UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI INGEGNERIA INDUSTRIALE
CORSO DI LAUREA MAGISTRALE IN INGEGNERIA DEI MATERIALI

TESI DI LAUREA MAGISTRALE IN INGEGNERIA DEI MATERIALI

HYBRID SCAFFOLDS FOR LOAD-BEARING BONE REGENERATION: BETA-TRICALCIUM PHOSPHATE SCAFFOLDS ENRICHED WITH SELF-ASSEMBLING PEPTIDES

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ANNO ACCADEMICO 2013 - 2014

Abstract

This thesis is part of a wider project called “Rebuilding Faces” which is a King’s College London project aimed at finding a way to rebuild critical-sized bone defects.
We enriched β-TCP scaffolds with self-assembling peptides in order to improve the cell adhesion and cell proliferation.
Self-assembling peptides are able to form 3D-nanofibrous water-rich structures useful as cell scaffolds. We have tested two different SAP sequences and three progressive enrichments of such hydrogels with RGD-functionalized SAP analogues for evaluate the role of 3D RGD-decoration in the first stages of cellular adhesion.
In addition, the growth factor BMP was added to the hydrogels to assess their capacity of modulate protein release.
Cell viability and proliferation were evaluated by two different biological assays. The analysis at fluorescence microscope was carried on in order to assess the cell morphology and the resistence of cells to trypsin treatment in presence or absence of SAPs.

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Chapter 1

Introduction

1.1 Biomaterials

Biomaterials are defined as materials designed to interface themself with biological systems or environments. They are able to support or replace any tissue, any organ and also some body function (Defined by: II International Consensus Conference on Biomaterials, Chester, UK, 1991).

The biomaterials history can be resumed in three most important steps, as follow:

- 1st generation of biomaterials: these materials are bio-inert, this means that they are able to minimize the physic-chemical interaction between the body and the material itself;
- 2nd generation of biomaterials: the material is bio-active, it means that in a short time it will integrate itself in the body, causing in the physiological environment some controlled actions and reactions. Otherwise these materials can be re-absorbable, therefore the material will be degraded in the body, without causing rejection or toxicity troubles;
- 3rd generation of biomaterials: the material is both bio-active and re-absorbable; this class of materials is the most promising one and represents the future for the biomaterials.

The materials of the last group are defined also as “bio-mimetic” materials because they interact with the cells of the surrounding tissues, using bio-molecular recognition processes [1]. In order to obtain these materials that “mimic” the natural behaviour, we need materials able to promote some specific cellular responses that, in turn, may lead to the formation of new tissues.

The cellular recognition of the materials mediated by the biomolecules could be obtained through two different strategies: the first one is using soluble molecules like growth factors or plasmid DNA embedded into carriers, in order to release the bio-active molecules from the carriers and promote or control the new tissue’s formation. The second strategy is the incorporation of bioactive proteins or peptides on the surface of bio-materials or into their core, using physico-chemical modifications.

The most important parameters in a bioactive material design are: the density, the spatial distribution and the orientation of bio-molecules.

In order to improve the performances of bioactive materials, allowing them to establish further favourable interactions with the biological environment, we resort to the biochemical functionalization: putting on the surface some organic molecules (like adhesive sequences or growth factors) which are able to communicate with the host tissue’s cells.

There are several different way to deliver proteins or short sequences of peptides to the interface between the material and the biological environment (Figure 1.1):

- By the absorption of biologically active molecules on the surface;
- Including biomolecules into a re-absorbable carrier (usually a polymeric material), the latter is used as coating on the surface of the scaffold. The bio-molecules release takes place during the carrier's degradation
- Anchoring molecules on the surface using a covalent bond.

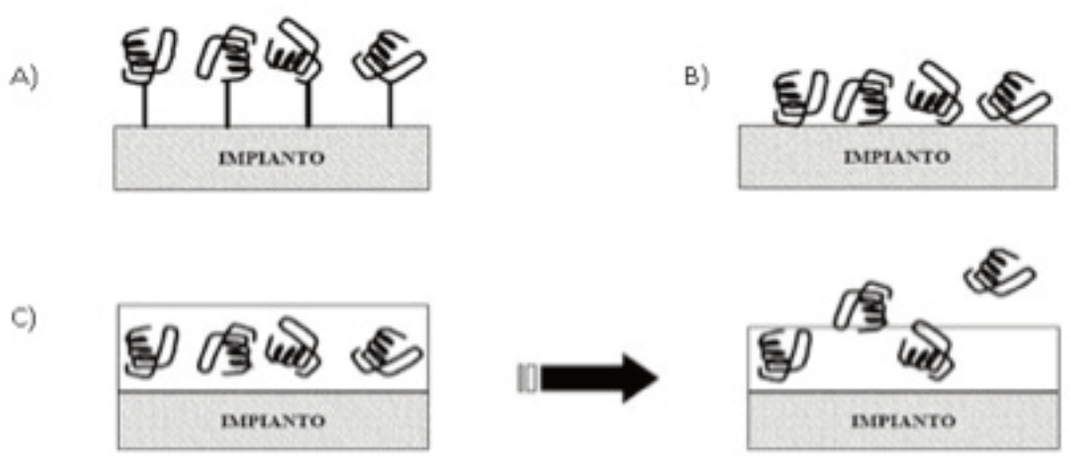


Figure 1.1: *Molecular anchorage, using covalent bond with spacer (A), by adsorption (B) and protein releasing process from a carrier (c).*

The molecules can be anchored directly on the surface or otherwise using a specific molecule called "spacer" which allows a better exhibition of the molecules, thus giving more freedom degrees in orientation [19]. Making use of particular techniques of specific covalent immobilization, we can also induce all molecules to bind with the surface using the same functional group (Figure 1.2).

Figure 1.2: *Covalent immobilization, non-specific (left) and specific (right).*

Biomaterials used in tissue engineering can be ranked by different criteria: degradation properties, mechanical properties and chemical-structural features. According to the latter criterion, for example, biomaterials could be split up in 5 categories: metals, polymers, ceramics, hybrid (or composites) and biological materials.

1.2 Critical-sized bone defects

The need for an appropriate bone substitute is significantly increasing: in 2004 alone, more than 1100000 surgical procedures involving the partial excision of bone, bone grafting and inpatient fracture repair were performed in the USA at an estimated total cost of more than $5 billion (Kretlow & Mikos 2007).

The critical-sized bone defect is defined as the smallest size intra-osseous wound that will not spontaneously heal completely with bone tissue, or the defect will heal by connective tissue during the lifetime of the animal (Schmitz & Hollinger 1986; Hollinger & Kleinschmidt 1990). In these cases auto-graft is regarded as the best choice because of its excellent combination of osteo-conduction and osteo-induction. However, it is always associated with several side effects (irregular rates of resorption, pain and morbidity of the donor site, requires additional surgical procedures). These limitations have already led to the pursuit of alternatives including allografts, xenografts and synthetic alloplasts. Most of them are osteo-conductive, while the lack of an intrinsic property of osteo-inductivity is always the main problem. To solve this problem, one of the strategies in bone tissue engineering has been the introduction of bone growth factors into a suitable scaffold.

The facial bone has significant functional as well as aesthetic importance. There has been an increase in the frequency of facial bone injuries due to road accidents and sports injuries. Many procedures are needed to restore facial bone structure surgically. Damaged bone heals by not only formation of scar tissue but also regeneration as seen in the epidermis. When regeneration cannot occur because of a bone defect caused by severe comminuted fracture or tumour excision, an autologous bone graft is recommended to repair the defect.

1.3 Tissue engineering

Bone tissue engineering is an interdisciplinary field that combines the knowledge and technology of material engineering and biological factors to regenerate damaged bone tissues. This innovative engineering field was defined by D. Williams in “The Biomedical Engineering Handbook” as: “The application of scientific principles to the design, construction, modification, growth and maintenance of living tissue”.

We can have two different types of approach:

- In vitro seeding: this is the traditional approach, which involves the use of a mechanical bio-reactor simulating the biological environment;
- Tissue guided regeneration: this recent approach uses the patient as bio-reactor, implanting directly the scaffold without the “in vitro” cells seeding.

An engineered tissue is made by three fundamental constituents: a matrix or scaffold, bio-chemical factors and the receiver’s cells. The scaffold can be both artificial (as a Polymeric bio-material) and “bio-derived”.

Bio-chemical factors are proteins, or, more commonly, short peptide sequences which are useful for the guided growth and for the cells development on the matrix/scaffold.

The tissue engineering has a great range of medical applications: for example, it was carried out the replacement of a trachea portion using a engineered tissue, obtained in vitro by a biological scaffold taken from a corpse; in this case the patient was dismissed 10 days after the surgery and it was not necessary any immunosuppressive therapy. The grafted tissue was also normally vascularized.

This field of engineering was also used to re-build a bladder in vitro, then successfully implanted in a paediatric patient.

Ultimately the tissue engineering is a new multidisciplinary field which could revolutionize medicine and surgery, allowing the organs and morbid tissue’s regeneration, not only stimulating the healing process. The development of this innovative discipline may lead to new healing possibilities, a better quality of patient’s life and overall it may lead to overcome the chronic lack of transplant organs.

1.4 β-TCP

The β-TCP is a particular type of calcium phosphate. This ceramic material is very interesting for the bone reconstruction because of his stoichiometric similarity to amorphous biologic precursor of bone minerals, and furthermore because it displays a Ca-to-P molar ratio of 1,5. The most widely studied calcium phosphates are TCP ($Ca_3(PO_4)_2$), Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and the newest tetracalcium phosphate [1]. The appeal of the calcium phosphates rests largely with their biocompatibility. Since they are proteins-free, minimal immunological reactions, foreign body reactions and systemic toxicity have not been reported with their use.

Although the inorganic ceramics have not shown osteoinductive ability, they certainly possess osteoconductive abilities as well as remarkable ability to bind directly to bone.

In 2004 Ogose and co-workers published an article about the comparison of hydroxy apatite and beta tricalcium phosphate as bone substitutes. The patients treated with β-TCP had shown

a better replacement of graft by forming natural bone, than the patients treated with HA.
Postoperative radiographs demonstrated radiolucent zones between implanted Beta-TCP and the surrounding bone immediately after surgery, which was similar to that of the HA cases. Over time, these radiolucent zones disappeared and all 30 patients showed new bone in a mean period of 9 weeks while the patients treated with HA developed new bone in 16 weeks. These processes appear to have begun on the periphery and progressed toward the core of the implanted material. These radiographic changes are believed to represent direct bone deposition on the ceramics, which is referred to as osteoconduction.
So they concluded saying that the best tricalcium phosphate is the β-TCP for the reduced time required for the resorption step and also for the better osteoconductive ability than the HA [20].
Furthermore, many reports have suggested that greater extent and faster rate of bone penetration are correlated with increasing macroporosity (i.e. pores>50 μm in size) in calcium phosphate ceramics.
Recent experiments indicated that manipulation of the level of microporosity within calcium phosphate ceramics can lead to the acceleration of bone formation.
Previous studies demonstrated that several synthetic calcium phosphates possess osteoconductivity. Moreover, this ability depends on both the species of animal and the type of ceramics in terms of different phasic composition as well as macropore and micropore structures.

1.5 Cell adhesion and cell proliferation

Cellular adhesion and proliferation on the scaffolds are mandatory in order to create a new tissue through the tissue engineering's strategies. These processes are fundamental for the formation and maintenance of the new tissue's structure, and thus for its proper functioning.
Moreover, the cell's adhesion is deeply linked to the growth, migration and differentiation of cells, actually several of them are "anchorage-dependent", this means that they cannot express their functions and their properties, and hence they cannot proliferate, until they adhere to the substrate.
The adhesion process can be stimulated by the presence of a lot of different proteins:

- Fibronectin, collagen, laminin, vitronectin from the ECM;
- Actin and vinculin from the cytoskeleton;
- Integrins from the cell membrane.

The interactions between these proteins and the receptors, affect cell adhesion and cell growth and also the tissue formation [1].
The integrins are membrane's receptors and they have an extremely important role in mechanisms of cellular recognition and cell-cell and cell-scaffold interaction.
Integrins are heterodimers which have two different chains: sub-unit alpha and sub-unit beta.

This glycoprotein family has an extracellular large domain: a hydrophobic zone soaked in the membrane and a short cytoplasmic tail. The latter plays an important role in the communications between the extracellular environment and the cytoplasm. Some integrins are able to recognize peptide sequences as signals placed in other proteins, especially in proteins belonging to the extracellular matrix. In addition to the integrins there are other cell adhesion molecules: cadherins, immunoglobulins and selectines.
Fibronectins are long and flexible adhesive proteins, usually formed by two dimers, the latters are linked each other by a disulphide-bridge.
The cell proliferation is deeply dependent by the adhesion process, and it is equally essential to the new tissue growth. Mitosis of cells, mandatory for the proliferation, is induced by growth factors (e.g. BMP proteins).

1.5.1 RGD: adhesive sequences

Some membrane's integrins are able to recognize only short peptide sequences, like the RGD sequence (Arg-Gly-Asp), also present in the main structure of the ECM's fribronectin.
This sequence (RGD), is actually part of the "adhesive sequences", which allows the cell adhesion on the matrix. The figure 1.3 briefly shows the integrin-fibronectin bond through the RGD sequence recognition.

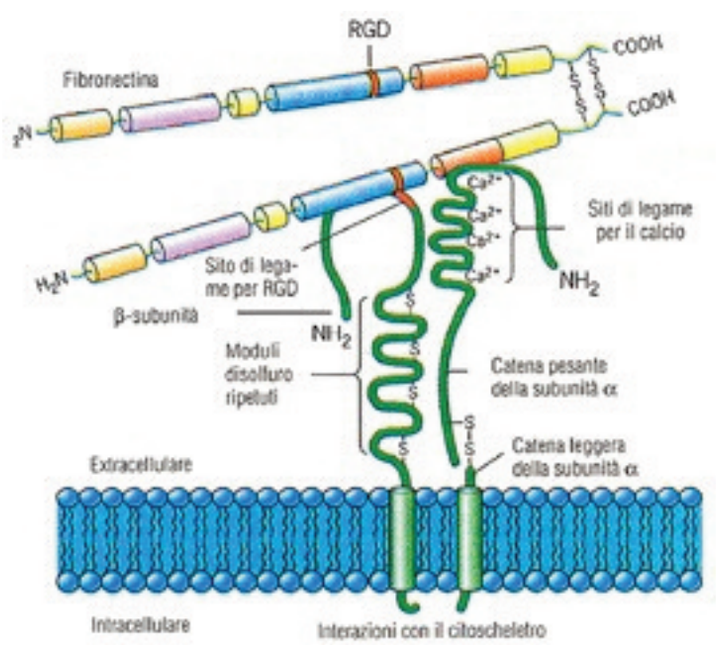


Figure 1.3: *Integrins of the cellular membrane are able to recongnize RGD adhesive motif in fibronectin sequence.*

The clinical usage of native proteins, biologically active, is hindered by the fact that usually they are unstable, insoluble and expensive. In order to avoid these problems, currently we can use the technic named as “peptide mimicry”, in which we can use bio-mimetic peptides with the signal sequence, the same of the native protein.
Several studies showed that the cell adhesion on scaffolds (synthetic and biological) is increased by “RGD-peptides” (peptides containing RGD sequence): amino-acid chains of different lengths, starting from simple tri-peptides, marked by a peculiar sequence in their structure: Arginine-Glycine-Aspartic Acid.
This is a fundamental sequence for the activation of different types of cell receptors for cell adhesion, and also for the biological impact on anchorage-dependent cells, especially with regard to both the adhesion and the general cell behaviour (proliferation and survival).
The tri-peptide activity is also deeply linked to the block of the carboxyl terminal or to the amino-acids flanking the adhesive sequence.
The activity could be ranked as follows:
GRGDSP > RGDS > RGD amide-terminale > RGD carboxyl-terminal (inactive).

1.5.2 Growth factors (BMP)

The growth factors are proteins that bind to specific receptors of cell membrane, in order to activate some specific cell functions, including: differentiation, proliferation and growth [1].
There are different types of growth factors and each one has different functions, which or may involve several types of cells or can be otherwise specific for only a single cell type.
During this experiment we used a protein called BMP-7 (Bone Morphogenetic Protein).
BMPs belong to the transforming growth factor-β superfamily and play an important role in osteogenesis and bone metabolism. It has also been reported that BMPs can induce bone formation in both ectopic and orthotopic sites in vivo assays.
It was been demonstrated that rhBMP-7 in a prefabricated β-TCP scaffolding is an effective method for bone regeneration in the mandibular critical-sized defect in the rabbit model [36].
In the same study the authors demonstrated that the healing process in a critical-sized defect is not enough fast and efficient if treated only with β-TCP scaffolds. The β-TCP scaffolds enriched with BMP-7 lead to a more complete healing process, with a good integration between the mineral scaffold and the original mandibular bone.
Another research group assessed the BMPs morphogenetic role in the case of their inclusion in self-assembling Peptides-Amphiphile (PA) [37]. They demonstrated that the direct injection of a saline solution containing BMP-2 or the injection of PA alone was not effective in inducing ectopic bone formation. On the other hand, histological analysis proved that there was bone regeneration around the site where the nanofibers within BMPs injection took place.

That study demonstrated that the osteo-inductive activity of BMP-2 was greatly influenced by incorporation of BMP-2 into self-assembled PA nano-fibers.
Haidar et al. [34,35] affirmed: “The best BMPs delivery system have to be insoluble under physiological conditions in order to retain the BMP yet absorbable by host tissue after implantation/administration so that be replaced by the regenerating bone”.
Actually the β-TCP scaffolding loaded with SAPs Hydrogels can reach all these “mandatory” properties.
SAPs hydrogels could create favourable environment for cells growth and proliferation, and the inorganic scaffolds increase the osteo-inductivity of this “new” delivery system.

1.6 Self-assembling peptides

The molecular self-assembling process is defined as the spontaneous molecular organization into well-defined and stable structures, in a thermodynamic steady-state (thermodynamic equilibrium). This phenomenon is caused by several non-covalent interactions as hydrogen-bonds, or Van der Waals bonds: taken individually these interactions are not very strong, but if they act all together they create very stable structures.
A very interesting class of self-assembling molecules is the Self-Assembling Peptides (SAPs) discovered by Zhang et al. (1993). These peptides became a new possibility in the scaffolding world for the cellular growth.
The self-assembling peptides are a particular class of bio-molecules characterized by the capability of organizing themselves into ordered 2D and 3D structures, used in order to promote multi-dimensional cells-cells interactions and to increase the cellular density.
The wide range of SAPs was divided by Zhang in 5 main classes [2]:

1. Type I: also called “molecular Lego”, they are able to create hydrogels which could be used in scaffolding process;
2. Type II: including the so called “molecular switches”;
3. Type III: these peptides are often used in surfaces coatings (“molecular hooks” and “molecular Velcro”);
4. Type IV: including the peptide nano-tubes and the “molecular capsules” used as carriers for genes or proteins;
5. Type V: “molecular hollows” used for the bio-mineralization [44].

During this experiment we will use four different peptides belonging to the first type, also called self-assembling ionic-complementary peptides. These particular peptides are characterized by charge alternations (positives and negatives) of basic and acid residues interspersed by hydrophobic residues (e.g., AA positively charged / AA Hydrophobic / AA negatively charged / AA Hydrophobic / etc.).

Peptides belonging to this class of molecules can take secondary beta-sheet structures. Each beta-sheet plane has an “ionic face” with positively and negatively charged side-chains, and a “hydrophobic face”.
Due to these interactions both ionic and hydrophobic, several beta-sheet planes can build up themselves in three-dimensional structures (Figure 1.4) and then assembled peptides can take many different shapes: fibres, membranes and hydrogels increasing the concentration or changing other physicochemical parameters [46].

