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DESIGN AND SYNTHESIS OF SELF-ASSEMBLING PEPTIDES WITH BINDING AFFINITY TO HYALURONAN

by
Rita Saraiva de Aragão da Silva Dória

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DESIGN AND SYNTHESIS OF SELF-ASSEMBLING PEPTIDES WITH BINDING AFFINITY TO HYALURONAN

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in
Biomedical Engineering

by
Rita Saraiva de Aragão da Silva Dória

Place: School of Engineering and Materials Science (SEMS) - Queen Mary University of London

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Co-Supervisor: Dominic Collis

January 2017

To my grandparents, my guardian angels

RESUMO

O ácido hialurónico é um polímero natural que, devido á sua viscoelasticidade, boa biocompatibilidade e conformação flexivel ,nos ultimos anos tem sido alvo de grande interesse or parte da comunidade científica sendo utilizado em inúmeras aplicações clínicas e biomédicas tais como engenharia de tecidos , engenharia de biomateriais e cosmética.

Este projecto teve como objectivo explorar processos de organização molecular para desenvolver biomateriais combinando o ácido hialurónico, com péptidos com afininidade para este polímero, como o péptido identificado por "phage display" (Pep-1, [NH_2-GAHWQFNALTVR-NH_2]).

Para tal, foram sintetizadas seis variações do Pep-1 com o objectivo de testar se estas formam nanoestruturas em conjunto com o ácido hialurónico. Os péptidos com cadeias alquilo permitem a auto-organização para formação de nanoestruturas altamente estáveis. Considerando este facto, foram sintetizados quatro péptidos com estas propriedades para tentar a interação entre eles e o ácido hialurónico. Por outro lado, os tióis (compostos que contêm um grupo SH) podem ligar-se ao ouro devido à sua capacidade de adsorção química em superfícies metálicas, formando uma ligação metal-enxofre muito estável. Foi, portanto, sintetizado um péptido com uma terminação constituída por ácido mercaptoetanol de forma a que este reagisse com o ouro deixando o Pep-1 livre para interagir com ácido hialurónico.

Foram usadas as técnicas de Espectrometria de Massa com Ionização Electrospray e and Cromatografia líquida de alta eficiência para caracterizar e purificar os péptidos.

Após o trabalho experimental e respectivos resultados, foi concluído que aumentando a percentagem do péptido Pep-1, é possível ver uma interacção entre este e o ácido hialurónico. Os péptidos com cadeia alquilo mais longa são muito hidrofóbicos para serem purificados e consequentemente é impossível estudar a interacção entre estes e o ácido hialurónico. Quando as cadeias alquilo se tornam mais curtas, os péptidos tornam-se menos hidrofóbicos e a sua purificação é possível. No entanto, os peptídeos com cadeias mais curtas não permitem a formação de estruturas construídas por processos de auto-organização molecular. O péptido que possui uma terminação constituida pelo ácido mercaptoetanol é hidrofóbico mas, embora difícil, a sua purificação é possível. Mais testes devem ser realizados com o objectivo de analisar e caracterizar se a interacção entre este último péptido e o ácido hialurónico é possível.

ABSTRACT

Hyaluronan (HA) is a natural polymer that due to its viscoelasticity, good biocompatibility and a conformational flexibility, has been a polysaccharide of great interest to the scientists that is used in various biomedical and clinical applications such as tissue engineering, biomaterials engineering and cosmetics.

This project aimed to explore the self-assembly between hyaluronan (HA) and HA-binding peptides derived from Pep-1[NH_2-GAHWQFNALTVR-NH_2], a peptide known to bind HA and identified by phage display.

Six variations of Pep-1 were synthesized to test if they can form new nanostructures with HA. Peptides with alkyl chains allow the formation of highly stable self-assembled nanostructures. Considering this fact, were synthesized four peptides with these properties in order to try the interaction between them and hyaluronan. On the other hand, thiols (compounds that contain a SH group) can bond to gold due to the capability to be chemisorbed on metal surfaces, forming a very stable metal-sulfur bonds. Therefore, it was synthesized a peptide with a tail constituted by Mercaptoethanol acid that is expected to react with gold, displaying the Pep-1 free to interact with HA.

Electrospray Ionization Mass Spectrometry and High-Performance Liquid Chromatography techniques were used to characterize and purify the peptides, respectively.

