mechanical, physical and/or chemical functions to the body. Commonly speaking, they are defined as biomaterials [1].

It is of paramount importance to enhance the bioactivity of materials scaffolds by combining biomaterials with biological cues to drive a particular tissue function [2]. In practice, biocompatibility can be achieved by first choosing bioinert solids and then coating them with bioactive molecules.

Interestingly, different biological effects may be targeted when implanting a biomaterial by combining peptides from diverse nature. In the field of bone regeneration, the use of BMP-2 derived peptides is of crucial importance to promote osteodifferentiation of mesenchymal stem cells (MSCs) and guide bone formation [3].

Peptides are easily modifiable for functionalization of surfaces, reaching, in some cases, similar biological activity to the total protein for a specific target [4].

A simple though useful approach to improve the performance of peptidic molecules is to combine peptide sequences with synergistic or complementary effects, enabling to address two or more biological effects. In this way, multifunctionality may be installed on biomaterials without using proteins.



Our goal is to develop a new family of safe & bioactive HEAs

High Entropy Alloys (HEAs) are a novel class of materials known for their exceptional mechanical properties and corrosion resistance. These alloys generally are composed of multiple principal elements in equimolar or non/near-equimolar ratios, with elemental concentrations ranging from 5% to 35%. Among these HEAs have been specifically developed for biomedical applications. Notable examples include equiatomic and non-equiatomic alloys like TiZrNbTaMo, which have attracted significant attention as a new generation of biomaterials. However the integration of such materials as implants into bone is only addressed as a passive process. In such an approach, the fixation is mainly mechanical, with anchorage failure. Therefore, the increase of the biointegration of the implant and the bone tissue is becoming a research hotspot. Such an active integration is usually achieved by grafting bioactive molecules on the surface of the metal implant.

## Proadhesion and osteogenic peptides synthesis for multifunctional alloy coating

### Peptide selection

	Peptide sequences	Description
P1	KIPKASSVPTELSAISTLYL	These 2 peptides sequences are the BMP-2(73-92) fragment of the Bone morphogenetic proteins BMP-2, that is the most investigated and potent osteoinductive growth factors in bone regeneration[5].
P2	KIPKASSVPTELSAISTLYL(KAMRA)PEG <sub>9</sub> -NH <sub>2</sub>	
P3	TAMRA-GGCRGD	RGD peptide has been demonstrated to be able to increase the expression of osteogenic markers and mineralization in hMSCs culture[6]

### Synthetic conditions:

Peptides were synthesized in solid-phase using a microwave-assisted protocol (MW-SPPS) on a Liberty Blue™ automated peptide synthesizer (CEM Corporation, Matthews, NC, United States), following the Fmoc/tBu strategy.

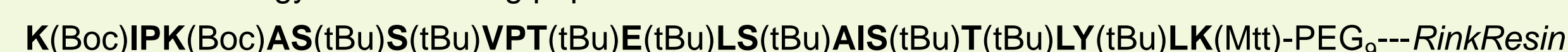
The introduction of an additional lysine (Lys<sup>93</sup>) in the BPM-2(73-92) fragment allows the introduction of the fluorescent probe in C terminus by coupling of the carboxylic moiety of TAMRA to the amino function of the side chain through the formation of an amide bond. For this purpose, we chose 4-methyltrityl group (Mtt) which is removed in very weak acid conditions. In order to increase the peptide solubility and to facilitate the on resin TAMRA coupling, a PEG<sub>9</sub> linker has been introduced in C terminus.

Peptide assembly was performed by repeating the standard MW-SPPS coupling cycle for each amino acid, using Fmoc-protected amino acids (5 equiv, 0.2 M in DMF), OxymaPure® (5 equiv, 1 M in DMF), and DIC (5 equiv, 0.5 M in DMF).