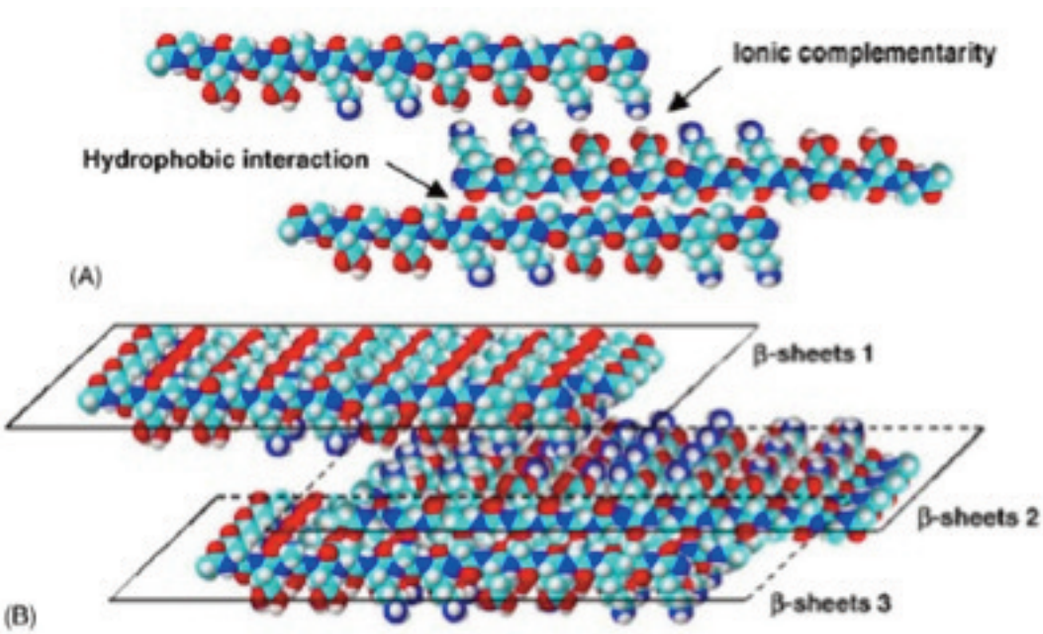


Figure 1.4: *(A) Self-assembly scheme for the self-assembling peptide EAK 16-II, using hydrophobic and ionic complementarity interactions. (B) Self-assembly model for peptide EAK 16-II, which formed β-sheet based aggregates. β-sheets are stabilized by hydrogen bonds, hydrophobic and electrostatic interactions.*

The self-assembling process is closely related to the peptide sequence, concentration, solution pH value, presence of different salts and time.
The first member belonging to this peptides class, discovered by Zhang et al. (1993), is called EAK (AEAEAKAKAEAEAKAK). This peptide assumes “beta-sheet” structure in solution and can self-assemble forming completely non-toxic membranes, after adding salts to the solution (specifically monovalent positive ions). The ion type used seems playing an important role inducing the self-assembling process: the efficacy in inducing the membranes formations can be ordered as follows: $Li^+ > Na^+ > K^+ > Cs^+$. According to the proposed model, the membranes are formed by different layers of “beta-sheet” structures, which are able to stick to each other. Every single layer can link to the previous one using ionic bridges, formed between the charged groups in the lateral chains (glutamic acid and lysine residues) and to the following layer using the hydrophobic interactions which take place between alanine side chains [45].

Recently, several self-assembling ionic-complementary peptides were designed in order to evaluate the abilities of different amino-acid residues to induce “beta-sheet” formation and consequently to form three-dimensional matrices. The proposed changes do not perturb the typical pattern of self-assembling peptides (alternation of polar and nonpolar residues and frequency of charged residues). The change of the hydrophobic residue Ala -> Abu preserves the peptides capability to form “beta-sheet” structures meanwhile the change Ala -> Tyr allows the formation of “beta-hairpin” structures. Both peptides are able to self-assemble in saline buffer. In this experiment we used two peptides EAK and EAbuK: EAbuK is an analogue of EAK in which the residue Ala (A) was replaced by α-amino-butyric acid (Abu).
Self-assembling peptides are used as:

(i) substrates for the growth of neuritis and for the synapse formation;
(ii) injectable nanofibres able to create intramyocardial microenvironments for endothelial cells;
(iii) scaffolds for the development and repair of cartilage.

Self-assembling peptides were studied as biomaterials for bone regeneration, actually they work better than Matrigel™ promoting bone formation in chronic defects in rat models.
Features of self-assembling peptides scaffolds are quite good in the healing process of small bone defects, but for critical-sized defects or for defects in loaded bones these scaffolds have not enough strength and resistance. But in these situations we propose the use of SAPs as filler for scaffolds with high mechanical properties.

1.7 Bone tissue’s cells

The bone tissue’s cells responsible for the synthesis of extracellular matrix components are osteoblasts: these cells are constantly engaged in all steps of bone formation including molecular components.
When they have exhausted their function, osteoblasts start a new condition, a state of quiescence, or they are trapped into the bone matrix which is calcified and these cells transform themselves in another type of less active cells, called osteocytes.
The latter represent the 90% of the total amount of bone cells contained in the human body. Therefore osteocytes are a subsequent functional step of the same cell (osteoblast).
Another type of cells of the bone tissue are the osteoclasts, these cells are able to product and secrete enzymes which deteriorate the calcified bone matrix, permitting the bone resorption. These enzymes play an important role both in the growth processes, when the immature bone tissue have to be substituted by the adult lamellar bone tissue, and also during the subsequent bone remodelling transformations.
In addition to cells, the bone tissue is formed also by a generous protein matrix, made by the

bone tissue cells, and in which the cells were dispersed. There are components both organic (30-35%) and inorganic also (65-70%).
The organic fraction of the bone matrix is formed by a fibrous part of protein nature, which consists of twisted bundles of protein fibres (collagen), and also by an amorphous component (proteoglycans).
The most abundant organic component is type I collagen, organized in fibres: it has to support the sedimentation of salts during the mineralization process. Other protein components (osteocalcin, osteonectin, osteopontin) are able to modulate several processes such as matrix formation and mineralization and cell adhesion process.
In addition to proteoglycans and collagen, in the matrix there are also adhesion proteins.
The most important adhesion proteins are:

- Osteonectin: it is the most abundant of the proteins of the bone tissue. It is believed that this protein has an important role in the nucleation process of the mineral crystals and then causes the calcium phosphate precipitation;
- Alkaline phosphatase protein (ALP): this is an enzyme and it is able to hydrolyze the phosphate groups bound to the organic substrates; this enzyme is active in an alkaline environment. Probably it plays an active role during the mineralization processes, providing phosphates ions then used to create mineral crystals;
- Fibronectin: it is an adhesion macro-molecule, mostly localized in the pericellular matrix. A sequence of fibronectin is able to bind with collagen. Probably this protein is involved in cellular migration processes, in cellular adhesion to the matrix and also in the bone cells organization.

The mineral elements of the bone matrix, confer upon the scaffold hardness and compactness for the support and locomotion functions of the bones. This mineral component is mainly made by calcium, oxygen, phosphorus and hydrogen; these elements together form a crystalline molecule called hydroxyapatite [1, 28, 29].

1.8 Aim of the thesis

The complexity of the biological matrices can hardly reproduced using materials of one nature. In particular, bone substitutes for critical-sized defects must be scaffolds with appropriate mechanical properties such as the ceramic scaffolds in β-TCP. The β-TCP is an osteo-conductive material but it is not osteo-inductive. To improve the cell colonization of such material we have proposed to enrich its porosity using a solution of self-aggregating peptides as filler. The self-assembling peptides are used to obtain hydrogels that support the proliferation of various cell types including osteoblasts. These hydrogels consist of nanofibers and are also widely used as matrices for the delivery of growth factors, cells and drugs.

Consequently samples of β-TCP were enriched with two different types of self-assembling sequences. These hydrogels were further decorated with adhesive RGD sequences in three different concentrations. Furthermore, the hydrogels have been tested as matrices for a modulated release of the growth factor BMP-7. The preparation of self-aggregating peptides used in this study was performed at the University of Padua, while the enrichment of β-TCP samples and the in vitro biological assays were performed at King's College London.

Chapter 2

Materials, instruments and methods

During the experimental part of this thesis we used several different chemical substances and laboratory instruments. In this chapter main reagents and used instruments are listed; then the used methods are described.

2.1 Materials

2.1.1 Reagents

Reagents supplied by Advanced Biotech Italia (Milan, Italy):
- HBTU;
- HOBt.

Reagents supplied by Biosolve (Valkenswaard, Holland):
- NMP;
- Piperidine;
- TFA;
- DCM.

Reagents supplied by Carlo Erba (Milan, Italy):
- Sodium Phosphate Monobasic.

Reagents supplied by Avantor Performance Materials (Center Valley, PA, U.S.A.):
- Methanol;
- Ethanol.

Reagents supplied by Sigma-Aldrich (Steinheim-Germania):
- Acetonitrile;
- Acetone;
- Glutaraldehyde;
- Lutidine;
- DMF;
- PLP;

- TES;
- P2O5.

Reagents supplied by Applied Biosystems (Perkin-Elmer, Waltham, MA, USA):

- Acetic anhydride

Reagents supplied by Novabiochem (Merck, Darmstadt, Germany):

- All Fmoc-protected and side-chain protected amino-acids used during the solid phase peptide synthesis.

Reagents supplied by Prolabo (Merck, Darmstadt, Germany):

- Diethyl ether.

Reagents supplied by Whatman (Schelicher&Schuell, Dassel, Germany):

- Absorbent/Filter paper.

2.1.2 Peptides for samples enrichments

Samples were enriched by different peptides, named: EAK, RGD-EAK, RGD-EAbuK, EAbuK and GE3M. The first three peptides were synthesized in the laboratory of chemical bio-engineering in the department of Industrial Engineering (University of Padua), during the thesis period the latters were just available in the lab.

2.1.2.1 EAK

This peptide belongs to the ionic-complementary self-assembling peptides class, in particular to the Type 1 class. Its molecular weight is 1614.796 Da and its sequence is:
H-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-NH_2

2.1.2.2 RGD-EAK

This peptide belongs to the ionic-complementary self-assembling peptides class, in particular to the Type 1 class. Its molecular weight is 1943.126 Da and its sequence is:
H-Arg-Gly-Asp-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-NH_2

2.1.2.3 EAbuK

This peptide belongs to the ionic-complementary self-assembling peptides class, in particular

to the Type 1 class. Its molecular weight is 1728.012 Da and its sequence is:
H-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-Abu-Lys-Glu-Abu-Glu-Abu-Lys-Abu-Lys-NH_2

2.1.2.4 RGD-EAbuK

This peptide belongs to the ionic-complementary self-assembling peptides class, in particular to the Type 1 class. Its molecular weight is 2055.376 Da and its sequence is:
H-Arg-Gly-Asp-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-Abu-Lys-Glu-Abu-Glu-Abu-Lys-Abu-Lys-NH_2

2.1.2.5 GE3M

This peptide does not belong to the self-assembling peptides class. It were used as a negative control.

2.1.3 Laboratory instrumentations

During this thesis project several different instrumentations were used for both the peptide synthesis and the biological characterization.

2.1.3.1 Solid phase peptide synthesis

The synthesizer Syro I (Figure 2.1) supplied by MultisynTech (Witten, Germany) was used for solid-phase peptide synthesis.

Figure 2.1: *Synthesizer Syro I (MultisynTech).*

The de-protection of peptides from solid support was carried out using a rotary evaporator (Laborota 4100-Efficient, Heidolph Instruments s.r.l., Milan, Italy) and the lyophilizer (Lioph. Lock 4.5, Labconco, Kansas City, MO, USA).

2.1.3.2 Mass Analysis

Spectrometric mass analysis was used for peptide characterization and molecular identity determination. The mass measurements were carried out at the Department of Pharmaceutical Science, University of Padua, by the Professor D. Dalzoppo, by an ESI-TOF, Mariner System (model 5220, by Applied Biosystem ;Perkin-Elmer).

2.1.3.3 Piperidine Test

Piperidine test was carried ut using a spectrophotometer UV/Vis Lambda 2 (Perkin-Elmer) (Figure 2.2).

Figure 2.2: *Spectrophotometer UV/Vis Lambda 2 (Perkin-Elmer).*

2.1.3.4 Chromatographic analysis

In order to separate, purify and analyse the synthetic peptides we utilized two different chromatographic systems:

1. HPLC Waters 600E, equipped with auto-sampler (model 717) and with a UV/Vis multichannel detector (model 2487). The acquisition and the integration of data were made using the Empower software (Waters, Milford, MA, U.S.A.);

2. HPLC Waters 600, equipped with an UV/Vis multichannel detector (model 2487). The data acquisition was performed by a recorder (Carlo Erba Strumentazione, Cornaredo, Italia) and made by Kipp&Zonen (Delft, Holland), model BD40/BD41 Recorder.

Several different chromatographic columns were used:

- Delta-Pak C_{18} column (15 µm, 100Å, 7.8 × 300 mm);
- Jupiter C_{18} column (5 µm, 300 Å, 250x10 mm);
- Symmetry Shield RP_8 column (5 µm, 300 Å, 250x4,6 mm);
- Jupiter C_{18} column (5 µm, 300 Å, 250x4,6 mm).

The separations were carried out in gradient made using two elements:

- Eluent “A”: 0.05% of TFA in MilliQ water;
- Eluent “B”: 0.05% of TFA in Acetonitrile.

The “milliQ” water was double-distilled with the distiller Distinction water still by Bibby Scientific Limited (Beacon Road, Stone, Staffordshire, ST15 OSA, UK), desalted and deprived of any organic substances using the “milliQ System” by Waters.

2.1.4 Biological analysis

All biological assessments were performed at King’s College London laboratories, placed at the Dental Institute in the Guy’s hospital building in London. Our attentions were focused on two main biological analysis: Alamar Blue staining and the MTT test. Before the cytotoxicity evaluation, and before the AB and MTT tests, the cells were cultured and grown for the seeding phase of the experiment.

2.1.4.1 Cells culture

For the cells culture we absolutely needed the Sterile Hood (Bioquell Lab, model ABS1200) in order to performed the cells culture media change and all other daily actions in a safe way.
The cells culture media receipt is the following:

DMEM (+ phenol red) (Sigma-Aldrich)	500 ml
HEPES 1M (Sigma-Aldrich)	10 ml
L-glutamine 200mM (Sigma-Aldrich)	5 ml (1 aliquot)
Pen/strep 100x (Sigma-Aldrich)	5 ml (1 aliquot)
Foetal calf serum (FCS) (Sigma-Aldrich)	50 ml (1 aliquot)
Minimal essential medium (MEM or NEAA) (Sigma-Aldrich)	5 ml
Ascorbate powder (Sigma-Aldrich)	0.075g (weigh out)

2.1.4.2 Alamar Blue test

To performed this test we used an Alamar Blue stock solution, made by 63 mg of Resazurin Sodium Salt (Sigma R7017-5g) in 500ml of Dulbecco PBS (Sigma D8537-500ml). To use this solution for Alamar Blue assay we needed to dilute 1:10 with a phenol-free cells culture media. To evaluate the absorbance of every single well we used a Opsys MR Microplate Reader, supplied by Dynex Technologies.

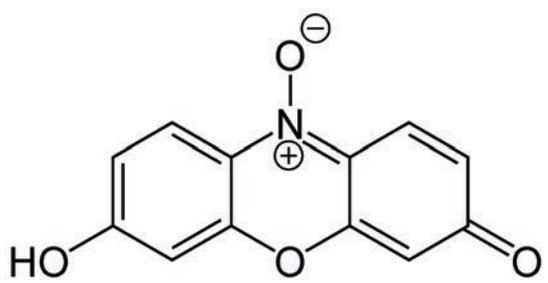


Figure 2.3: *Molecule of Resazurin, commercially available as Sodium salt.*

2.1.4.3 MTT test

The most important reagent used in this analysis is the (3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyl tetrazolium bromide, which is converted to a blue formazan by mitochondrial dehydrogenases. For the absorbance evaluation we use the same instruments used for the AB test.

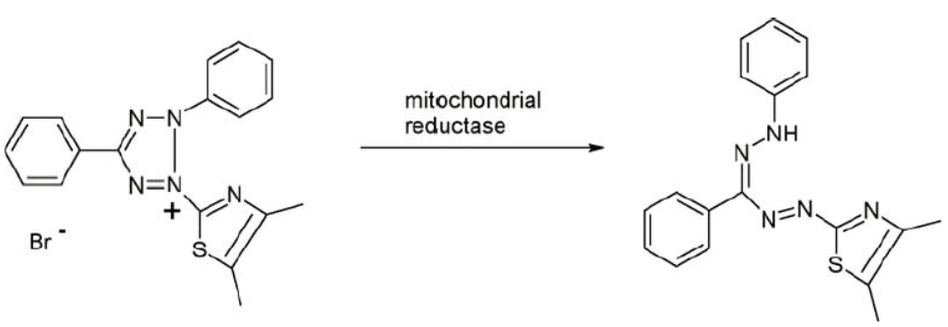


Figure 2.4: (3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyl tetrazolium bromide is shown on the left and the blue formazan is shown on the right.

2.2 Methods

2.2.1 Solid Phase Peptide Synthesis

Peptides were synthesized using “Solid Phase Peptide Synthesis” (SPPS), this method was developed in 1963 by Bruce Merrifield [5, 6]. The chemistry used was “Fmoc-Chemistry”.
The SPPS technique nowadays is the most useful method for the small-and medium-scale peptide synthesis, it allows to obtain peptides through 5 main steps:

1. Amino-acid chains assembly on a resin (solid support);
2. The peptides are unlocked from the resin and the protecting groups of the lateral chain are removed;
3. Purification of the target product;
4. Post-synthesis chemical changes;
5. Characterization.

During the synthesis the peptide chain grows from the C-terminal towards the N-terminal site; the carboxylic group of the last amino-acid is anchored to the solid support through a linker.
The most important advantage of SPPS is the solid matrix insolubility. The solid support allows us to isolate the growing peptide at each reaction step, using a simple filtration process; consequently the whole process can take place in a single reactor. Using this technique we can reduce the amount of needed material thanks to less handling of reagents and to high automation of the process with a simultaneous reduction of the production time.
Usually polymer-based resins are used as solid support, for example co-polymers such as polystyrene with 1% of divinylbenzene, or polyacrylamide resins [3]. These resins have a peculiar property: they can swell themselves up to 10 times the initial volume, when dipped in solvents like N,N-dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP) or dichloromethane (DCM).
Different type of peptides can be achieved: acid peptides, amide terminal peptides or also peptides with specific functional groups. The differences are due to the different linkers used to anchor the synthetic peptide to the polymeric resin. Different resins are commercially available with a wide variety of linkers or also with the C-terminal amino-acid already attached; acid-labile resins allow to unlock the peptide from the solid support, without side-chains de-protection, resins which are resistant under conditions of acidolysis and which reduce the anchored peptide’s losses, and many other substrates in order to meet specific chemical needs.
During this thesis project we used the RinkAmide MBHA resin. This support is an ideal tool for the Fmoc SPPS of peptide amides. Cleavage from this resin can be carried out by a single step treatment with 95% TFA, providing peptide amides in high yields and purities. The resin’s structure is shown in the figure 2.5.

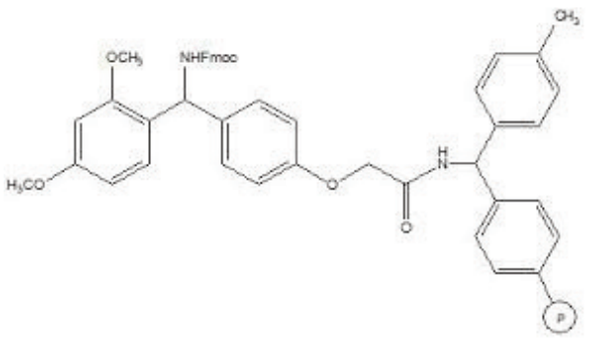


Figure 2.5: *Structure of the Rink-Amide resin we used during this thesis project.*

The peptide synthesis involves reaction cycles, each of which consists of de-protection and condensation. The de-protection is the removal of the Fmoc protecting group from α-aminic functional group. The treatment for de-protection is 40% piperidine in DMF for 3 minutes and 20% piperidine in DMF for 12 minutes. The condensation takes place between the free α-amino group on the resin and the activated α-carboxyl function of another amino-acid leading to a new peptide bind formation. The figure below schematizes the whole synthesis process.