After the experimental work and its results, it was concluded that by increasing the percentage of Pep-1 is possible to see an interaction between it and hyaluronan. Peptides with longer alkyl chain are to hydrophobic to be purified and consequently it was impossible to study the interaction between them and HA.When the alkyl chains become shorter, the peptides become less hydrophobic and their purification is possible. Though, the peptides with shorter chains can´t allow the formation of self-assembled structures. The peptide that has a Mercaptoethanol acid is hydrophobic but, its purification is possible. More tests should be done in order to study and analyze if the interaction between this last peptide and HA is possible.

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LIST OF ABBREVIATIONS

A	
Ala or A	Alanine
Arg or R	Arginine
Asn or N	Asparagine
ACN	Acetonitrile
B	
BOC	t-Butoxycarbonylamino
C	
COOH	Carboxyl Group
D	
DCM	Dichloromethane
DIC	N,N-diisopropylcarbodimide
DMF	Dimethylformamide
E	
ECM	Extracellular matrix
ESI-MS	Electrospray Ionization Mass Spectrometry
EDT	Ethanedithiol
F	
9-Fluorenylmethoxy carbonyl	FMOC
G	
Gln or Q	Glutamine
Gly or G	Glycine
H	
HA	Hyaluronic Acid
His or H	Histidine
HPLC	High-Performance Liquid Chromatography
HOBT	1-Hydroxybenzotriazole hydrate
L	
Leu or L	Leucine
M	
Mw	Molecular weight
N	

NH_2	Amine Group
NH_2-GAHWQFNALTVR-$CONH_2$	PEP-1
P	
PAS	Peptides Amphiphiles
Phe or F	Phenylalanine
S	
SPPS	Solid Phase Peptide Synthesis
T	
TFA	Trifluoracetic Acid
Thr or T	Threonine
TIS	Triisopropylsilane
Trp or W	Tryptophan
V	
Val or V	Valine

Chapter 1 - INTRODUCTION

1.1- SELF-ASSEMBLY

Health has been the world´s major concern over the past decade. Lots of investigations were developed in order to find new strategies for the practice of Medicine. This fact in association with the arising of the bionanotechnology has resulted in its integration into various medical techniques revolutionizing the traditional methods with the discovery of lots of new nanostructures, synthetic molecules, tissue engineering procedures, drug deliver. Nanotechnology develops structures with specific and predictive properties to build biomaterials that will be used for a desired medical function. A process called self-assembly allows the construction of these structures with a control of their chemical and physiological properties (Cui et al., 2010).

Molecular self-assembly is a process that consists in the spontaneous organization of molecules without external control (Bensaude-Vincent, 2006

; Grzybowski et al., 2009; Mendes et al., 2013; Whitesides & Grzybowski, 2002; Whitesides et al., 2005; Whitesides et al., 1991). This procedure occurs based on the principle of building blocks that comprises in the organization of small structures, into another structure with a higher organization and functional features (Fichman & Gazit, 2014; Mendes et al., 2013; Whitesides & Boncheva, 2002) (Fig.1.1). This new organization occurs due to the fact that each block has a specific shape, spatial distribution, hydrophobicity and other interactions that allows the formation of new nanostructures (Mendes et al., 2013).

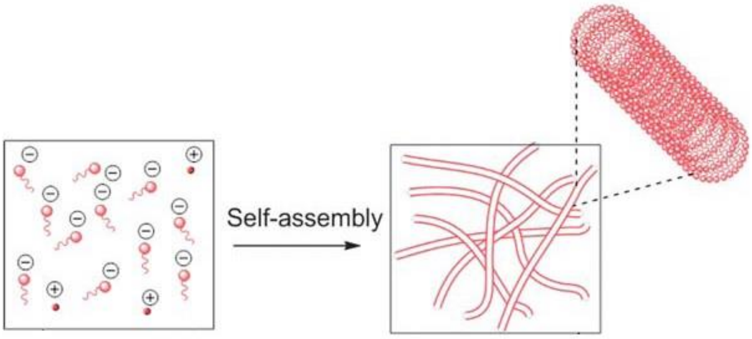


Figure 1.1: Examples of self-assembled structures (Ferreira, Reis, et al., 2013)