Polyethylene glycol-based spacer (PEG), containing 9-atoms chain (Fmoc-8-amino-3,6-dioxaoctanoic acid) (2.5 equiv) and TAMRA were coupled in C and N terminal position of peptide P2 and P3, respectively, as follows: Fmoc-8-amino-3,6-dioxaoctanoic acid (2.5 equiv) and TAMRA (2.5 equiv), TBTU (2,5 equiv) and DIPEA (3,5 equiv) were dissolved in DMF with a final dilution of 1 mL DMF for 100 mg of resin. Couplings were performed 45 min at room temperature

### Mtt deprotection

After the Lys(Mtt) loading, the synthesis was carried out with MW-assisted SPPS strategy. The following peptidil-resin was then washed several times with DCM and dried under vacuum:

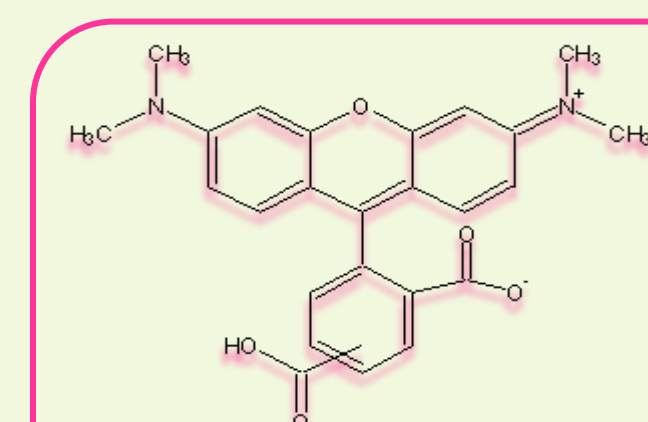


A satisfactory deprotection of Lys(Mtt) was accomplished in just three cycles of 1% TFA in DCM. The reaction was monitored by RP-HPLC from collected solutions.

Cleavage from the resin and side-chain deprotection were achieved by treatment with a TFA/TIS/water solution (95 : 2.5 : 2.5 v/v/v, 1ml/100mg of resin-bound peptide).

Lyophilized crude peptides were purified by semi-preparative Waters RP-HPLC (Milford, MA, USA) on a Phenomenex Jupiter C18 (10 μm, 250mm× 10mm) column at 4 ml/min. The solvent systems used were 0.1% TFA in H<sub>2</sub>O (A) and 0.1% TFA in CH<sub>3</sub>CN (B), and the gradients used are presented in Table 1. All peptides were obtained with a purity >95%.

Characterization of the peptides was performed by analytical RP-UPLC ESI-MS (Waters Acquity UPLC coupled to a Waters 3100 ESI-SQD MS) supplied with an Aeris Widepore C1 200A (3.6 μm 250 × 4.6 mm) column at 25 °C at 0.6 mL/min with solvent systems A (0.1% TFA in H<sub>2</sub>O) and B (0.1% TFA in CH<sub>3</sub>CN). Data were acquired and processed using MassLynx software (Waters, Milford, MA, USA).



### Fluorescent Probe

5(6)-Carboxytetramethylrhodamine (TAMRA).  
λ<sub>ex</sub> = 555 nm et λ<sub>em</sub> = 580 nm

	HPLC Rt (min)	HPLC gradient (%)	ESI-MS found (calc)
P1	1min85	10 - 90	[M+H] <sup>+</sup> =2118,31 (2118,53)
P2	2min12	30 - 50	[M+H] <sup>+</sup> =2805,12 (2805,23)
P3	1min35	20-60	[M+H] <sup>+</sup> =979,96 (976,06)