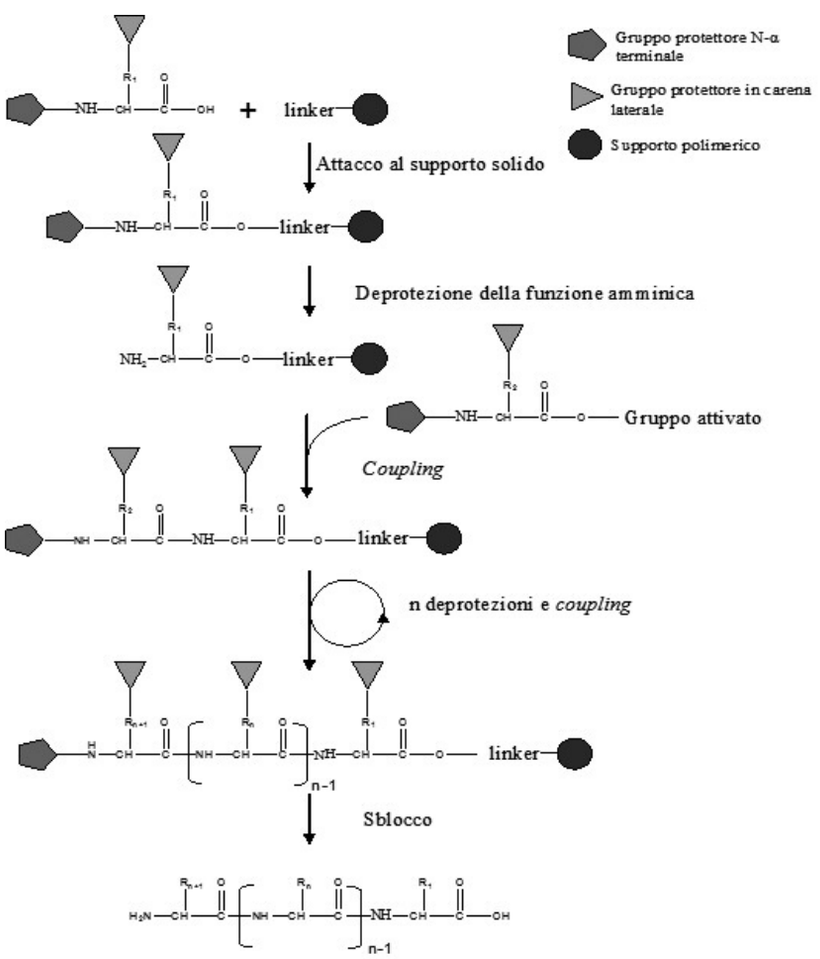


Figure 2.6: *General scheme of the synthesis process.*

The protecting groups of side chains must be stable during de-protection and couplings, avoiding side chains' reactions. On the other hand the side chains are used acid-labile protecting groups, generally removed by trifluoroacetic acid (TFA).
The protecting group of the amino functional group is 9-fluorenyl methyloxycarbonyl (Fmoc) which is usually removed by piperidine treatment [7, 8].

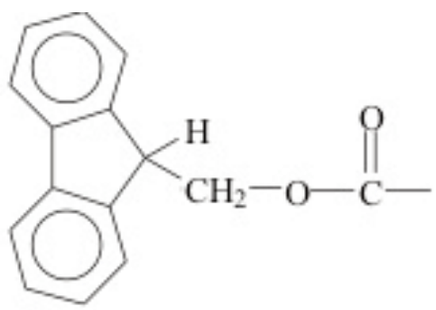


Figure 2.7: *9-fluorenyl methyloxycarbonyl molecule. This is the protecting group of the amino functional group.*

During solid phase peptide synthesis several by-products could be produced, such as incomplete sequences (without one or more residuals), or deleted sequences: this situation forces a difficult purification process because the molecules are very similar. In order to individuate the most difficult couplings the "ninhydrin test" ("Kaiser test") can be used, this assay allows to monitor the coupling reaction yield during the synthesis (the yield is considered acceptable if over the 98%). In case of a low yield (<98%) a further coupling cycle for the same amino-acid can be made or, alternatively, a "capping" reaction with benzoic anhydride or acetic anhydride can be carried out.

2.2.1.1 Test on resin loading

The procedure that follows [3] is used in order to evaluate the resin loading with the first amino-acid, that shows protection of the α-amino group (Fmoc) of the side-chain (if necessary).
A little amount of loaded resin (between 4 and 8 mg) is taken from the reactor, it has to be washed several times with methanol and it must dry under vacuum for 1 hour. The dried sample has to be carefully weighed and treated with 0.5mL of a 20% piperidine solution in DMF. This solution is left in contact with the resin for 15 minutes and manually stirred. The Fmoc de-protection gives the 1(-9-florenylmethyloxy carbonyl) piperidine that absorbs at 301 nm ((ε = 7800 $M^{-1}cm^{-1}$) (figure 2.8).

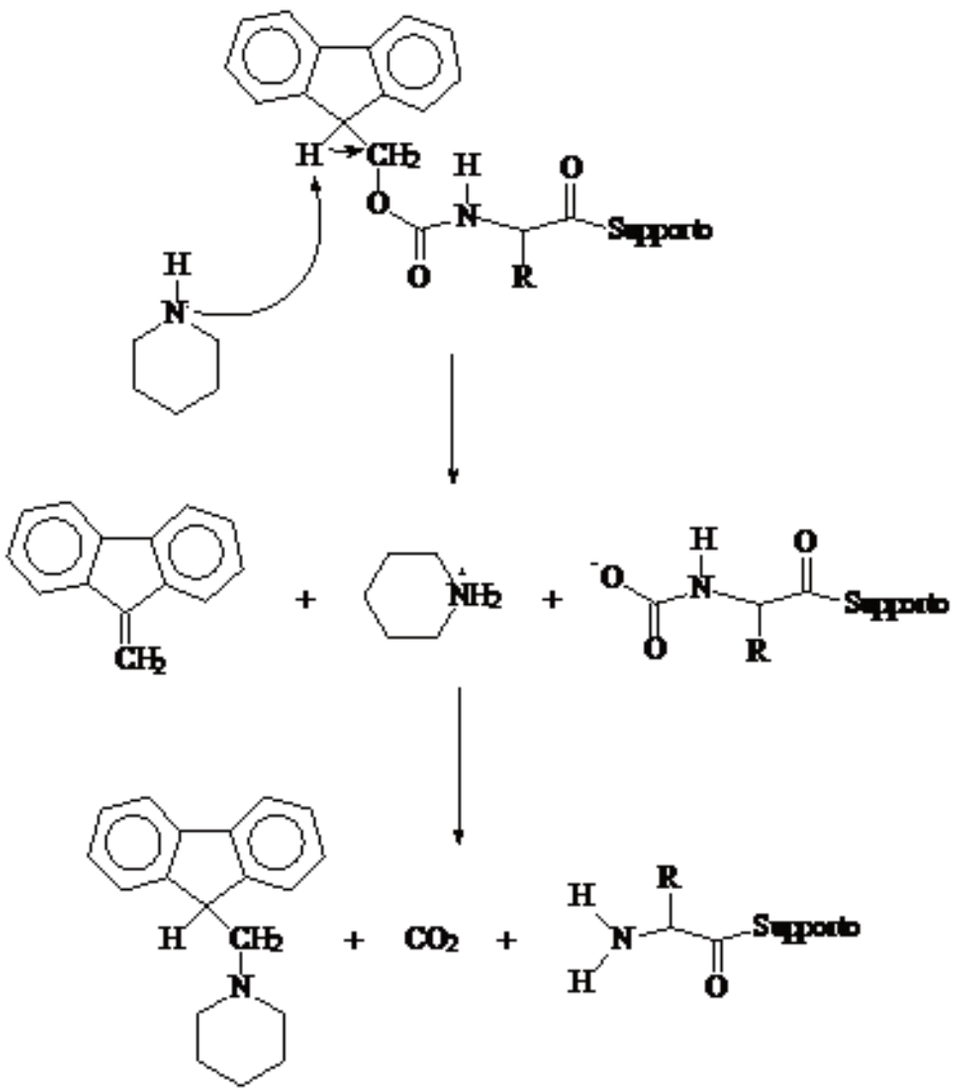


Figure 2.8: *De-protection process of the amino-acid, during the resin loading test.*

DMF is added to a final volume of 50 mL. The absorbance at 301 nm of the sample is measured by a UV/Vis spectrophotometer.
The substitution, expressed in [mmol/g] is calculated using the following formula:

$$Substitution = (A_{301} \times V) / (7800 \times wt) \quad [1]$$

- A_{301} is the absorbance at 301 nm;
- V is the mixture volume expressed in [mL];
- wt is the weight of the resin sample [g] after the drying process.

2.2.1.2 Activation of the carboxylic group

During the peptide synthesis, in the conjugation reactions, the accessibility and the reactivity of the resin-anchored free amino functions are very important, but also the reactivity of the carboxylic group of the next amino-acid is important. The carboxylic group of the amino-acid which must be linked to the main growing peptide chain has to be activated to ensure that reactions reach completion [46].
There are different ways to activate the carboxylic group, but now the most commonly used in automatic SPPS is the “in situ” activation. This step involves the use of an activating agent: 2(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), in presence of 1-hydroxybenzotriazol (HOBt) and of a base like the tertiary amine N-N-diisopropylethylamine (DIPEA) (Figure 2.9) [9, 10].

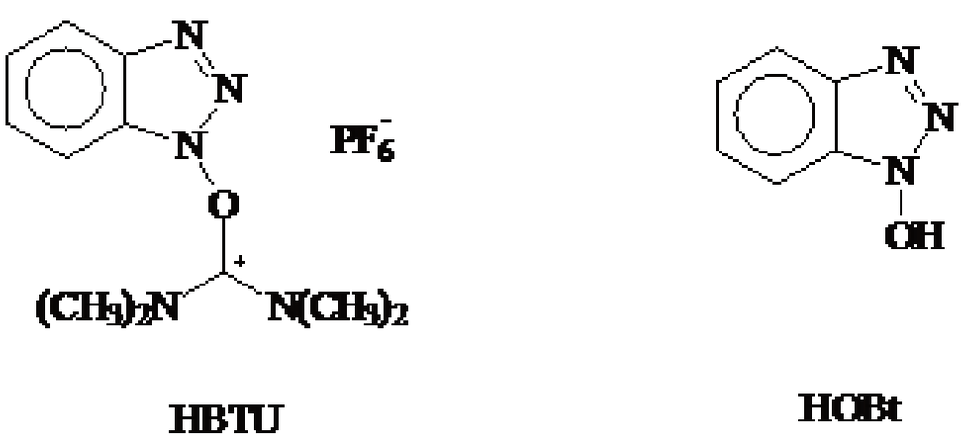


Figure 2.9: *Structures of the reagents used for the carboxylic group activation: HBTU and HOBt.*

The automatic procedure requires the use of 1mL of 0.5M Fmoc-protected amino-acid solution in DMF, 1.2 mL of 0.45M HBTU/HOBt solution in DMF and 0.5 mL of coupling solution of DIPEA in NMP. The reaction time range for a coupling reactions goes from 20 minutes to 2 hour. The standard procedure requires 45 minutes for a single coupling and 90 minutes for a double coupling.

2.2.1.3 Cleavage

The combination of side-chain protecting groups and anchoring linkages commonly used in Fmoc chemistry are simultaneously de-protected and cleaved by TFA.
The cleavage of side-chain protecting groups result in cations formation.

These species are responsible for modifications of target peptide which can be minimized during TFA cleavage by utilizing effective scavengers [6, 12].
The mixture of scavengers depends from the amino-acids present in the sequence and also depends on the type of the protecting groups used for their side-chains. The choice of a cleavage mixture instead of another one, and the time needed depend on the number and on the type of protecting groups.
General cleavage procedure:

1. After the end of the synthesis N-terminal Fmoc protecting group is cleaved with 20% piperidine/DMF solution for 20 minutes;
2. The peptide on the resin is washed with DCM and dried under vacuum for 2 hours (till a stable weight);
3. The peptide on the resin is treated with a 95% TFA solution and H2O MilliQ 2,5% and TES 2.5% (v/v/v) [14];
4. The mixture is magnetically stirred for 90 minutes; the exact reaction time depends on the number and on the amount of side-chain protecting groups;
5. The resin is filtered off using a Gooch 3 filter. The solution containing the peptide is concentrated to a small volume using a rotary evaporator;
6. The peptide is precipitated by addition of cold diethyl ether. The peptide is filtered with a Gooch 4, dried, dissolved in MilliQ water and finally lyophilized.

2.2.2 Chromatographic purification and characterization

The purification of target peptide from wrong and deleted sequences was by Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) [15].
This technique, born as preparative technique, it is also a very powerful analytic technique. It is suitable for the separation of non-volatile and thermally labile species, like peptides.
The Reverse-Phase HPLC is a partition chromatography, where the stationary phase is non-polar, and the mobile-phase can be made mixing of different percentages of water and organic solvents (acetonitrile).
The interaction which allows the retention of the peptides in the stationary phase is an hydrophobic one. The chromatography can be performed in “isocratic mode” or in “gradient mode”. In the latter case, the organic solvent concentration is gradually increased, modifying the mobile phase’s hydrophobicity. This causes a “competition” between the two phases, consequently the analyte is de-adsorbed at a specific concentration of the organic solvent.
Generally, peptides are better separated in an acid environment, for this reason the most of the mobile phases contain low concentration of TFA (during this thesis project we used 0.05% TFA for all eluents). This acid acts as ionic couple, increasing the retention, getting a better peptide

solubility and decreasing the electrostatic interactions between the latters and silanol residues in the stationary phase.

All the analysis during this thesis were performed using as eluents:

- 0.05%TFA in MilliQ water (Eluent A);
- 0.05% TFA in acetonitrile (Eluent B).

Both eluents were carefully degassed with Helium.

The solution which contains the sample was injected using a solvent delivery line in the purification process or by a syringe (small volumes) for analytic runs.

Figure 2.10 shows a scheme of a chromatographic system.

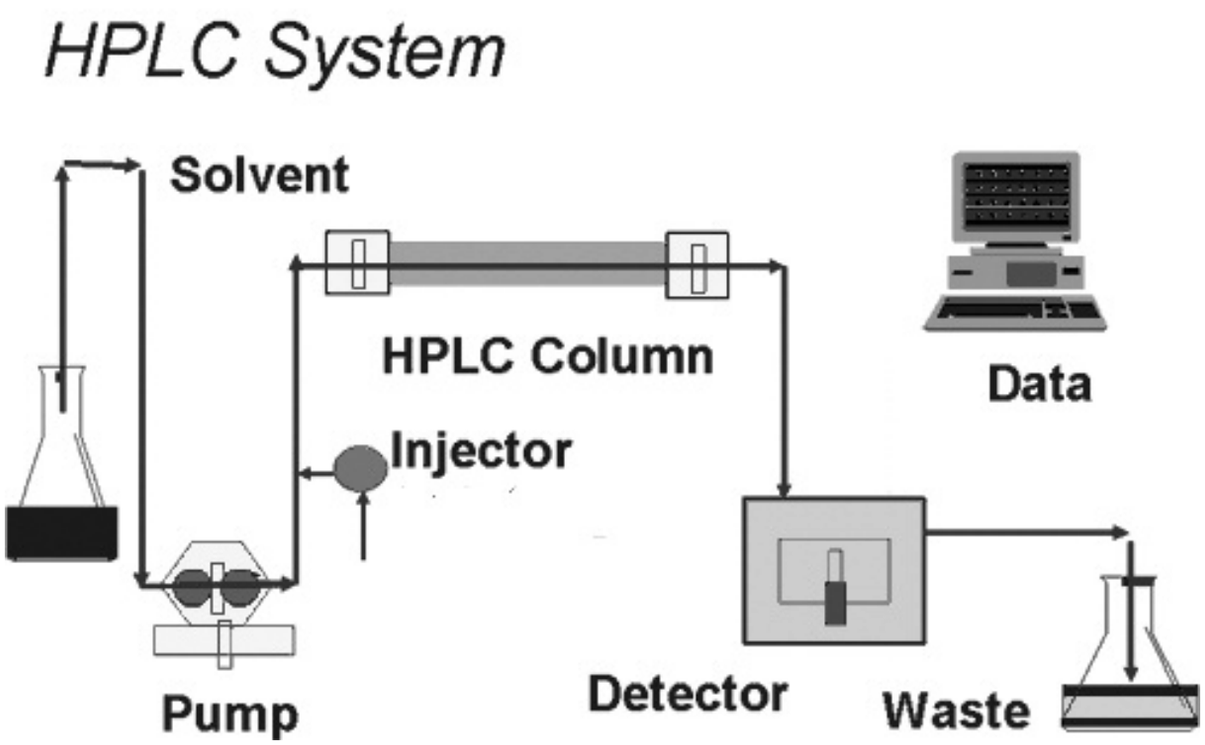


Figure 2.10: *General scheme of a liquid chromatographic system.*

Once fixed chromatographic conditions (column, mobile phase, gradient), the retention time is a finger-print of the molecule. The area under the chromatographic peak is directly proportional to the sample's amount contained in the mixture; so, integrating the whole chromatogram, we are able to evaluate the homogeneity grade of the purified product.

2.2.3 Mass spectroscopy

The Mass Spectroscopy (MS) is a destructive analysis method, it allows us to measure the molecular masses and to evaluate the structure formula of unknown compounds, also using little amounts of samples.

The spectrometer is made of three main parts:

1. Ionization chamber or source;
2. Analyser;
3. Detector.

The figure 2.11 shows the scheme of a mass spectrometer.

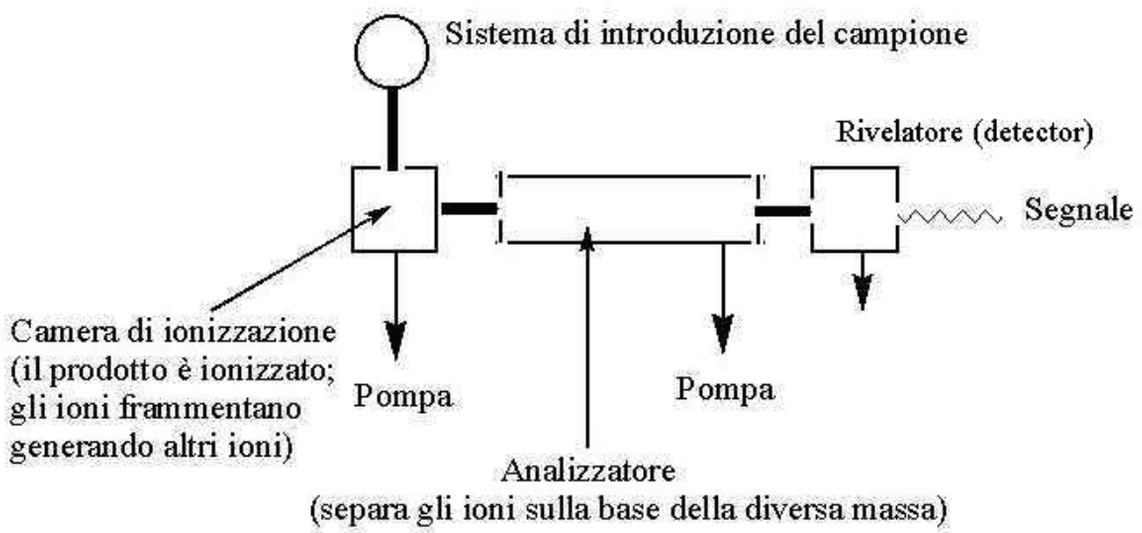


Figure 2.11: *General scheme of a mass spectrometer.*

The sample is ionized in a special chamber, the ionization chamber exactly, by the impact of an electrons, atoms, ions or photons beam, depending on the used ionization technique. Some ionization techniques are very powerful, they work at high energy levels and they cause a high fragmentation (“hard techniques”), while other techniques work at low energy levels and they produce less ions (“soft techniques”).

The ionization techniques can be divided by the source used.

- The ESI-TOF instrument utilizes a ionization technique known as Electro Spray Ioniza tion (ESI) [17] which allows samples’ ionization at room temperature creating multi-charged species. This instrument is also equipped with a “Time Of Flight” (TOF) analyser. It is able to select the different ions assessing their mass/charge (m/z) ratio. This type of analyser can measure also great values of mass with a high sensibility. At

the end a detector acquires the ions' flow and it turns the current flow into a signal which can be amplified, sent to a computer and then registered using the "Data Explorer TM" software. The ESI ionization technique needs an inert gas (i.e. nitrogen) in order to facilitate the process of atomization which is caused by a big potential difference applied on the metallic needle of the injector which releases some droplets of solution (analyte and solvent). The ESI (figure 2.12a) has two essential features:

i. The ionization takes place at room pressure;

ii. A multi charged analyte is produced.

The whole process takes place in solution (aqueous or organic) which is atomized in a thermo-stated chamber (usually 80°C), and introduced in an electric field.