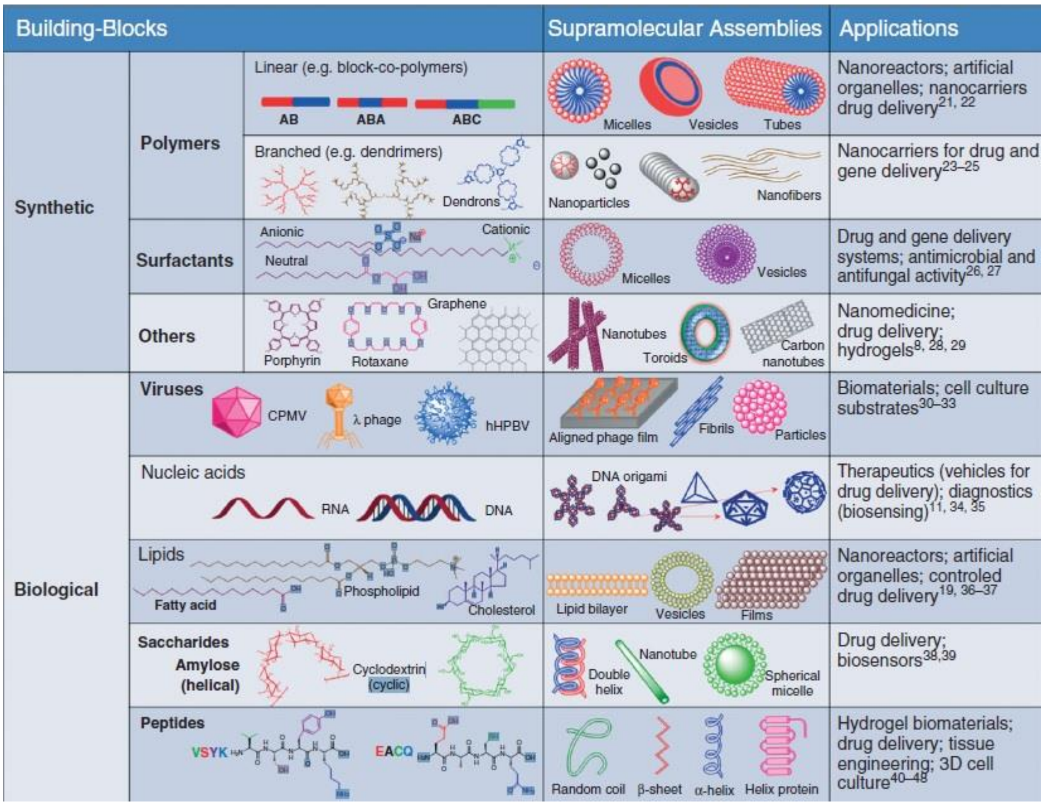
Molecular self-assembly is based on noncovalent forces. Electrostatic interactions, hydrophobic, $\pi - \pi$ stacking, and hydrogen bonding are considered noncovalent forces.(Mendes et al., 2013).

Electrostatic interactions occur between cations and anions, and can generate attractive or repulsive effects depending on the charges. These interactions allow the organization of different structures that are charged, such as surfactants, peptides and lipids (Mendes et al., 2013).

Hydrophobic process is the effect caused by the interaction between a non-polar molecule and a solvent (Mendes et al., 2013).

$\pi - \pi$ stacking is a process that influences the stability of the molecules and occurs between aromatic rings that are face-to-face to each other (Mendes et al., 2013).

Hydrogen bonding is the link of an electronegative atom (such as N, O or F) and a hydrogen atom attached to a second electronegative atom (Mendes et al., 2013). These type of interactions can be intermolecular or intramolecular and their strength depends on the nature of donor or acceptor and on the number of the combinations of some hydrogen bonds (Mendes et al., 2013).