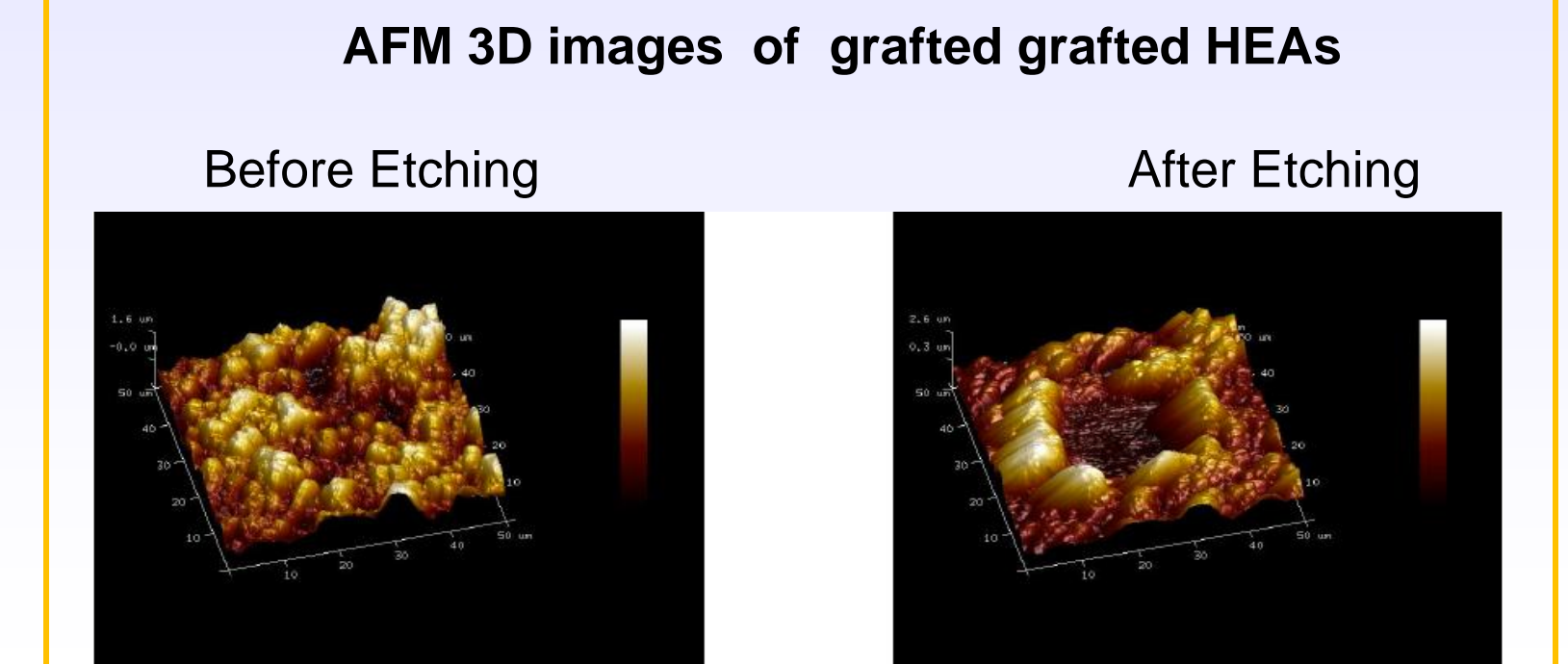
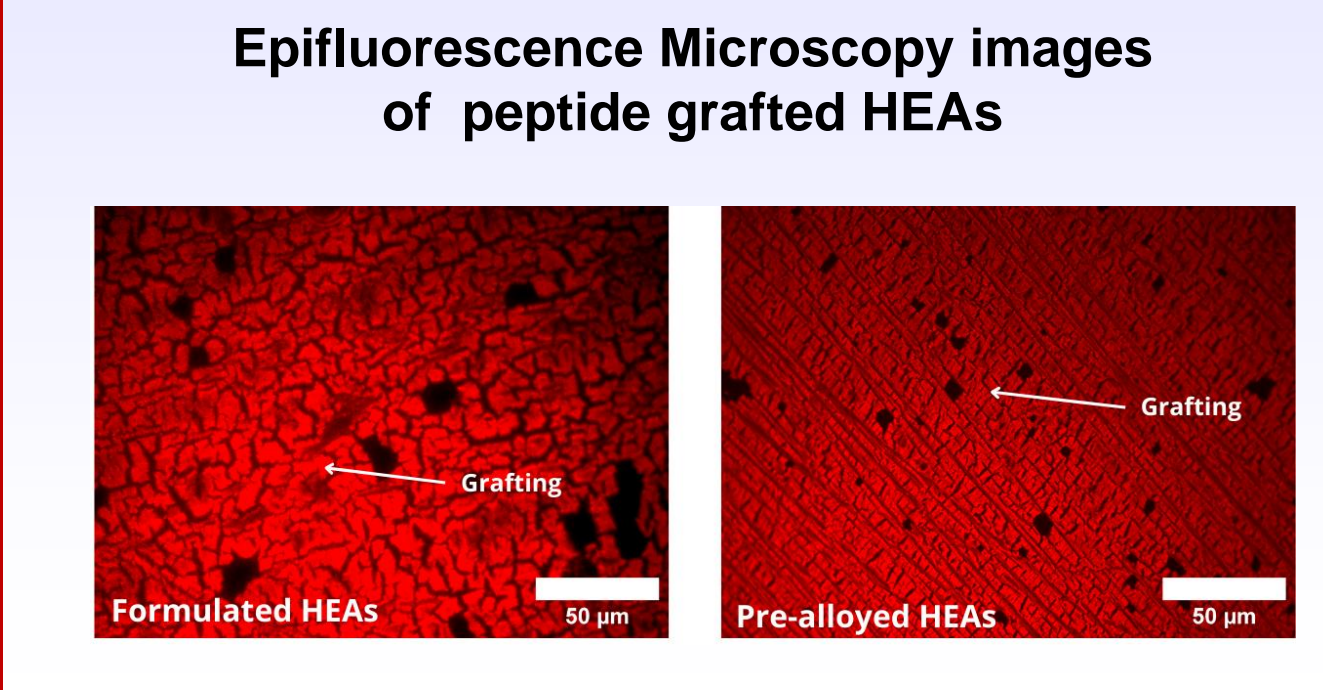