– For the MALDI (Matrix Assisted Laser De-sorption Ionization) [18] ionization technique the sample is soaked in matrix and it is hit by a laser beam. The laser's energy is sufficient to create molecular ions MH+ but creation of clusters of analyte-matrix protonated can also take place. The laser hit the analyte which, absorbing the UV light, turns itself into an exited status causing also a fast heating of the interested area. So we can obtain the expulsion of the aggregated analyte which is solvated from the matrix's molecules and also a desolvation which causes a proton transfer (acid-base reaction between the molecules of analyte and those of the matrix; figure 2.12b).

The reprocessing of the output signals from the detector provides a mass spectrum: it is a series of peaks with a variable intensity, whose position is linked to a specific m/z value. In the ordinate axis the "relative abundance" values of the analysed ions are plotted. The peaks' intensities are expressed as percentage of the most intense peak, called "base peak", to which it was arbitrarily assigned the value of 100. The resolving power (RP= m/Δm), or resolution gives a measurement of the instrument sensibility to the mass differences and it depends on the analysed sample's mass.

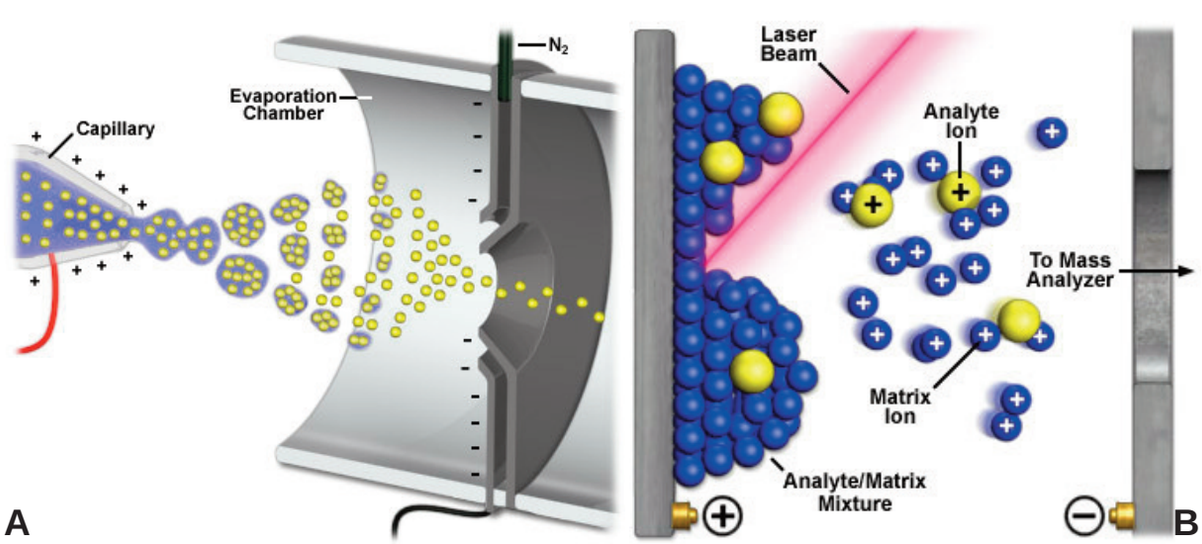


Figure 2.12: *Scheme of the ionization process; on the left ESI-TOF technique (A), on the right the MALDI technique (B).*

2.2.4 UV/Vis Spectroscopy

The UV/Vis radiation belongs to a large part of the electro-magnetic spectra between 700 and 10 nm; the most used radiations for scientific purpose are the visible radiation (between 700 and 400 nm) and the UV radiation (between 400 and 200 nm).
The UV/Vis spectroscopy is based on the electrons excitement. Because this electronic transition takes place, the absorbed energy must be exactly equal to the energy gap between the two orbitals.
In a classic double ray UV/Vis spectrophotometer, the polychromatic radiation is generated by a source (Hydrogen or Deuterium lamp for UV and a Tungsten lamp for Visible), this radiation is sent to a mono-chromator. This device selects the set wavelength and, at the same time, sends it to two different cuvettes: the first is filled with the solution containing the sample and the second with the solvent (both cuvettes are made of quartz, which is transparent to the radiation). In this way, we can easily delete the absorbance bands due to the solvent and obtain the absorbance bands of the analyte.

2.2.5 MTT test

We can use the MTT test in order to determine the cellular growth in a specific cell colture. Cells were counted evaluating the colour changing, this phenomenon is due to a particular chemical substance, which is sensible to cellular vitality [25, 26, 27].
The MTT is a yellow compound, it is reduced by mitochondria of living cells and it is converted in formazan by mitochondrial dehydrogenases, a blue/violet substance. This particular enzyme is present only in intact and living cells hence the blue colour produced should be proportional to the number of viable cells present.
The MTT method is used in cytotoxicity assays, to determine cells growth responding to mutagens, to antigenic stimuli, to growth factors and also in adhesion assays.
An MTT solution is added to tested material, on which preliminarily cells were seeded for an appropriate period. In this way the scaffold, the material become yellowish. The mitochondrial dehydrogenases of the adherent (and living) cells can cut the tetrazolium ring, producing formazan crystals which are violet and they are water-insoluble. The formazan instead is not able to go through the cytoplasmic membrane and once it is produced it is accumulated into the cells. Then an incubation period is planned in order to allow the cells to adhere. At the end of this period we can add to the cell culture, enriched by MTT solution, another solution which is able to dissolve the formazan.

The well plate is put on the shaker in order to ensure the complete dissolution of the crystals and to create an homogeneous solution. At the end we can evaluate the optical density of the final solution, using a spectrophotometer and selecting an appropriate wavelength (usually 570 or 630 nm).
The absorbance value is directly proportional to the formazan amount and hence to the number of living cells. Usually the results are compare to some controls, primarily are two: the negative control is media only, and the positive control is 10% alcohol diluted in media.

2.2.6 Alamar blue

Alamar Blue is a REDOX indicator i.e. it responds to reduction or oxidation of the surrounding medium. In this assay it both fluoresces and changes colour in response to the chemical reduction of culture medium, which results from cell growth and division [22, 23, 24]. The Alamar Blue can then be removed and replaced with fresh medium so that monitoring can be continued.
The method we used can be divided into 4 steps:

1. Prepare an “ad hoc” solution from the stock solution: dilute 10X Alamar Blue with phenol free media and then this solution have to be filtered to sterilize it before use.
2. At Day1, 24 hours after the cells seeding, remove media and discard/or keep. Add 1 ml of diluted Alamar Blue solution and then incubate at 37°C in a CO2 incubator for 4 hours.
3. Remove 100ul from each sample to 96 well plates and read on the plate reader. Test wavelength 570nm and reference wavelength 630nm.
4. Once results are obtained, wash the wells with PBS three times each sample and then replace with 1ml of complete DMEM (cells culture media).

We repeat this process for Day1, Day3, Day7, Day14, Day21 and Day28.
At Day1 and Day3 we did not discard the medium of each sample, we kept it in order to use those liquids to perform the MTT test.

Figure 2.13: *The Opsys MR Microplate Reader used during Alamar Blue and MTT analysis.*

2.2.7 Immunofluorescent staining

Immunofluorescence (IF) is a common laboratory technique used in almost all aspects of biology. Applications include the detection of specific proteins. In IF techniques, antibodies are chemically conjugated to fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). These labelled antibodies bind to the antigen of interest which allows for antigen detection through fluorescence techniques. Methods of staining may be either indirect as describe above (we used this method) or direct, i.e. the F-actin conjugated to a fluorochrome is attached to a fluorescent probe in the case of Phalloidin. The fluorescence can then be quantified using a confocal laser scanning microscope. In particular, we used a Olympus, model 1X51 equipped with the burner Olympus model U-RFL-T (figure 2.14).

Figure 2.14: *Microscope Olympus, model 1X51 equipped with the burner Olympus model U-RFL-T.*

2.2.7.1 Procedure

1. Fix cells seeded on scaffold at the selected time in 4% formaldehyde containing 2% sucrose in PBS in the fume cupboard, leave overnight at 4°C. Wash samples once in PBS;
2. Permeabilise cell membrane using PBS + 0,1% TritonX-100 for 15 minutes at 4°C;
3. Block non-specific sites using blocking buffer consisting of 1% BSA/PBS by incubating at 37°C for 20 minutes;
4. Add primary antibody (Monoclonal anti-vinculin CLONhVn-1) at the required concentration (1:100, in this case 10ul in 990ul), made up in 1% BSA/PBS to cells and incubate for 1 hour at 37°C;

5. Wash cells 3 times with washing buffer consisting of PBS + 0,5% Tween20 at 5 minutes intervals;
6. Add secondary antibody (FITC IGC whole, rabbit molecule) at the required concentration (1:200, in this case 10µl in 1990µl) made up in 1% BSA/PBS and incubate for 1 hour at 37°C;
7. Wash three times with PBS + 0,5% Tween20 at 5 minutes intervals;
8. Counterstain nuclei with DAPI (1:1000 in PBS) for 5 minutes at 37°C;
9. Wash briefly using PBS + 0,5% Tween20;
10. Visualise cells under a confocal laser scanning microscope;
11. Store samples at 4°C which maintains the fluorescence.

Chapter 3

Experimental part

In this chapter we are going to explain the different application of instruments and methods described in the previous chapter.

3.1 Peptide synthesis

3.1.1 EAK: peptide synthesis

3.1.1.1 Sequence

H-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-NH_2
MW = 1614,796 Da

3.1.1.2 Solid phase peptide synthesis

The self-assembling peptide EAK is obtained by the solid phase synthesis process, using Syro I synthesizer and the Fmoc chemistry. Side-chain protecting groups were: OtBu, Glu; Boc, Lys. The support used during this process is the Rink Amide mBHA resin (substitution 0,7 mmol/g); in this specific case we used 181,5 mg, which is equal to 0,125 mmol.

1. We prepared a 0,63M solution of each of the following amino-acids:
    a. Fmoc-Glu(OBut)OH;
    b. Fmoc-AlaOH;
    c. Fmoc-Lys(Boc)OH;
2. The synthesis began with 4 washing cycles with DMF;
3. A double coupling for the first amino-acid loading step was carried out;
4. The “Piperidine test” was performed in order to evaluate the resin functionalization;
5. Then the following 15 couplings were set: 4 with a single coupling, and the remaining with double couplings;
6. In order to remove the Fmoc group we treated the peptide with 40% piperidine/DMF solution for 3 minutes;

7. The activation of the carboxylic group of the protected N-alfa-Fmoc amino-acid, during the condensation reaction, was obtained dissolving 1 mL of amino-acid solution in 1,4 mL of 0,45M solution of HBTU/HOBt in DMF, and then adding 0,625 mL of DIPEA in NMP;
8. At the end, the resin was washed with dichloromethane 3-4 times;
9. Then the resin was dried under vacuum for 90 minutes: 434,59 mg of crude peptide on resin were obtained.

3.1.1.3 Peptide cleavage from the resin

Once the resin was dried we prepared the cleavage solution:

- 0,125 mL of MilliQ water;
- 0,125 mL of TES;
- 4,750 mL of TFA.

Each reactor was kept under gentle magnetic stirring for 90 minutes. When the releasing reaction was finished we filtered the resin and collected the solution into a round bottom flask. We performed also four washing cycles with TFA of the reactor in order to remove the entire amount of peptide.

The solution containing the crude peptide was concentrated to a small volume (5mL) using a Rotavapor.

The product was precipitated adding cold ethyl ether (4°C) and the solution was left in fridge for few minutes. The peptide obtained was filtered using a Gooch 4 and dried under vacuum for 1 hour.

Once dried, the weight of crude peptide was 65 mg, this weight was too little considering the weight of the resin with the peptide after the synthesis. So we supposed that some of the peptide was trapped into the porous septum of the gooch (filter) so we continued with other filtering sessions. After several washing cycle for the filter we obtained 91 mg of crude peptide.

3.1.1.3 Crude peptide purification

In order to characterize the peptide 1,03 mg of crude peptide were dissolved in 1mL of MilliQ water. The sample was used for two RP-HPLC analysis under the following conditions:

1. First analysis:
    a. Column: Jupiter C18 (5µm, 300Å, 250x10 mm);
    b. Injected volume: 50 uL;
    c. Flow rate: 1 mL/min;

d. Detector wavelength: 214 nm;
e. Eluent A: 0,05% of TFA in ultra-purified water;
f. Eluent B: 0,05% of TFA in CH3CN;
g. Gradient: from 0% to 20% of eluent B in 40 minutes. (figure 3.2).

2. Second analysis:
   a. Column: Jupiter C18 (5µm, 300Å, 250x10 mm);
   b. Injected volume: 50 uL;
   c. Flow rate: 1 mL/min;
   d. Wavelength: 214 nm;
   e. Eluent A: 0,05% of TFA in ultra-purified water;
   f. Eluent B: 0,05% of TFA in CH3CN;
   g. Gradient: from 5% to 25% of eluent B in 20 minutes. (figures 3.1).

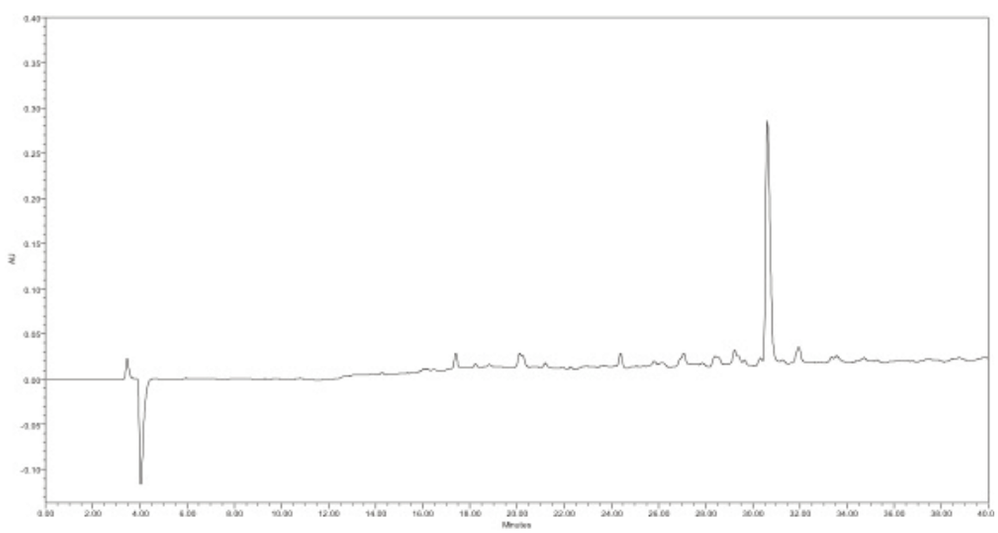


Figure 3.1: *Chromatogram of the crude peptide EAK (gradient: from 0% to 20% of eluent B in 40 minutes).*

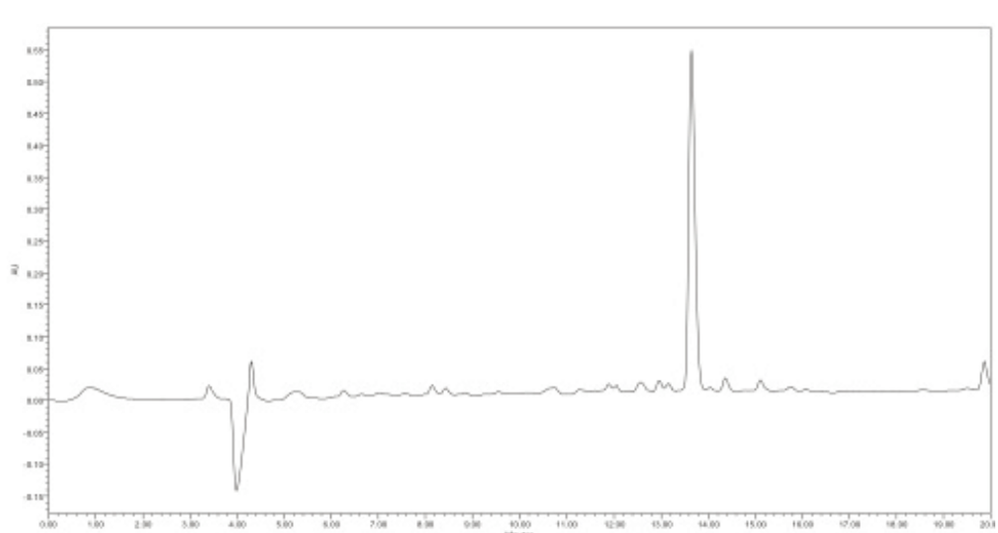


Figure 3.2: *Chromatogram of the crude peptide EAK (Gradient: from 5% to 25% of eluent B in 20 minutes).*

Three HPLC runs were performed in order to purify the crude peptide. All the crude peptide was dissolved in 50 mL of eluent A.

The purification was performed using the following conditions:

- Column: Delta Pack C18 (15µm, 300Å, 300x7,8 mm);
- Injected volume: 15 mL of a solution 1,3 mg/mL;
- Flow rate: 4 mL/min;
- Detector wavelength: 214 nm;
- Eluent A: 0,05% of TFA in ultra-purified water;
- Eluent B: 0,05% of TFA in CH3CN;
- Full-scale: 4.00;
- Gradient: from 0% to 16% of eluent B in 64 minutes.

We obtained several chromatogram and all of them had the same pattern, showed in figure 3.4. The collected fractions were analysed by analytical HPLC in order to evaluate the quality of each fraction. At the end we obtained 46,52 mg of peptide with a purity grade of 96,63%. The figure 3.3 shows the analytical HPLC we performed to control the purity of the collected fractions.

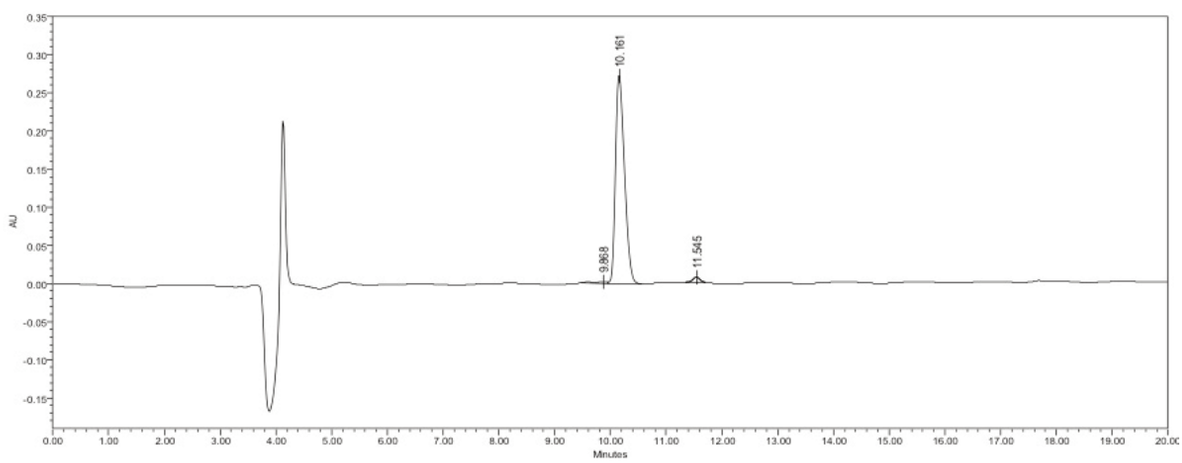


Figure 3.3: *HPLC analysis of purified EAK, performed in order to evaluate the purity of the peptide.*

Retention time (min)	% Area	Height (µV)
9,868	1,37	2430
10,161	96,63	273201
11,545	2,01	6829

Table 3.1: *Measurement of the peaks area, to evaluate the purity of the peptide.*