Building-Blocks			Supramolecular Assemblies	Applications
Synthetic	**Polymers**	Linear (e.g. block-co-polymers) AB ABA ABC	Micelles Vesicles Tubes	Nanoreactors; artificial organelles; nanocarriers drug delivery[21, 22]
		Branched (e.g. dendrimers) Dendrons	Nanoparticles Nanofibers	Nanocarriers for drug and gene delivery[23–25]
	Surfactants	Anionic Cationic Neutral	Micelles Vesicles	Drug and gene delivery systems; antimicrobial and antifungal activity[26, 27]
	Others	Porphyrin Rotaxane Graphene	Nanotubes Toroids Carbon nanotubes	Nanomedicine; drug delivery; hydrogels[8, 28, 29]
Biological	**Viruses**	CPMV λ phage hHPBV	Aligned phage film Fibrils Particles	Biomaterials; cell culture substrates[30–33]
	Nucleic acids	RNA DNA	DNA origami	Therapeutics (vehicles for drug delivery); diagnostics (biosensing)[11, 34, 35]
	Lipids	Fatty acid Phospholipid Cholesterol	Lipid bilayer Vesicles Films	Nanoreactors; artificial organelles; controled drug delivery[19, 36–37]
	Saccharides **Amylose (helical)**	Cyclodextrin (cyclic)	Double helix Nanotube Spherical micelle	Drug delivery; biosensors[38,39]
	Peptides	VSYK EACQ	Random coil β-sheet α-helix Helix protein	Hydrogel biomaterials; drug delivery; tissue engineering; 3D cell culture[40–48]

Figure 1.2: Types of building blocks, possible supramolecular assemblies and their applications (Bromley et al., 2008; Hirst et al., 2008; Mendes et al., 2013; Scanlon & Aggeli, 2008; Schnur, 1993; Ulijn & Smith, 2008; Whitesides et al., 1991; Zayed et al., 2010)

It is also important to refer the structures that work as the base of the process of self-assembly. Self-assembly's building blocks can already exist in nature (viruses, nucleic acids, lipids, saccharides, and peptides) or can be developed synthetically (polymers, surfactants) (Bromley et al., 2008; Hirst et al., 2008; Mendes et al., 2013; Scanlon & Aggeli, 2008; Schnur, 1993; Ulijn & Smith, 2008; Whitesides et al., 1991; Zayed et al., 2010). These blocks can assemble and form a great variety of structures with a high level of precision and complexity that can be the base of many biomaterials (Ferreira, Reis, et al., 2013; Mendes et al., 2013) (Fig.1.2).

1.2- THE APPLICATION OF PEPTIDES IN SELF-ASSEMBLY

Nature is full of structures that are constructed by self-assembly processes. Protein folding is a good example of this process can form different structures by only using pre-existing blocks and noncovalent forces (Fig.1.3).