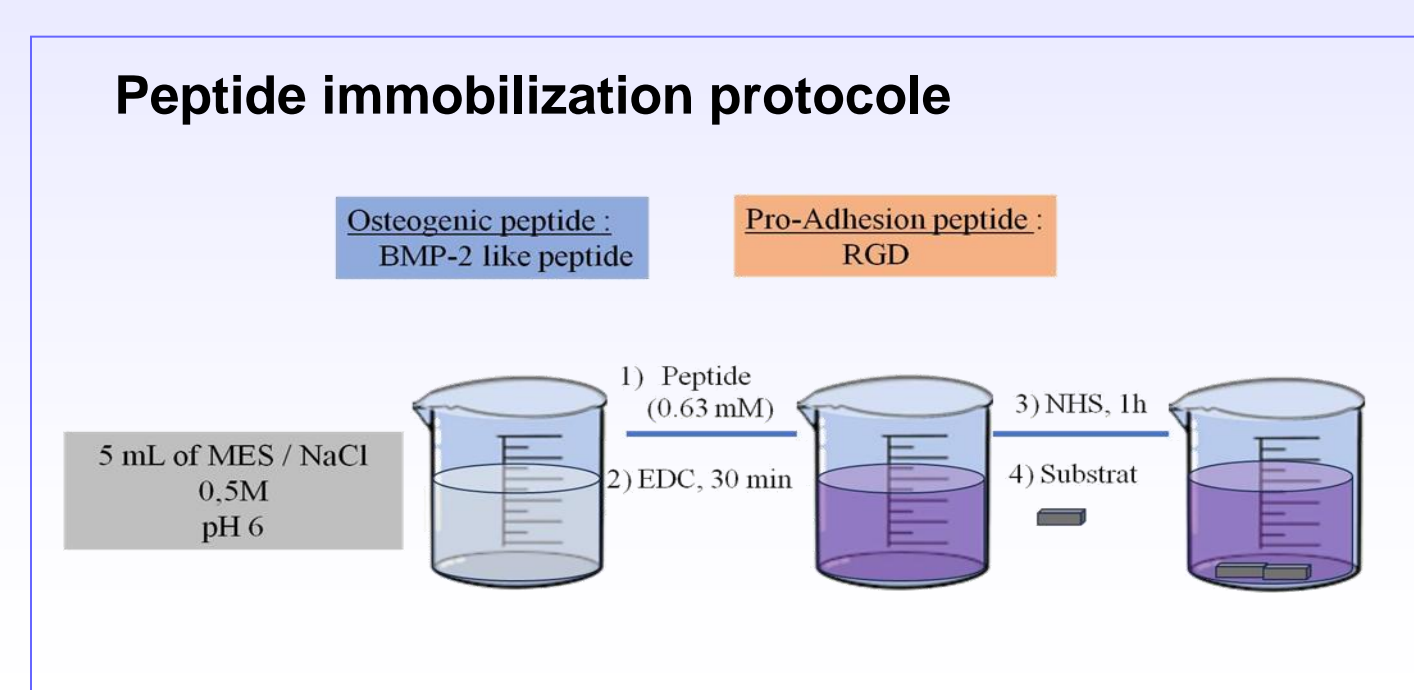
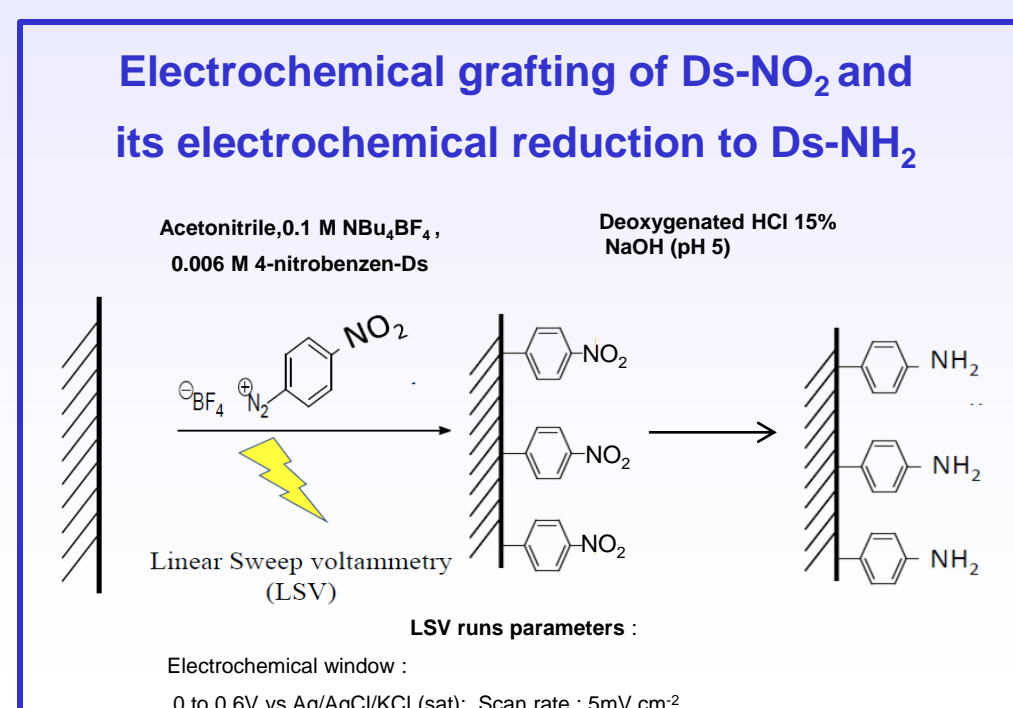
## HEAs Surface functionalization & characterization