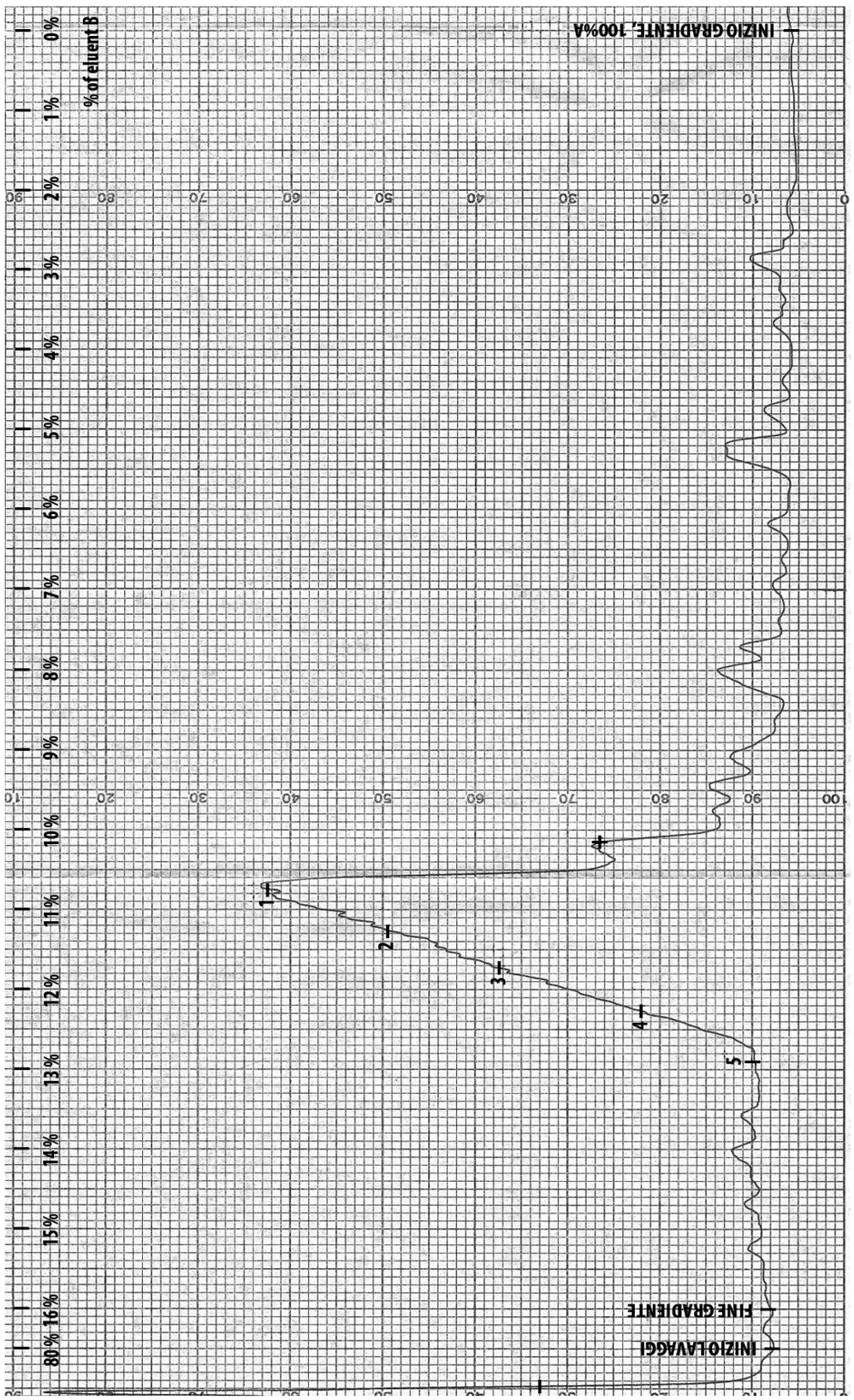


Figure 3.4: *Peptide EAK chromatogram. We obtained these results with the following conditions: column: Delta Pack C_{18} (15μm, 300Å, 300x7,8 mm); injected volume: 15 mL of a solution 1,3 mg/mL; flow rate: 4 mL/min; detector wavelength: 214 nm; eluent A: 0,05% of TFA in ultra-purified water; eluent B: 0,05% of TFA in CH_3CN; full-scale: 4.00; gradient: from 0% to 16% of eluent B in 64 minutes.*

3.1.2 RGD-EAK: peptide synthesis

3.1.2.1 Sequence

H-Arg-Gly-Asp-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys-NH_2
MW = 1943,126 Da

3.1.2.2 Solid phase peptide synthesis

The peptides RGD-EAK was synthesized using the same protocol indicated for EAK. Side-chain protecting groups were: Pbf, Arg; OtBu, Asp; OtBu, Glu; Boc, Lys. At the end of the synthesis the weight of the resin with the peptide was 452,78 mg

3.1.2.3 Peptide cleveage from the resin

In this case the unlocking process of the peptide from the resin worked very well and we obtained 228,45 mg of rough peptide.

3.1.2.4 Crude peptide purification

From the crude peptide we took 0,87 mg and we prepared a solution 1 mg/mL; with that solution we performed two analytic RP-HPLC in order to evaluate the peptide quality. The first one had a wide gradient and the second one has a gradient more focused. The settings are described below:

1. First analysis:
   - Column: Jupiter C18 (5µm, 300Å, 250x10 mm);
   - Injected volume: 50 uL;
   - Flow rate: 1 mL/min;
   - Detector wavelength: 214 nm;
   - Eluent A: 0,05% of TFA in ultra-purified water;
   - Eluent B: 0,05% of TFA in CH3CN;
   - Gradient: from 0% to 80% of eluent B in 80 minutes.

2. Second analysis:
   - Column: Jupiter C18 (5µm, 300Å, 250x10 mm);
   - Injected volume: 50 uL;
   - Flow rate: 1 mL/min;
   - Detector wavelength: 214 nm;
   - Eluent A: 0,05% of TFA in ultra-purified water;
   - Eluent B: 0,05% of TFA in CH3CN;
   - Gradient: from 10% to 25% of eluent B in 30 minutes.

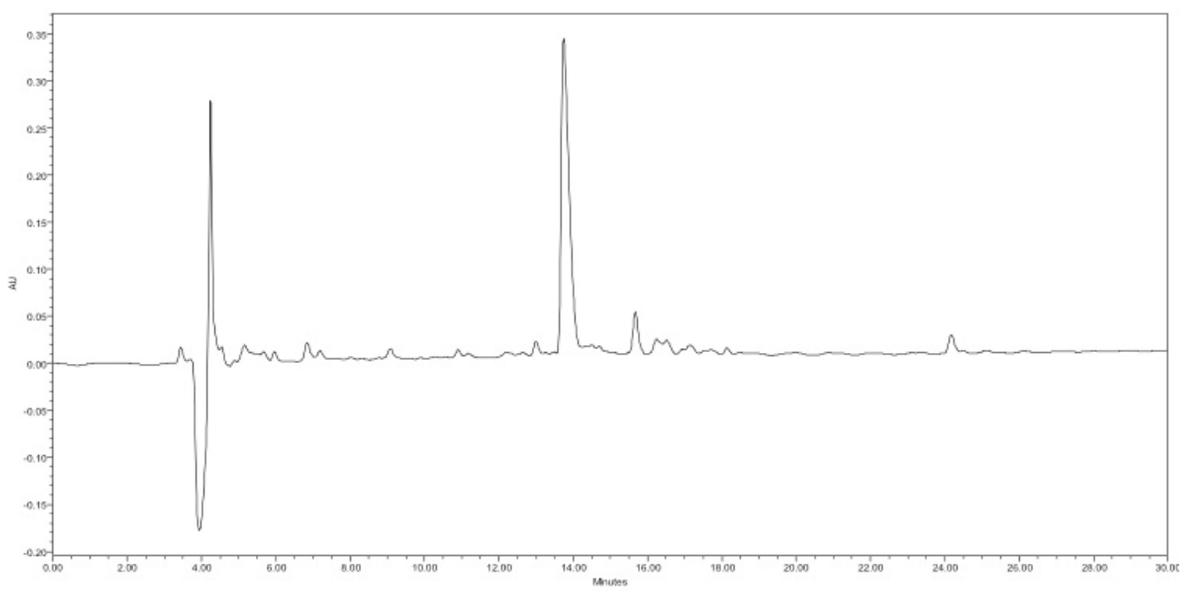


Figure 3.5: *Chromatogram of the crude peptide RGD-EAK (Gradient: from 10% to 25% of eluent B in 30 minutes).*

Once we were sure about the peptide (this peptide was already synthesized in our laboratory, so we knew its elution time) we continued with 4 RP-HPLC analysis in order to purify the crude peptide.

For the first analysis we dissolved 26,2 mg in 25 mL of eluent A. For the second one we dissolved 51,34 mg of peptide in 35 mL of eluent A. We could not weight the remaining peptide because of the presence of solid impurities and also of the gluey featuring of the rough peptide. So we aspired the entire remaining amount of peptide (about 90 mg) and we used it for the third and for the fourth analysis.

All these RP-HPLC analysis were performed using the following settings:

- Column: Jupiter C18 (5µm, 300Å, 250x10 mm);
- Injected volume: 15mL of a solution 1,3 mg/mL;
- Flow rate: 4 mL/min;

- Detector wavelength: 214 nm;
- Full-scale: 4.0;
- Eluent A: 0,05% of TFA in ultra-purified water;
- Eluent B: 0,05% of TFA in CH3CN;
- From 0% to 10% of eluent B in 2 minutes, then from 10% to 20% of eluent B in 30 minutes.

We kept the same conditions for all the HPLC, so we obtained the same pattern in all chromatograms. Figure 3.7 shows the pattern of RGD-EAK chromatogram.

When we finished all RP-HPLC analysis we performed several analytic HPLC analysis in order to evaluate the purity of the fractions collected. Once dried out all fractions of pure peptide we obtained 17,66 mg of peptide with a purity grade of 94,20 (Figure 3.6). This attested the low yield of the HPLC purifying process.

We did not need the mass spectroscopy for these peptides (EAK and RGD-EAK) because they are two “well known” peptides in our laboratory, so we know very well the retention time in HPLC analysis. We recognized those peptides using the analytic HPLC analysis and comparing them with previous analysis.

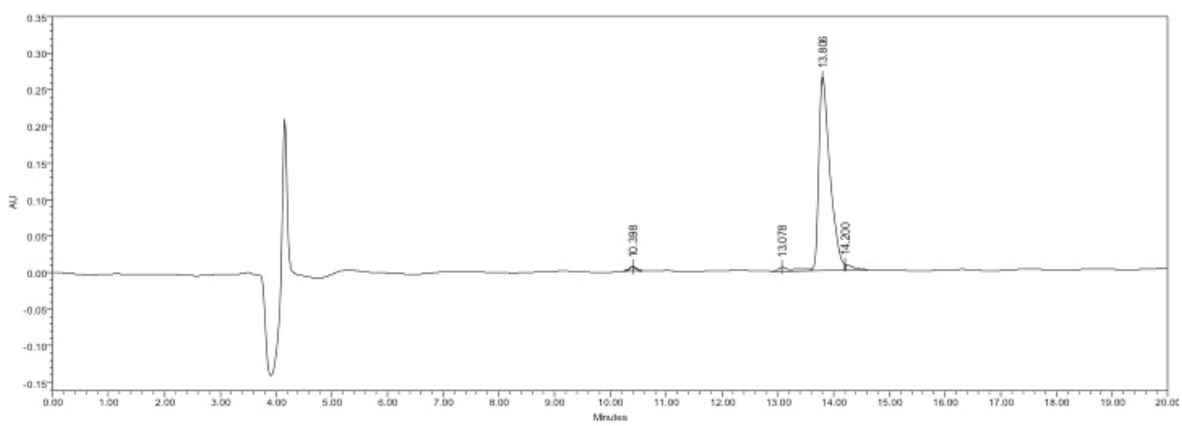


Figure 3.6: *HPLC analysis of purified RGD-EAK, performed in order to evaluate the purity of the peptide.*

Retention time (min)	% Area	Height (µV)
10,398	1,30	5735
13,078	2,54	4858
13,806	94,20	265296
14,200	1,95	7454

Table 3.2: *Measurement of the peaks area, to evaluate the purity of the peptide.*

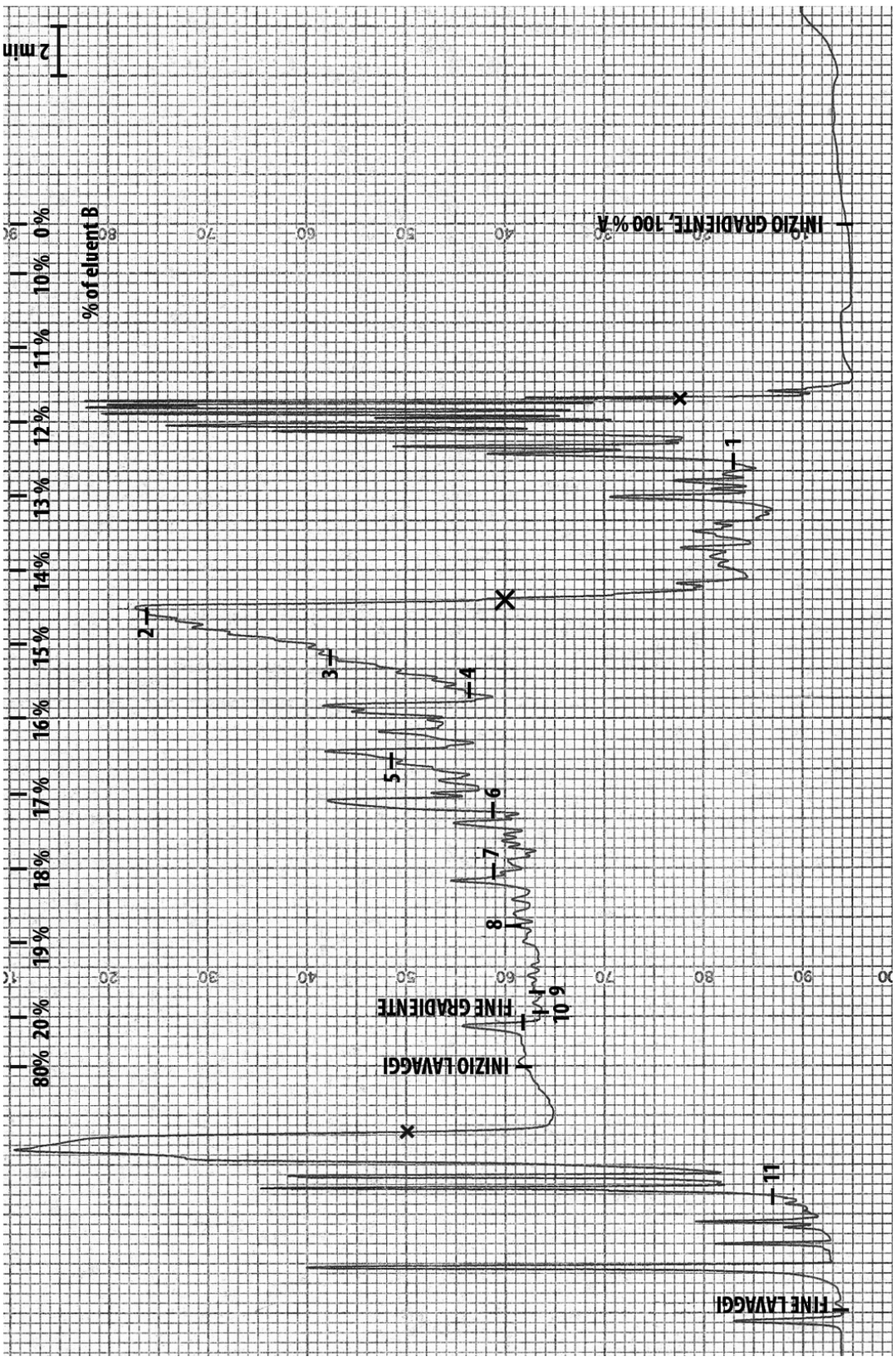


Figure 3.7: *Peptide RGD-EAK chromatogram. We obtained these results with the following conditions: column: Jupiter C_{18} (5μm, 300Å, 250x10 mm); injected volume: 15mL of a solution 1,3 mg/mL; flow rate: 4 mL/min; detector wavelength: 214 nm; full-scale: 4.0; eluent A: 0,05% of TFA in ultra-purified water; eluent B: 0,05% of TFA in CH_3CN; from 0% to 10% of eluent B in 2 minutes, then from 10% to 20% of eluent B in 30 minutes.*

3.1.3 EAbuK: peptide synthesis

3.1.3.1 Sequence

H-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-NH_2
MW = 1728,012 Da

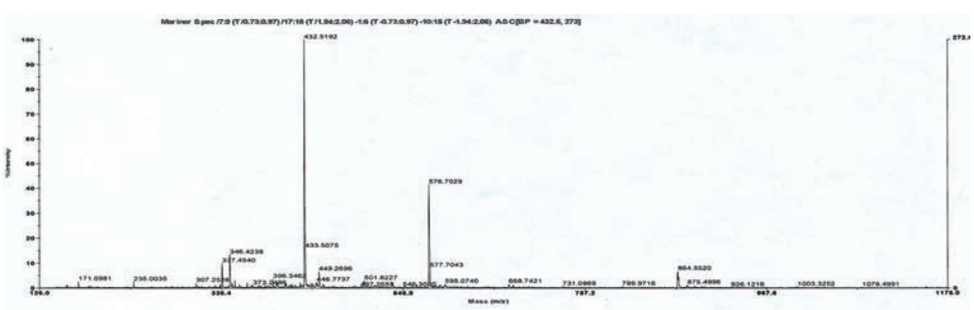
3.1.3.2 Solid phase peptide synthesis

This peptide was already made during previous synthesis.
Side-chain protecting groups were: OtBu, Glu; Boc, Lys.

3.1.3.3 Peptide characterization

This peptides was already made during previous synthesis and we performed a mass spectroscopy in order to ensure the correct molecular weight. The mass analysis results are shown in figures 3.8a and 3.8b.
These mass analysis confirmed the presence of the peptide with a accettable purity grade.

a)



b)

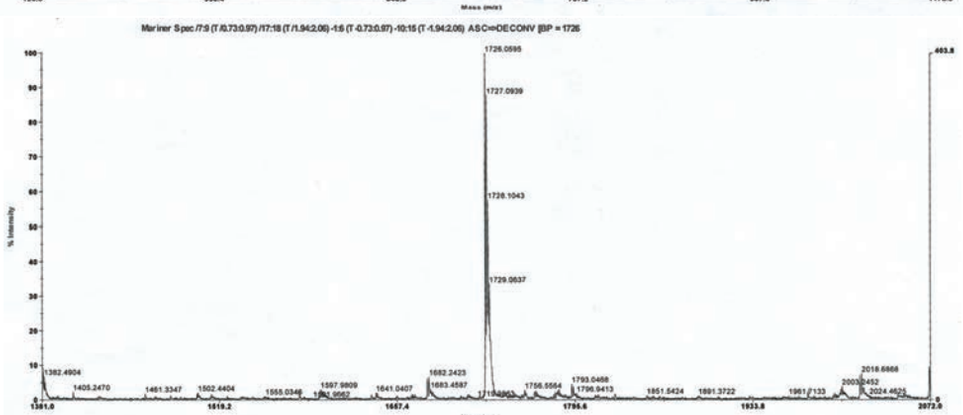


Figure 3.8: a) *Mass analysis (ESI-TOF) for the peptide EAbuK (theoretic weight 1728,012 Da);* **b)** *deconvolution of the same measurement.*

Then, in order to control the purity and to avoid the presence of by-products, we performed an analytic HPLC analysis of the fractions we used during the scaffolds enrichment process.
The analytic HPLC analysis we performed was set as follow and the chromatograph is shown below (figure 3.9):

- Column: Symmetry Shield RP8 5um;
- Injected volume: 180 mL;
- Flux: 1 mL/min;
- Wavelength: 214 nm;
- Eluent A: 0,05% of TFA in ultra-purified water;
- Eluent B: 0,05% of TFA in CH3CN;
- Gradient: from 10% to 30% of eluent B in 40 minutes.