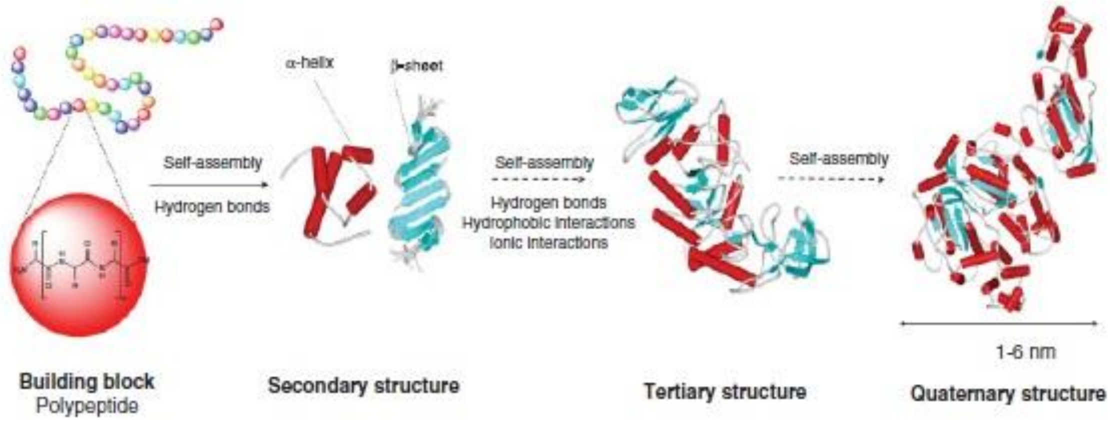


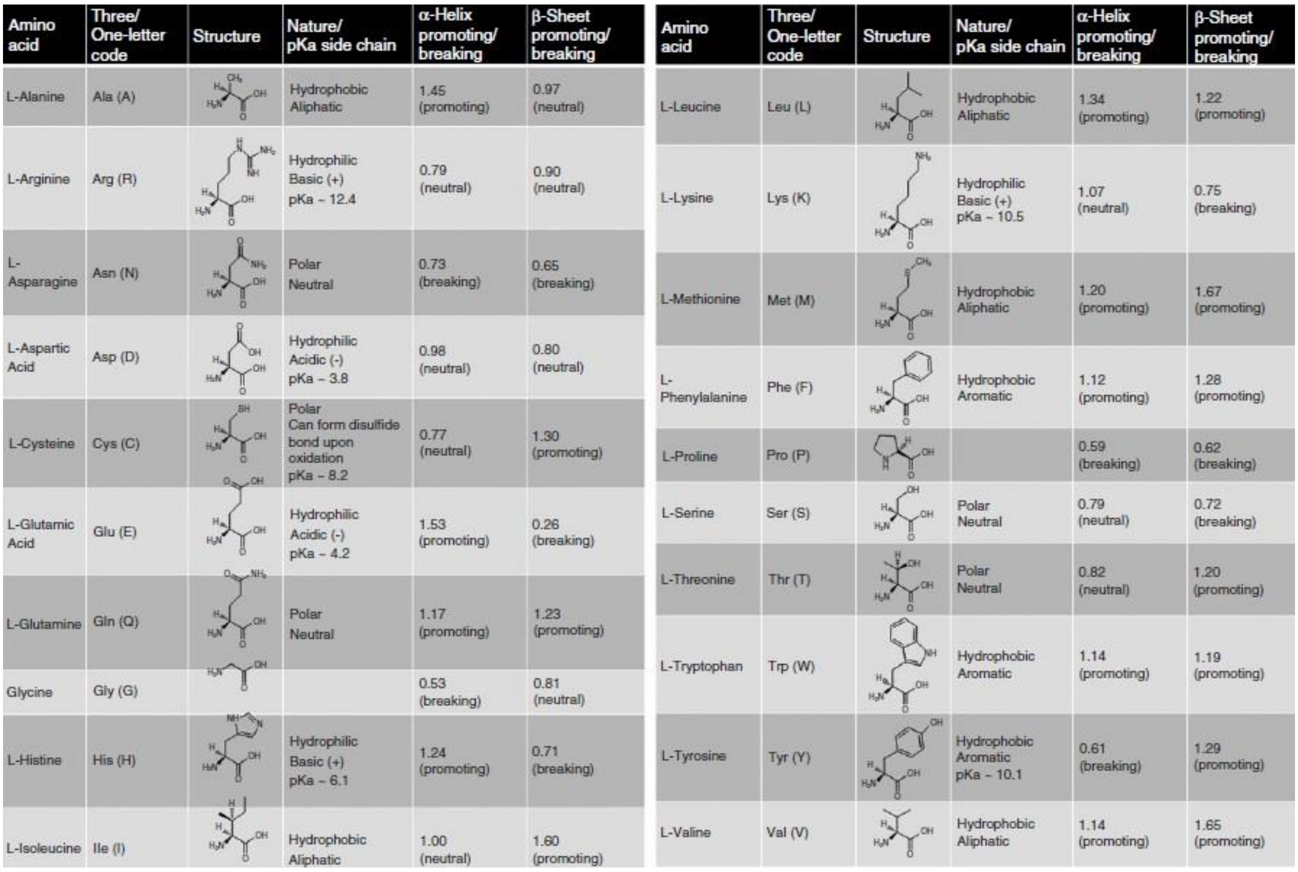
Figure 1.3: Protein folding as a biological example of self-assembly (Dicker et al., 2014; Mendes et al., 2013).

After studying this natural process of self-assembly, in the past decades, many scientists have begun to investigate the properties of peptides and concluded that they have properties (such as their bioactivity, biodegradability, their conformation) that are very useful to obtain a great variety of self-assembled structures. Furthermore, they are easy to synthesize and have the ability to spontaneously self-assemble, which make them an excellent choice to be used as structural units of nanobiomaterials (such as hydrogels, drug deliver, tissue engineering, etc) (Ferreira, Reis, et al., 2013; Gazit, 2010; Mendes et al., 2013; Sadava et al., 2011). Peptides are molecules made up of sequences constructed by amino acids. (Sadava et al., 2011).