The final goal of the project is the development of safe, non-destructive and biocompatible strategies to modify the surface of HEAs for applications in the field of medical implants. The main aim of this research is to enhance the osseointegration of two types of HEAs (formulated and pre-alloyed) by immobilizing peptides providing a favorable medium to biological reactions on the alloy surface. This was accomplished by modification of the alloy surfaces by the aryl amine coating via diazonium salt (Ds) electrochemical grafting and then covalent bonding of the peptide.

### 2 types of HEAs:

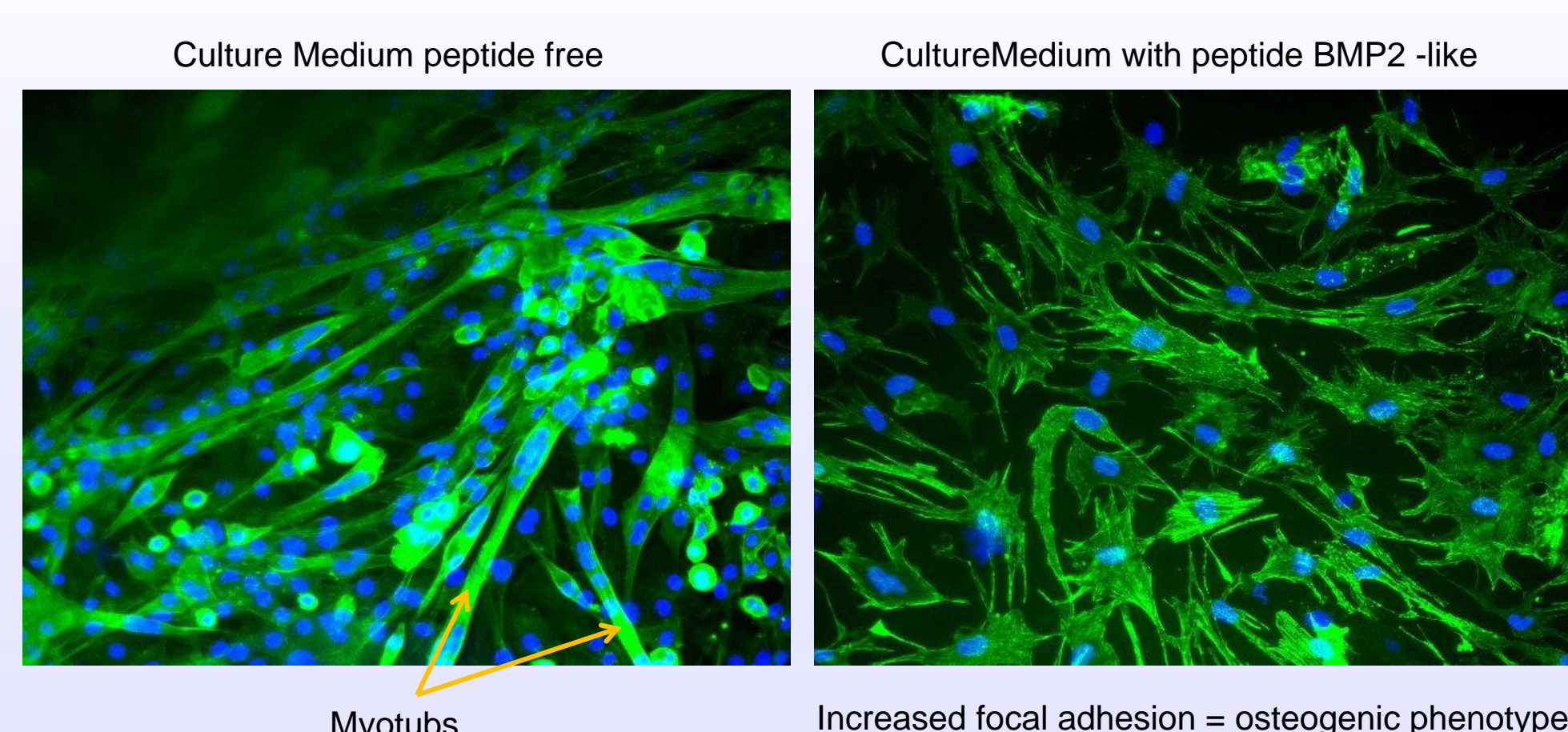
Ti<sub>35</sub>Nb<sub>25</sub>Zr<sub>25</sub>Ta<sub>7.5</sub>Mo<sub>7.5</sub> Formulated