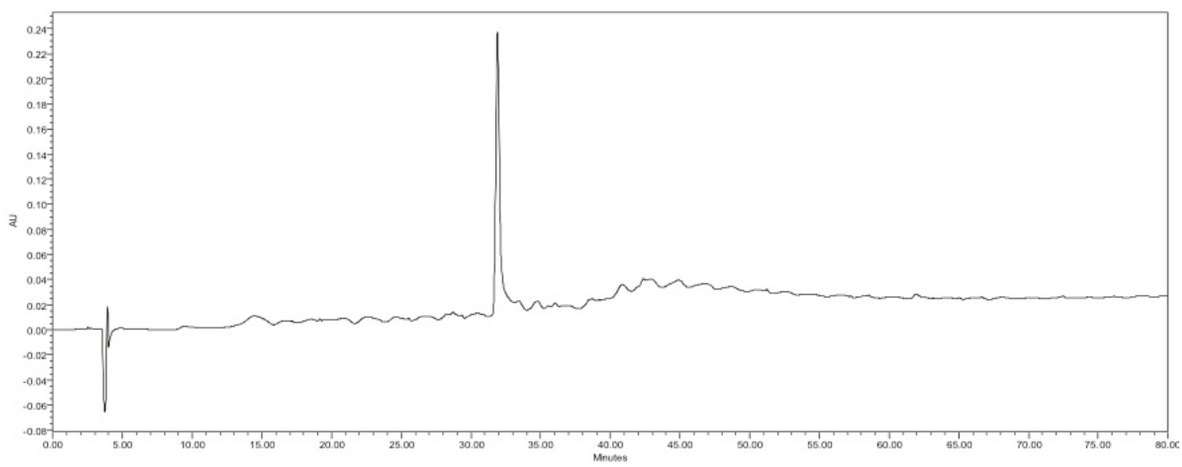


Figure 3.9: *Chromatogram of the crude peptide EAbuK (Gradient: from 10% to 30% of eluent B in 40 minutes).*

3.1.4 RGD-EAbuK: peptide synthesis

3.1.4.1 Sequence

H-Arg-Gly-Asp-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-Abu-Glu-Abu-Glu-Abu-Lys-Abu-Lys-NH_2
MW = 2055,376 Da

3.1.4.2 Solid phase peptide synthesis

The self-assembling peptide RGD-EAbuK was obtained by the solid phase synthesis process,

using Syro I synthesizer and the Fmoc chemistry. Side-chain protecting groups were: Pbf, Arg; OtBu, Asp; OtBu, Glu; Boc, Lys. The support used during this process is the Rink Amide mBHA resin (substitution 0,7 mmol/g); in this specific case we use 180,16 mg, which is equal to 0,125 mmol.
The synthesis proceeds as for the other peptides (EAK and RGD-EAK). Once the first amino-acid was bound to the resin we performed the "piperidine test" as required by the process, but in this case the yield was too low to continue the synthesis. In order to improve the reaction yield we performed the acetylation reaction.
The acetylation is a chemical process used to block free sites of the resin where amino-acids could attach. Hence if we block these sites when the first amino-acid is bind to the resin, the following amino-acids should attach in the right succession.
In order to performed this chemical process we made a "acetylation solution" made of:

- 10% acetic anhydride;
- 5% lutidine;
- DMF.

We added this solution to the reactor and we left it in the reactor for 20 minutes. Once the reaction was ended we performed 4 washing with DMF in order to clean the resin and to remove all the solution's residuals.
Once the synthesis was ended we obtained 426,03 mg of resin with peptide.

3.1.4.3 Peptide cleavage from the resin

For this peptide we used the same protocol proposed for the other peptides.
So we prepared the cleavage solution:

- 0,125 mL of ultra-purified water;
- 0,125 mL of TES;
- 4,750 mL of TFA.

The resin was kept under gentle magnetic stirring with the unlocking solution for 90 minutes.
When the cleavage reaction was finished we filtered the resin and collected the solution into a round-bottom flask. We performed also three washing cycles with TFA of the reactor in order to remove the entire amount of peptide.
This solution containing the peptide was concentrated to a small volume (5 mL) using a Rotavapor.
The product was precipitated adding cold ethyl ether (4°C) and the solution was left in fridge for few minutes. The peptide obtained was filtered using a Gooch 4 and dried under vacuum for 1 hour.
Once the crude peptide was dried out its weight was 196,82 mg.

3.1.4.4 Crude peptide purification

In order to perform some analytic HPLC analysis, 0,62 mg of crude peptide were dissolved in 0,65 mL of MilliQ water. We performed a HPLC analysis using the following conditions;

- Column: Symmetry Shield RP8 (5µm, 300 Å, 250x4,6 mm);
- Injected volume: 100 mL;
- Flow rate: 1 mL/min;
- Detector wavelength: 214 nm;
- Eluent A: 0,05% of TFA in ultra-purified water;
- Eluent B: 0,05% of TFA in CH3CN;
- Gradient: from 0% to 40% of eluent B in 80 minutes (Figure 3.10).

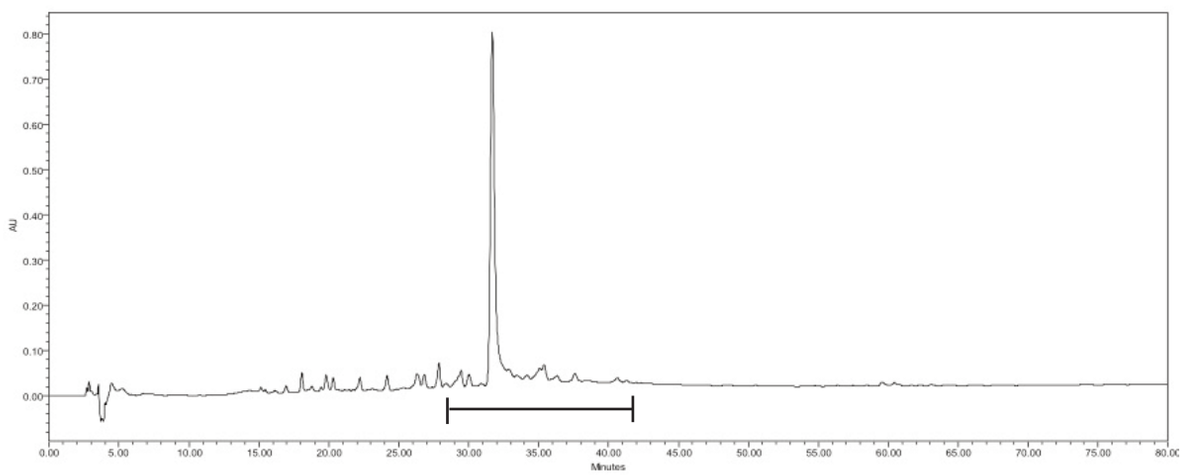


Figure 3.10: *Chromatogram of the crude peptide RGD-EAbuK (Gradient: from 0% to 40% of eluent B in 80 minutes).*

During this analysis we also collected the eluted solution during the period highlighted in the figure 3.10. The collected solution was used to performed a mass spectroscopy. We used that mass analysis in order to confirm that its molecular weight was close enough to the theoretic weight. (Figures 3.11a and 3.11b).

Once we were sure about the presence of the correct peptide, we began the purification process. We performed several HPLC purification runs with different amount of dissolved peptide.

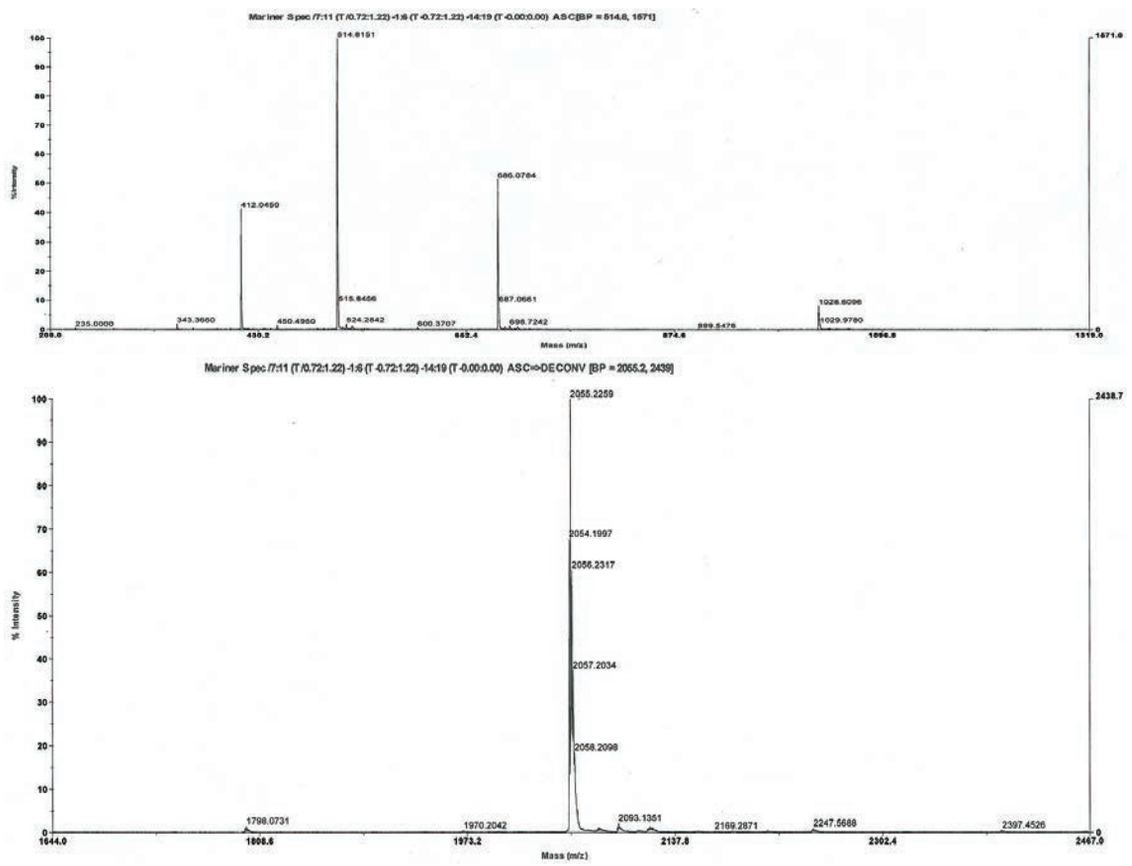


Figure 3.11: a) *Mass analysis (ESI-TOF) for the peptide RGD-EAbuK (theoretic weight 2055,376 Da);* **b)** *deconvolution of the same measurement.*

For all the purification runs we used the following conditions:

- Column: Delta Pack C18 (15 μm, 100 Å, 7,8x300 mm);
- Injected volume: 20 mL of a solution 1,2 mg/mL;
- Flow rate: 4 mL/min;
- Detector wavelength: 214 nm;
- Eluent A: 0,05% of TFA in ultra-purified water;
- Eluent B: 0,05% of TFA in CH3CN;
- Gradient: from 0% to 5% of eluent B in 2 minutes, then from 5% to 20% of eluent B in 45 minutes.

The figure 3.12 shows the chromatogram of a HPLC analysis we performed in order to purify the crude peptide. This is the standard pattern for all the purification analysis we made. Unfortunately the absorbance values are not very high, so there is not a tight and sharp peak. The peptide was eluted during a large period of time (about 9 minutes) and during a wide range of percentage (approximately between 14% and 20% of eluent B).

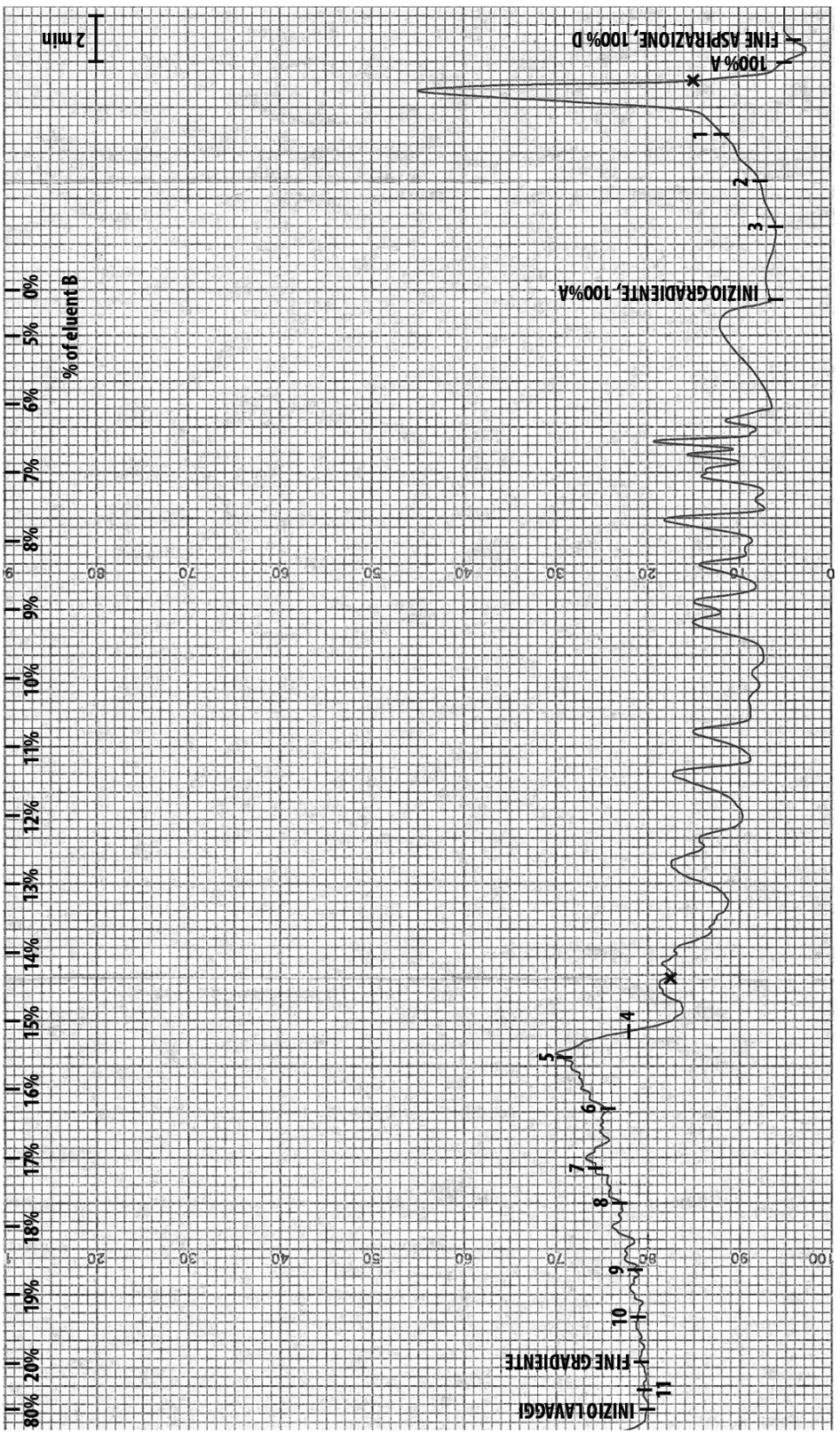


Figure 3.12: *Peptide RGD-EAbuK chromatogram. We obtained these results with the following conditions: column: Delta Pack C_{18} (15 μm, 100 Å, 7,8x300 mm); injected volume: 20 mL of a solution 1,2 mg/mL; flow rate: 4 mL/min; detector wavelength: 214 nm; eluent A: 0,05% of TFA in ultra-purified water; eluent B: 0,05% of TFA in CH_3CN; gradient: from 0% to 5% of eluent B in 2 minutes, then from 5% to 20% of eluent B in 45 minutes.*

For each fraction we performed an analytic HPLC analysis in order to evaluate the purity of the peptide. A typical pattern of these analysis is shown in the figure 3.13.
In this case the purity grade is of 92,23%.

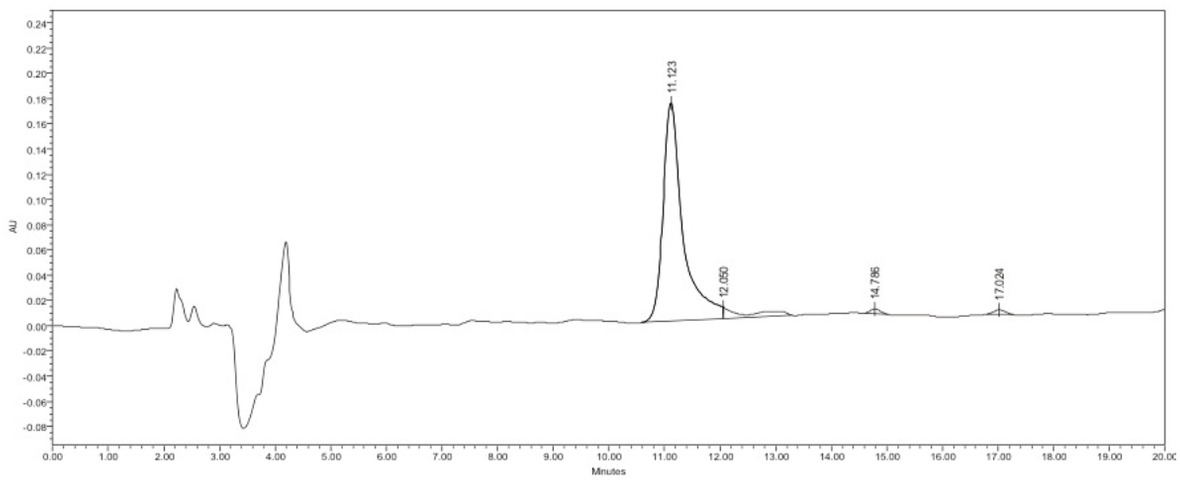


Figure 3.13: *HPLC analysis of purified RGD-EAbuK, performed in order to evaluate the purity of the peptide.*

Retention time (min)	% Area	Height (µV)
11,123	92,23	173316
12,050	5,68	9120
14,786	0,97	3664
17,024	1,13	3543

Table 3.3: *Measurement of the peaks area, to evaluate the purity of the peptide.*

Once we evaluated the purity of each collected fraction, we put together the fractions with similar purity and we lyophilized them. Once this process was ended we obtained 54,2 mg of the most pure peptide fraction.

3.2 Preparation of β-TCP disks

3.2.1 Liquid penetration evaluation

In order to be sure about the penetration of SAPs solutions into the β-TCP porosity, we evaluated this material feature performing a “dye test”. We prepared a solution with a cellular dye (the same used for cells counting) and we soaked our scaffolds into this aqueous solution. This simple test is quite significant because in the enrichment processes we used low peptide concentrations, so the viscosity of water and that of our solutions are quite close to each other. Once we completely immersed the scaffolds, we left them in the oven at 37°C for 4 hours. In our experiment we left the treated samples at the same temperature but for 24 hours, in order to allow the complete penetration.
After 4 hours we cut the “coloured” sample and we noticed that the dye was penetrated to the core of the ceramic material. The interconnected porosity allows the complete absorption of the liquid. Once we saw this result we prepared the SAPs solution for the enrichment processes.

3.2.2 SAPs treatment

We prepared different low concentration SAP solutions. The low concentration was mandatory in order to avoid the self-assembling process before the penetration of the solution into the scaffolds. We used four different self-assembling peptides: EAK, RGD-EAK, EAbuK and RGD-EAbuK, further more we prepared different concentrations in order to evaluate their influence. We set up eight different enrichment situations:

- 100% EAK;
- EAK + 10E-5M RGD-EAK;
- EAK + 10E-6 RGD-EAK;
- 50% EAK + 50% RGD-EAK
- 100% EAbuK;
- EAbuK + 10E-5M RGD-EAbuK;
- EAbuK + 10E-6M RGD-EAbuK;
- 50% EAbuK + 50% RGD-EAbuK.

Once SAPs are dried out, they are very electrostatic, so we prepared a 0,1% w/v stock solution with ultra-purified water, in order to avoid any peptide losses. Using this stock solution we prepared the different concentrations listed above in 1 mL for each sample (1 mL is the volume we needed to completely cover the β-TCP disks).
Once we prepared all samples, we left them in the oven at 37°C for 24 hours in order to allow the liquid penetrate inside the β-TCP porosity. Then we took out all samples and we put them

in PBS in order to induce the hydrogel formation. And again we left sample in the oven at 37°C for 24 hours.
When we took out the scaffolds they were ready to be seeded with HOBs.

3.3 Biological assessments

3.3.1 Cells culture

During our biological assessments we had to use several different cell culture techniques like the trypsinising process or the cryopreservation.

3.3.1.1 Trypsinising adherent cells

When a flask was full of cells, and they were confluent, we had to divide them in order to allow cells to grow. The method we used is described below:

1. Remove medium from flask(s) and discard;
2. To the new flask add 10 mL of PBS to culture, rinse round and discard PBS;
3. Add 1 mL trypsin/HEPES (x 1) mixture, gently and incubate in CO_2 incubator for 5 minutes;
4. Gently tap the flask to dislodge the cells. Observe under the microscope and control the cells detached from the flask. The cells should be floating;
5. Add 5 mL of cell culture media, swirl flask and remove cell suspension to a sterile universal. Rinse flask with further 5 mL of media and add rinsings to universal;
6. Centrifuge at 2000 rpm for 5 minutes;
7. Remove medium by tipping the fluid off, taking care not to disturb cell pellet;
8. A small amount of medium is left in the vial, flick the vial until the pellet is resuspended;
9. Add 10 mL of medium and divide cells between required number of flasks;
10. Add medium to make up to final incubation volume as appropriated.

3.3.1.2 Cryopreservation of cells

Cells are stored frozen in a mixture of FCS and dimethyl sulphoxide (DMSO); the addition of DMSO to the freezing mixture reduces ice crystal formation during the freezing process and prevents rupture of cell membranes.

The method we used to freeze down cells is described below:

1. Remove medium from flask(s) and discard;
2. To the flask add 10 mL of PBS to culture, rinse round and discard PBS;
3. Add 1 mL of trypsin/HEPES (x 1) mixture, swirl gently and incubate in CO_2 incubator for 5 minutes;
4. Gently tap the flask to dislodge the cells. Observe under the microscope and control the cells detached from the flask. The cells should be floating;
5. Add 5 mL of cell culture media, swirl flask and remove cell suspension to a sterile universal. Rinse flask with further 5 mL of media and add rinsings to universal;
6. Centrifuge at 2000 rpm for 5 minutes;
7. Remove medium by tipping the fluid off, taking care not to disturb cell pellet;
8. A small amount of medium is left in the vial, flick the vial until the pellet is resuspended;
9. Add 1 mL of freezing mixture and transfer cell suspensions to individual cryovials;
10. Put the vials into a tray and leave them in the -70°C freezer overnight for the first stage of the freezing process;
11. Transfer the vials to the appropriate tray in the liquid nitrogen storage vessel.

Vials of cells had to be transferred to liquid nitrogen within 24 hours of initial freezing, leaving them at -70°C for longer than this will result in a loss of viability.

3.3.2 Cell seeding

Once we counted the number of cells in the cell suspension (number of cells/mL) we seeded 10E5 cells for each sample. We used a repeating pipette and we seeded cells onto the top of the disks in order to allow a better cell adhesion.

Chapter 4

Results and discussion

During this thesis project, we assessed the cellular responses by using mainly two different biological tests: Alamar Blue and MTT. In this chapter the results of both analyses are shown and then discussed. Furthermore, once we illustrated all the biological outcomes we will concentrate on a particular bio-physical result discovered by using the immunofluorescent imaging.

4.1 Alamar Blue results

One day after seeding, the samples treated with the peptides showed higher absorbance values (indicating an increase of the cell proliferation) compared to the untreated β-TCP (indicated with “water”). However this effect was not as high as that observed on the control (THX). The difference between peptide-treated β-TCP and untreated β-TCP was significant (p-value<0,05). From the obtained results, it appears that the peptides are influencing the early stages of cell interaction with the substrate. The differences between the two self-assembling peptides are not significant (p-value>0,05). In this phase the self-assembling peptides showed the same bioactivity of non-self-assembling peptides (GE3M).
The enhanced proliferation observed for all peptide-treated samples is probably due to the surface modification caused by peptides [47] rather than the 3D structure of the self-assembling peptides. It is noteworthy that the self-assembling peptides have the advantage of gel formation and thus they are not subjected to desorption, as it is possible for the peptide GE3M (non-self-assembling peptide) to undergo.
In the surface modification process, the actions observed with GE3M may be the direct effect of its ionic interactions with negative charged β-TCP as it presents a net charge of 2^+ whereas SAPs presents a net charge of 1^+.
Four days post seeding, the samples treated with the peptides showed an enhanced cellular response compared to the untreated β-TCP (water), and significantly greater than the control THX.
The positive effect of β-TCP scaffold enrichment was similar for all the three peptides used.
7 and 14 days post seeding, no differences in cellular response were observed between the samples treated with the peptides and the control (water). However, all samples treated with the

peptides showed higher values compared to the control (THX). The drop in metabolic activity and cell proliferation corresponds to the normal decline in cell growth as the cells enter a differentiation stage.

It can be concluded that cells seeded on β-TCP scaffolds (both enriched or non-enriched with peptides) show better proliferation response when compared to the optimized (these surfaces are known to be optimized to enhance cell adhesion and growth) cell culture control THX.

At days 21 and 28, all β-TCP (both treated and untreated) showed very similar proliferation, lower than the THX control.

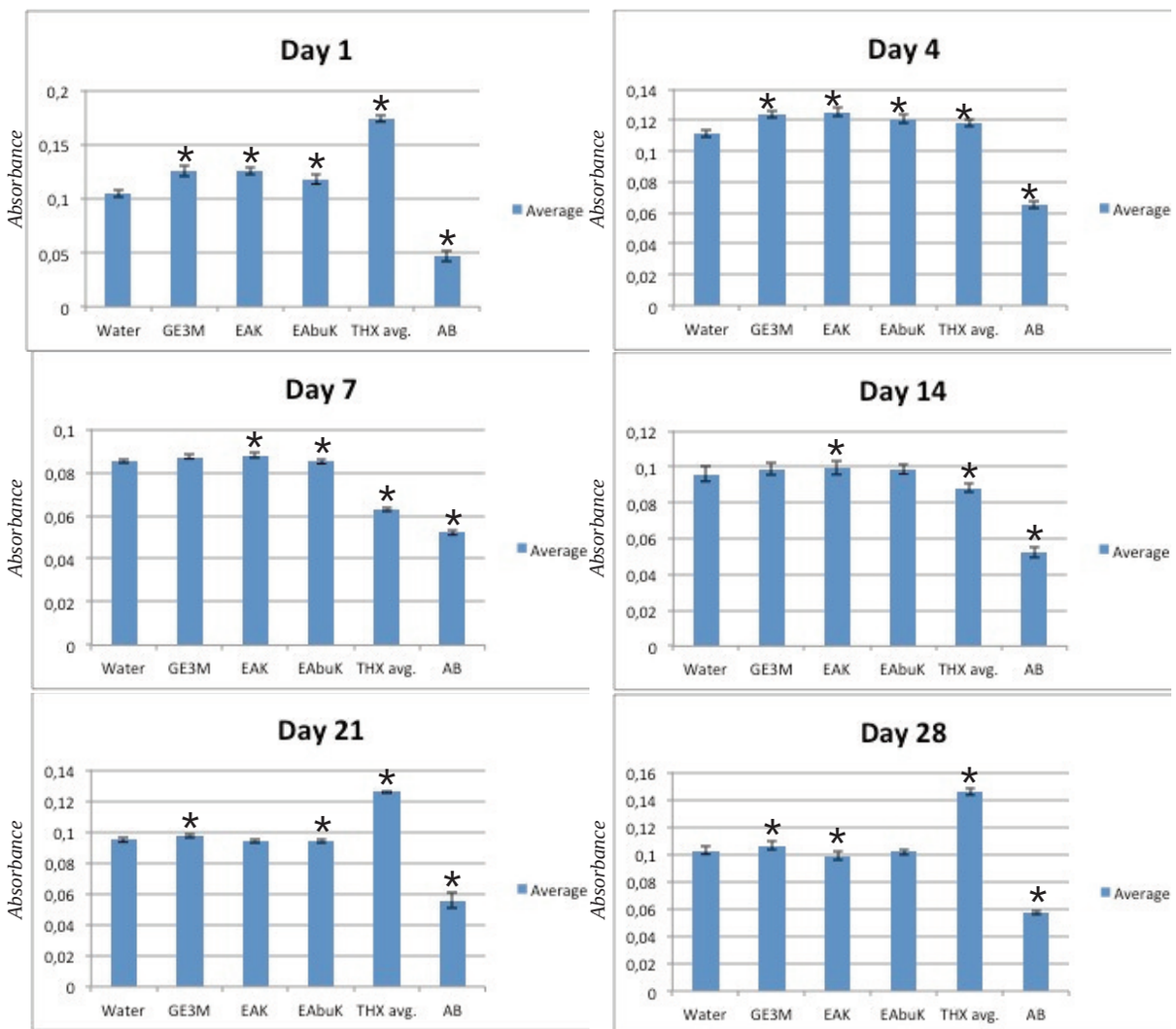


Figure 4.1: *Histograms of Alamar Blue data. In each table we marked the significant data compared to water (first column).*

Considering the further enrichment of SAPs with sequences carrying on the RGD motif, we can make the following considerations.

Twenty four hours post seeding cellular proliferation observed on the after seeding, peptide treated β-TCP sample was significantly (P-value<0,05) greater than the control water-treated β-TCP: in those scaffolds enriched with RGD sequences, cellular proliferation was significantly higher compared to the peptide alone or to β-TCP treated with water. The optimal response to RGD treated samples was observed at the concentration of 10E-5M, similar results were observed for both EAK and EAbuK peptides.

These differences became less evident as time passes and, after 28 days, proliferation was observed to be higher on hydrogels that had not been treated with RGD sequences. It should be noted, however, that RGD motif is an adhesive sequence whose effect is predominantly seen during the very early stages of cellular interactions with surfaces/scaffolds, and is associated with adhesion of cells rather than proliferation.

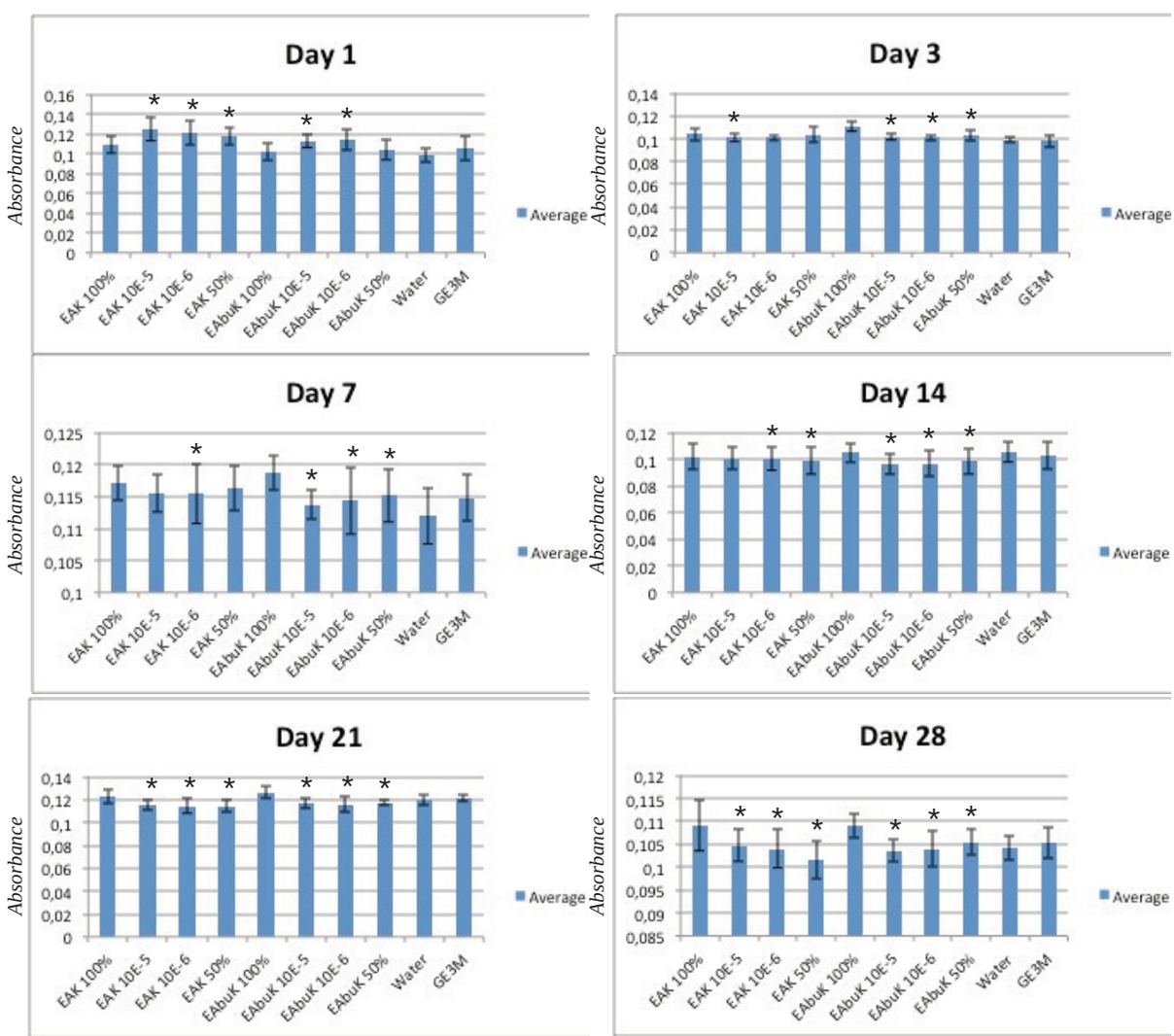


Figure 4.2: *Histograms of Alamar Blue data. In each table we marked the significant data compared to peptide without RGD motif, respectively for EAK and EAbuK.*

4.2 MTT results and discussion

A variable response was observed in cells exposed to the different tested peptide concentrations. In general, a drop was observed in metabolic activity at 24 hrs for cells cultured in the presence of EAK. However, cellular response was enhanced after 72 hours in all cases. The response to EAbuk was more varied between the different peptide concentrations at 24 hours but the response increased after 72 hours in the presence of EAbuk 100% and 50% and also GE3M, indicating that cellular activity was affected by both concentration and exposure time.

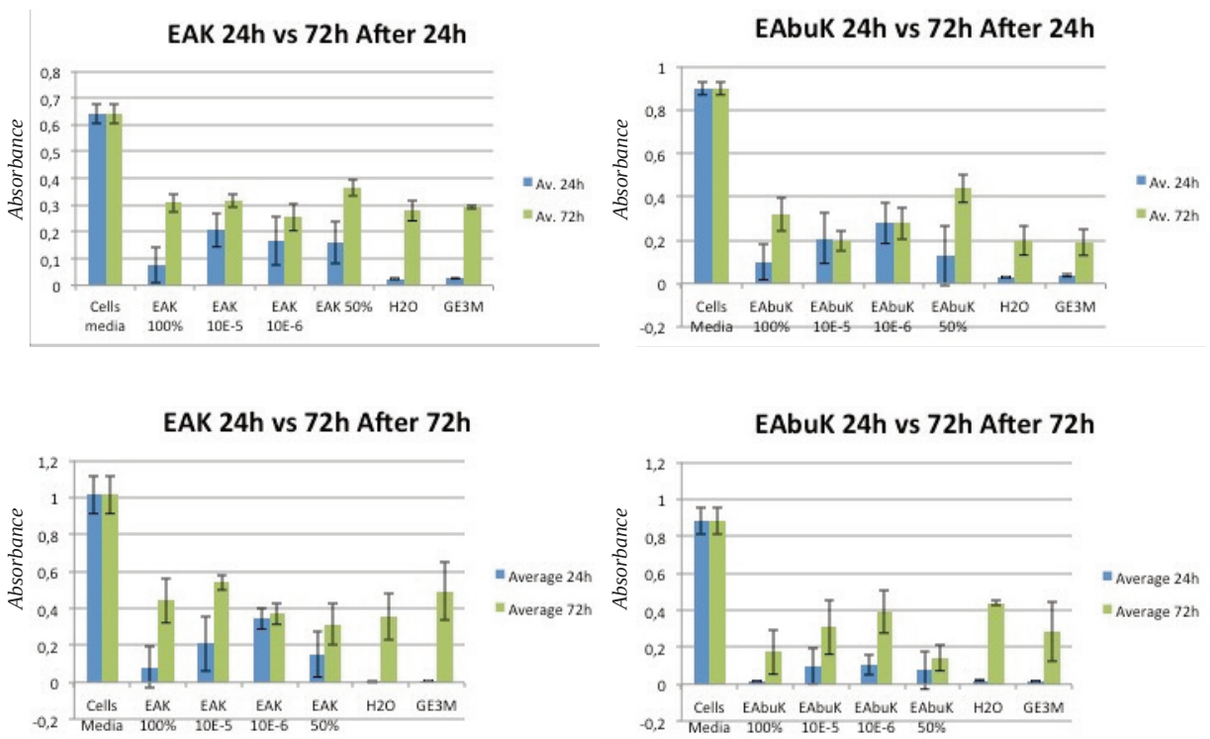


Figure 4.3: *Histograms of MTT data.*

If one examines the mean response of cells at 24 and 72 hrs, it appears that there was no significant (p-value>0,05) RGD motif dose effect on cells cultured in the presence of EAK. In the case of EAbuk, both 50 and 100% dose at 72 hrs showed a significant (p-value<0,05) increase in cell metabolic activity.

4.3 Imaging results

The fluorescent microscope images show cells on β-TCP scaffolds both treated and non-treated with self-assembling peptides: the cells appear roundish shape. The morphology of the cells does not show substantial differences in the presence of the peptides even in the case they present the adhesive RGD motif.

Worthy of note is the effect of trypsin on the samples. This enzyme commonly causes detachment of the cells from the substrates. This expected effect was obtained for the scaffolds not treated with the self-assembling peptides as evidenced by the images shown in figures 4.4 and 4.5, completely devoid of cells. In contrast trypsin was not able to remove the cells on the samples pre-treated with the self-assembling peptides (figures from 4.6 to 4.12). This can only be explained by the penetration of cells within the peptide hydrogel which, thanks to its three-dimensional structure, is particularly resistant to the action of various proteolytic enzymes.

This observation suggests possible uses of SAPs in the contexts in which cells should be protected from attack by proteolytic enzymes such as the protection of pancreatic cells from the activation and the action of trypsin, which they produce as inactive precursor (trypsinogen).

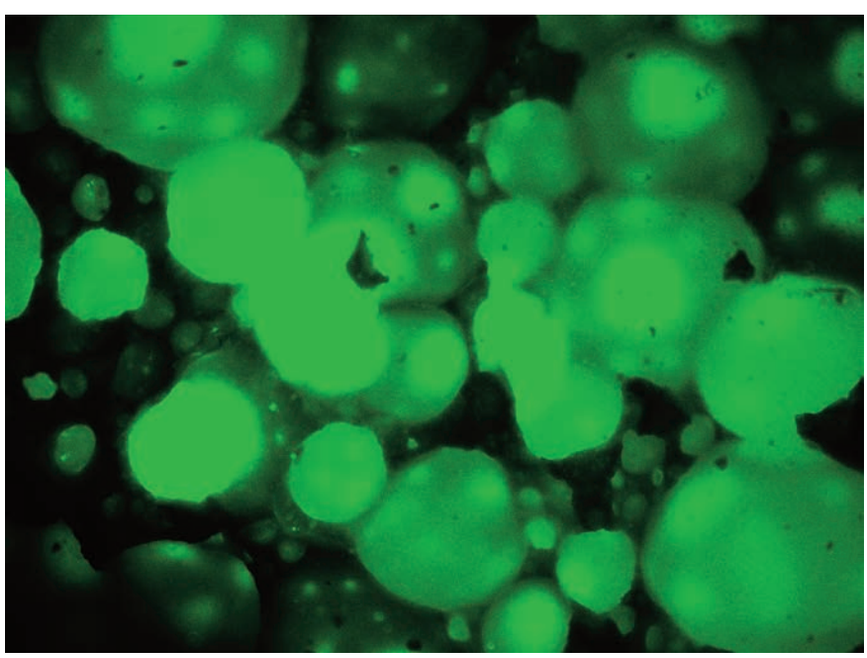


Figure 4.4: *Immuno-staining image of the sample treated with GE3M (non sel-assembling peptide) after the action of trypsin.*

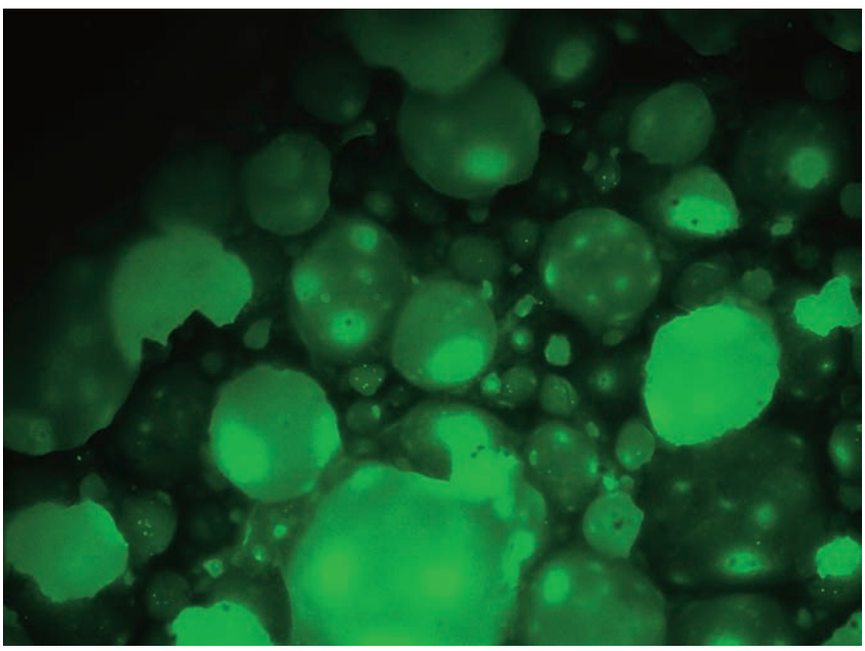


Figure 4.5: *Immuno-staining image of the sample non-treated with self-assembling peptides, nor with any other peptide; picture taken after the trypsin action.*