Ti<sub>35</sub>Nb<sub>25</sub>Zr<sub>25</sub>Ta<sub>7.5</sub>Mo<sub>7.5</sub> Pre-alloyed



## Biological data

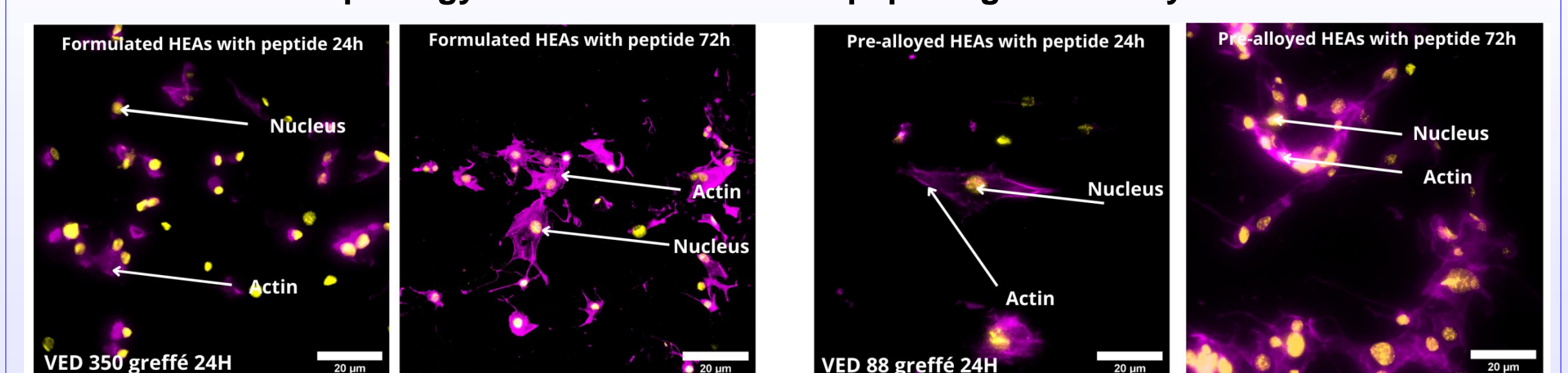
### BMP2 like peptide bioactivity → change in C2C12 morphology (21 days)



Assessment of the safety and biological activity of the BMP2 like peptide in cultured cell line models

In vitro cytotoxicity evaluation of biofunctionalized alloy surfaces and assessment of their biological performances toward osseointegration

### Morphology of cell incubated with peptide grafted alloys



Epifluorescence Microscopy images

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[1] Will Williams, D.F. *Biomaterials* 2009, 30, 5897–5909. [2] Förster Y, Rentsch C, Schneiders W, et al. *Biomater*. 2012; 2(3): 149- 157. [3] Mas-Moruno C. et al. *ACS Appl. Mater. Interfaces* 2014, 6, 9, 6525–36. [4] Williams DF. *Biomaterials*. 2011;32(18):4195-4197. [5] A. Saito, et al. *J. Biomed. Mater. Res. A* 2005, 72 77–82 [6] A.K. Jha, et al *PLoS One* 2014, 9, e98640