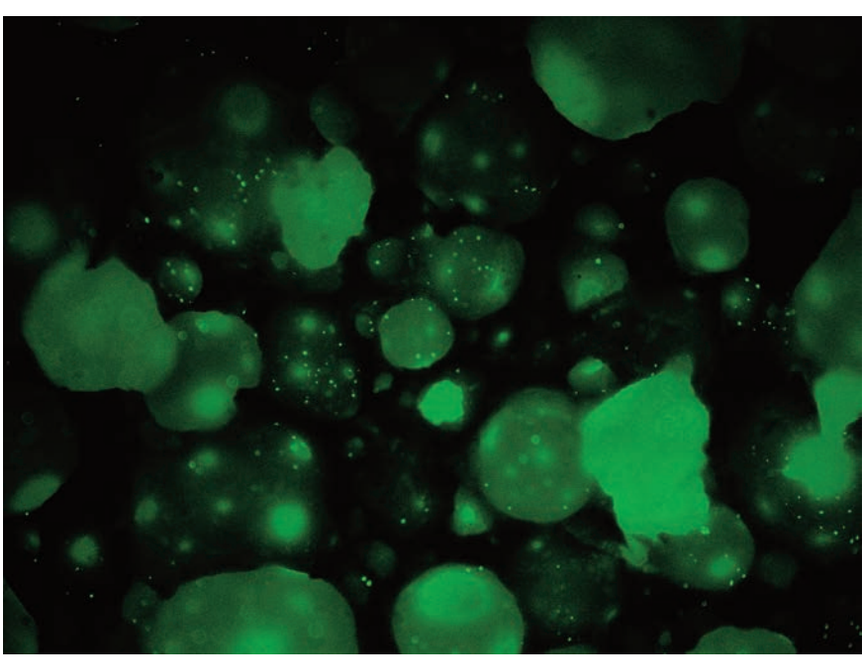


Figure 4.6: *Immuno-staining image of the sample treated with peptide EAbuK with RGD-EAbuK (the molar concentration of RGD motif in this case is 10E-6).*

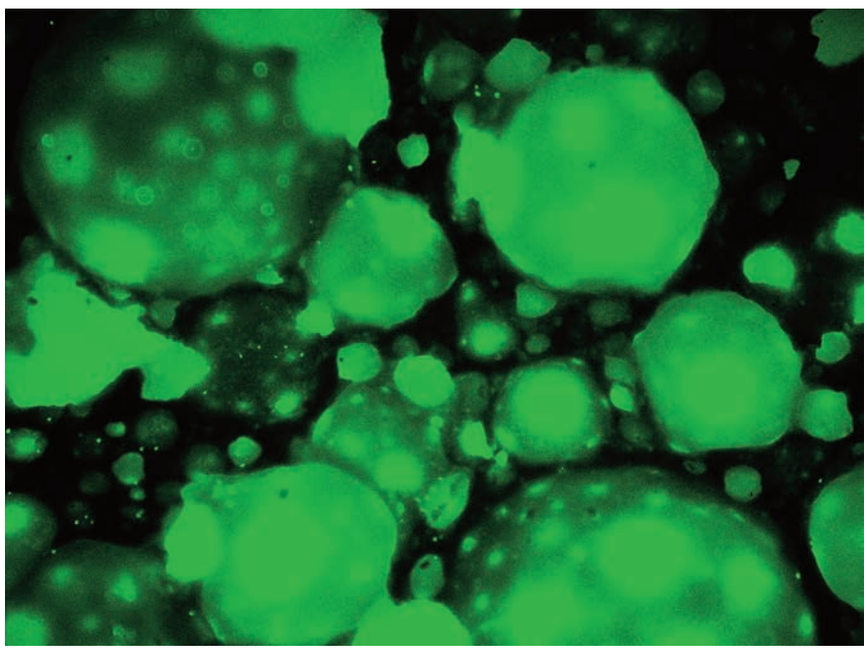


Figure 4.7: *Immuno-staining image of the sample treated with peptide EAbuK with RGD-EAbuK (the concentration of RGD motif in this case is 50% in volume).*

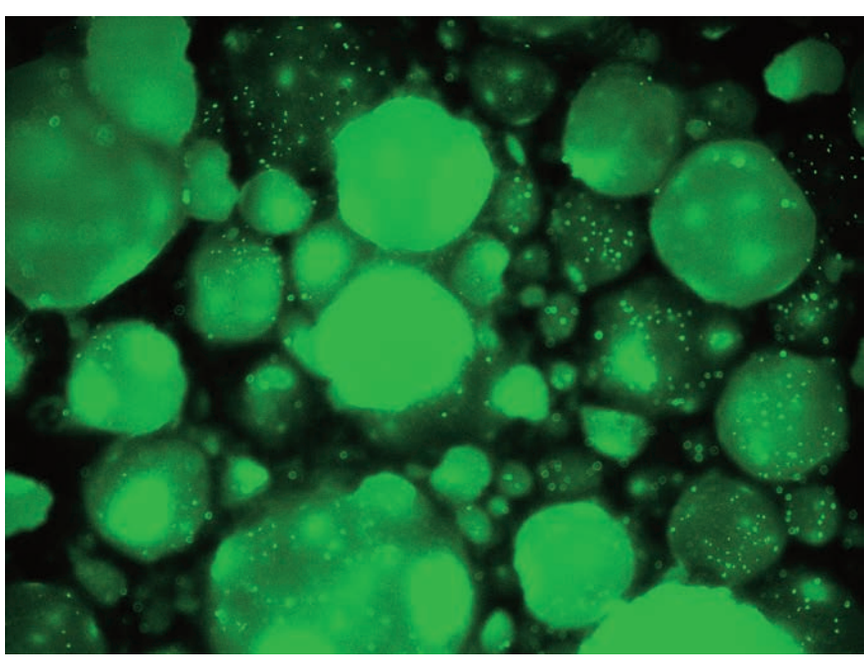


Figure 4.8: *Immuno-staining image of the sample treated with peptide EAbuK; in this case the RGD motif was not used for the sample enrichment.*

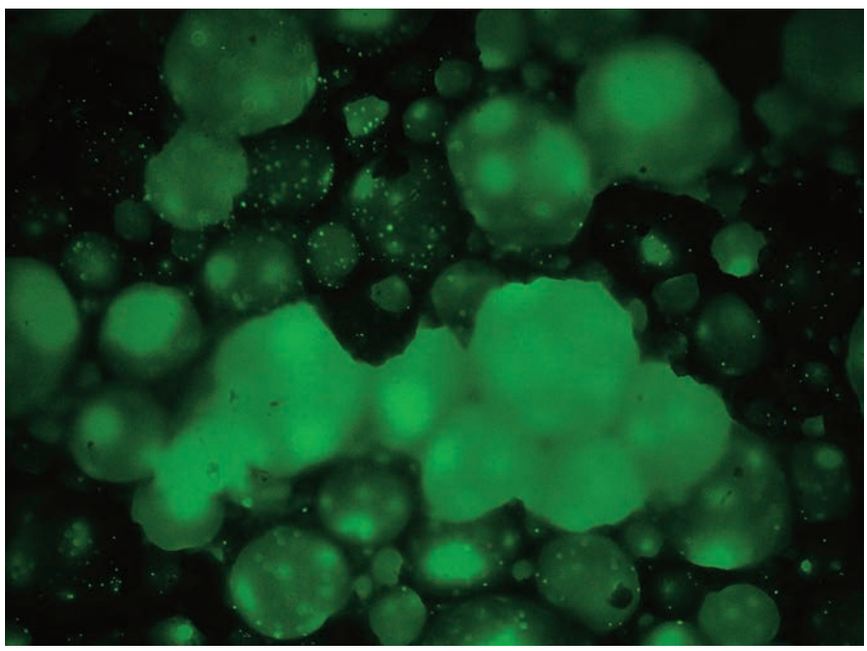


Figure 4.9: *Immuno-staining image of the sample treated with peptide EAK with RGD-EAK (the molar concentration of RGD motif in this case is 10E-5).*

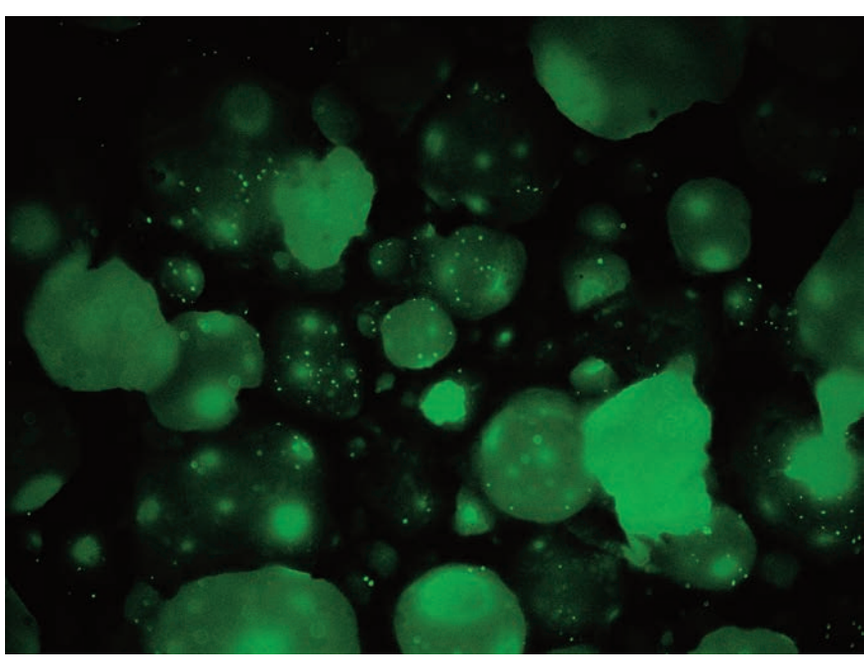


Figure 4.10: *Immuno-staining image of the sample treated with peptide EAK with RGD-EAK (the molar concentration of RGD motif in this case is 10E-6).*

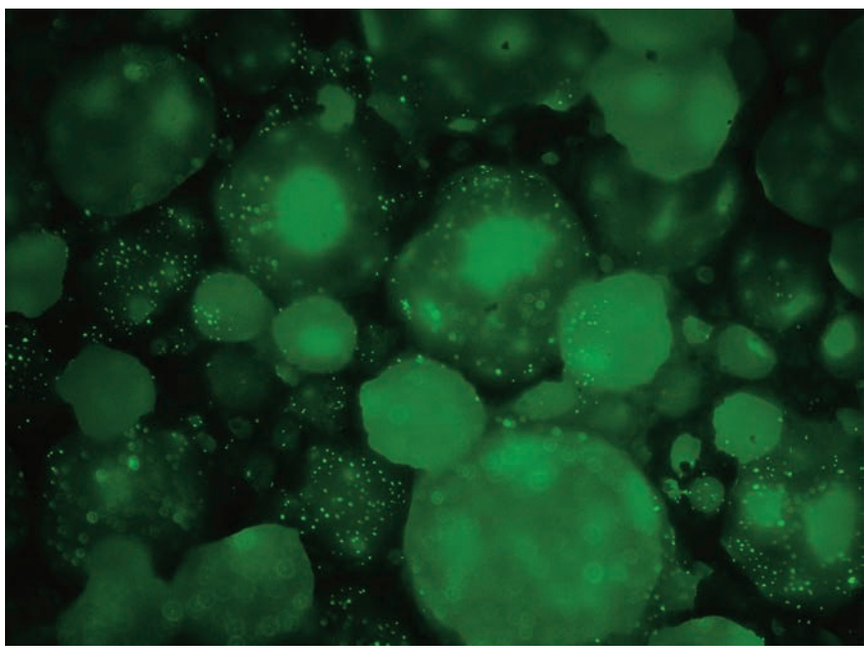


Figure 4.11: *Immuno-staining image of the sample treated with peptide EAK with RGD-EAK (the concentration of RGD motif in this case is 50% in volume).*

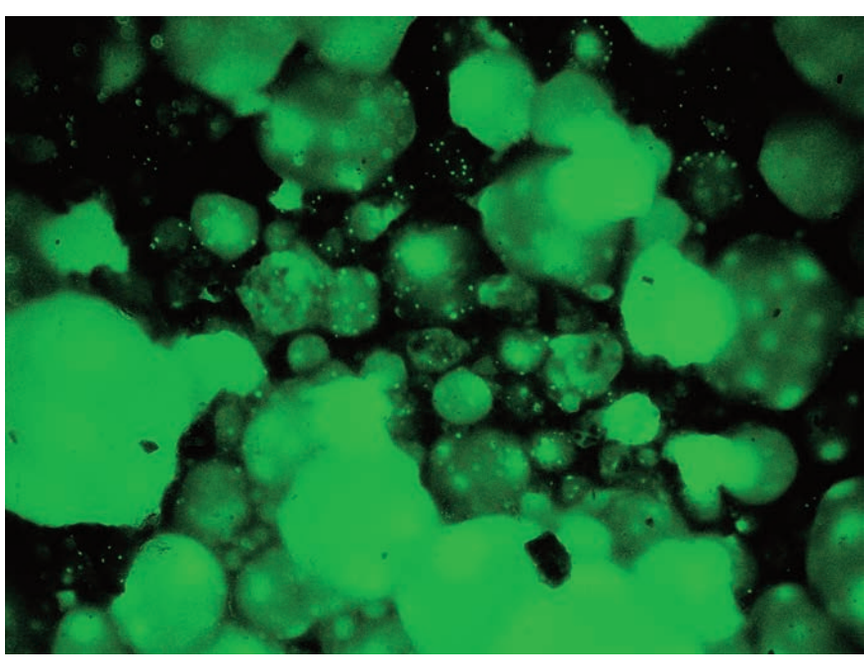


Figure 4.12: *Immuno-staining image of the sample treated with peptide EAK; in this case the RGD motif was not used for the sample enrichment.*

4.4 Conclusions

In summary:

1. The treatment with the peptides induces a better proliferation at 1, 4 and 7 days compared to β-TCP treated with water. The effect is present for both the self-aggregating peptides (EAK and EAbuK) and for the control peptide (GE3M, non-self-assembling peptides). The proliferation of these samples is less than that of the positive control (THX) at day 1 while it is higher than the positive control at day 7.
2. The enrichment of the self-assembling peptide (both EAK and EAbuK) with RGD sequences leads to further enhancement of proliferation which, although modest, is significant.

There were, however, some limitations to the cellular studies that warrant further investigations:

i) From 7th to 14th day the deceleration of cell growth can be considered physiological with cells resuming their usual proliferation at days 21-28. However, this trend was not observed on untreated surfaces (β-TCP and THX), this may be due to cell loss during the experimental procedure;

ii) Variable proliferation rates were observed on the two standard surfaces (THX) a further control should be introduced (e.g tissue culture plastic), to determine whether it is an operator variability or a surface effect .

iii) A rapid gelation of peptides only when in contact with the surface of β-TCP was observed.

Concluding

SAPs is a material that is highly likely to be used in clinical applications because it has several interesting characteristics:

1. It promotes favourable bone conduction properties;
2. It forms hydrogel able to act as a support for various different cells;
3. It possesses the capacity to modulate growth factor release;
4. It doesn’t show infection risk compared with materials of animal origin;
5. It eases of use, e.g. can be used as filler due to its capacity to fill defects with complex shapes and different nature.

The main issue with regard to the clinical use of SAPs is their strength. However, these peptides have the potential to be used as substitute for bone tissue if combined with a bone scaffold material. The interconnected porosity of β-TCP with bone regeneration potential combined with the development of novel scaffolds of a hybrid nature is becoming increasingly attractive in the quest to mimic natural tissues, furthermore, the conduction properties of SAPs makes this a useful approach for bone tissue engineering.

Preliminary findings have demonstrated that peptide pretreatment can improve h-osteoblast like

cell proliferation in the initial stages of cell adhesion and growth (1-4 days). The effect is present also for non-self-assembling sequences and is probably due to the modification of surface wettability. The further enrichment of peptide hydrogel with RGD-containing sequences adds beneficial effects.

Future works will consist in:

1. investigating the rate of permeation of β-TCP scaffold by SAPs;
2. investigating the effect of the nanostructure of the hydrogel on the β-TCP surface.

Abbreviations and acronyms

DMF = N,N-dimethylformamide
TES = triethylsilane
PBS = phosphate buffered saline
HBTU = 2(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt = 1-hydroxybenzotriazol
NMP = N-methyl-2-pyrrolidone
TFA = trifluoroacetic acid
DCM = dichloromethane
DIPEA = N,N-diisopropylethylamine
A / Ala = alanine
D / Asp = aspartic acid
E / Glu = glutamic acid
G / Gly = glycin
K / Lys = lysine
R / Arg = arginine
Abu = 2-aminobutyric acid
Fmoc = 9-fluorenyl methyloxycarbonyl
OtBu = ter-butyloxy
t-Boc = terz-butyloxyicarbonil (Boc)
Boc = see t-Boc
BMP = bone morphogenetic protein
HPLC = High Performance Liqud Chromatography
RP-HPLC = Reversed Phase High Performance Liqud Chromatography
UV/VIS = ultraviolet/visible
ESI = ElectroSpray Ionization
TOF = Time Of Flight
MALDI = Matrix Assisted Laser Desorption Ionization
SPPS = Solid Phase Peptide Syntesis
A_{λ}= absorbance [-]
wt = sample weight [g]
m = mass [g]
n = number of moles [mol]
V = volume [cm^3] or [mL]
λ = wavelength [nm]

TCP = tri-calcium phosphate

ε_λ = coefficiente di estinzione molare [$M^{-1}cm^{-1}$]

THX = thermanox

MTT = (3-[4,5-dimethylthiazol-2-yl])-2,5-diphenyl tetrazolium bromide

SAP = self-assembling peptides

BSA = bovine seerum albumin

DMEM = dulbecco’s modified eagle's medium

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Ringraziamenti

Ringrazio: la professoressa Monica Dettin per la disponibilità, la gentilezza e la professionalità che ha sempre dimostrato nei miei confronti; la dottoressa Annj Zamuner per avermi insegnato con pazienza le procedure di laboratorio che mi erano utili.
Ringrazio: la professoressa Lucy Di Silvio per avermi dato la possibilità di fare l'esperienza all'estero, in un ottimo laboratorio e con persone molto preparate, la ringrazio anche per la sua disponibilità e cortesia nell'aiutarmi durante il mio periodo a Londra; lo staff del Dental Institute del King's College London, in particolare Cristian, Bernadine e Paula per avermi fatto sentire meno solo in città straniera.
Un ringraziamento speciale va ai miei genitori, sempre capaci di credere in me e di darmi il loro più incondizionato sostegno; senza di loro non sarei mai potuto arrivare a questo traguardo.
Vorrei ringraziare mia sorella Silvia per esserci sempre stata in questi anni, nonostante le litigate, quando sono con lei il mio umore migliora sempre.
Vorrei ringraziare tutti i miei zii, perchè nei momenti difficili loro sono sempre stati come dei genitori, capaci di consigliarmi, consolarmi e guidarmi; in particolare vorrei ringraziare la zia Francesca e lo zio Luca per il loro apporto "accademico" a questa tesi, senza dimenticare il supporto morale sempre dimostratomi in questi anni; inoltre un ringraziamento speciale va alla zia Emanuela che da quando sono nato è sempre stata la mia seconda mamma e la quale mi ha aiutato nella stesura del presente lavoro.
Un altro ringraziamento speciale va a Beatrice perchè "c'è sempre stata" e perchè assieme abbiamo fissato le cose importanti della vita; la ringrazio inoltre per il suo continuo e costante supporto che mi ha aiutato nei momenti più difficili.
Vorrei ringraziare Enrico, Samantha, Davide, Laura, Chiara, Elena, Matteo e Riccardo per essermi accanto da tutti questi anni e vorrei complimentarmi con loro per essere riusciti a non stancarsi. Li ringrazio perchè, come gli zii sono stati per me dei genitori, loro sono stati come fratelli con i quali ho condiviso la gioia dei momenti felici e il peso di quelli più difficili.