1.2.1- AMINO ACIDS

There are 20 natural amino acids. All amino acids (excepting glycine) are chiral, which means that are constituted by the same structure: a carboxyl group and an amino group attached to the same carbon atom known as α carbon; bonded to this α carbon there is a hydrogen group and a side chain also called R group that differs from amino acid to another amino acid (Nelson et al., 2008). The nature of these side chains define how amino acids will interact (Mendes et al., 2013). There are different types of R groups: aliphatic residues, aromatic residues, polar, charged and non-polar. The aliphatic residues include the several amino acids L-Alanine, L-Isoleucine, L-Leucine, L-Methionine, L-Valine which contribute for the hydrophobicity of the molecule (Mendes et al., 2013).The aromatic ones, L-Phenylalanine, L-Tryptophan and L-Tyrosine may form π-π stacking (Mendes et al., 2013). L-Asparagine, L-Glutamine, L-Serine, L-Threonine are neutral polar residues which can allow the formation of hydrogen bonds (Mendes et al., 2013). Cysteine is a polar amino acid that can bind to golden surfaces and contains SH groups that can form intra or intermolecular disulfide bonds through an oxidation reaction (Mendes et al., 2013). Basic (L-Histidine, L-Lysine, L-Arginine) and acidic (L-Aspartic acid, L-Glutamic acid) residues are charged positively and negatively, respectively; These hydrophilic amino acids are responsible for creating electrostatic interactions (Mendes et al., 2013). L-Proline is a non-polar amino acid that has a side chain linked to the amino terminus which provides structural rigidity (Mendes et al., 2013; Ulijn & Smith, 2008). Glycine is a non-chiral amino acid that can offer a good flexibility comparing to the other residues (Mendes et al., 2013).

Amino acids, depending on their nature, influence peptides’ conformation, which means that they can interact in a way that can be neutral, promote or break α-helix or β-sheet formation (Mendes et al., 2013).



Amino acid	Three/ One-letter code	Structure	Nature/ pKa side chain	α-Helix promoting/ breaking	β-Sheet promoting/ breaking
L-Alanine	Ala (A)		Hydrophobic Aliphatic	1.45 (promoting)	0.97 (neutral)
L-Arginine	Arg (R)		Hydrophilic Basic (+) pKa ~ 12.4	0.79 (neutral)	0.90 (neutral)
L-Asparagine	Asn (N)		Polar Neutral	0.73 (breaking)	0.65 (breaking)
L-Aspartic Acid	Asp (D)		Hydrophilic Acidic (-) pKa ~ 3.8	0.98 (neutral)	0.80 (neutral)
L-Cysteine	Cys (C)		Polar Can form disulfide bond upon oxidation pKa ~ 8.2	0.77 (neutral)	1.30 (promoting)
L-Glutamic Acid	Glu (E)		Hydrophilic Acidic (-) pKa ~ 4.2	1.53 (promoting)	0.26 (breaking)
L-Glutamine	Gln (Q)		Polar Neutral	1.17 (promoting)	1.23 (promoting)
Glycine	Gly (G)			0.53 (breaking)	0.81 (neutral)
L-Histine	His (H)		Hydrophilic Basic (+) pKa ~ 6.1	1.24 (promoting)	0.71 (breaking)
L-Isoleucine	Ile (I)		Hydrophobic Aliphatic	1.00 (neutral)	1.60 (promoting)
L-Leucine	Leu (L)		Hydrophobic Aliphatic	1.34 (promoting)	1.22 (promoting)
L-Lysine	Lys (K)		Hydrophilic Basic (+) pKa ~ 10.5	1.07 (neutral)	0.75 (breaking)
L-Methionine	Met (M)		Hydrophobic Aliphatic	1.20 (promoting)	1.67 (promoting)
L-Phenylalanine	Phe (F)		Hydrophobic Aromatic	1.12 (promoting)	1.28 (promoting)
L-Proline	Pro (P)			0.59 (breaking)	0.62 (breaking)
L-Serine	Ser (S)		Polar Neutral	0.79 (neutral)	0.72 (breaking)
L-Threonine	Thr (T)		Polar Neutral	0.82 (neutral)	1.20 (promoting)
L-Tryptophan	Trp (W)		Hydrophobic Aromatic	1.14 (promoting)	1.19 (promoting)
L-Tyrosine	Tyr (Y)		Hydrophobic Aromatic pKa ~ 10.1	0.61 (breaking)	1.29 (promoting)
L-Valine	Val (V)		Hydrophobic Aliphatic	1.14 (promoting)	1.65 (promoting)

Figure 1.4: Amino acids structure and properties (Barrett & Elmore, 1998; Mendes et al., 2013; Ulijn & Smith, 2008)

1.2.2- PEPTIDES

A peptide is formed by the attachment of the two reactive groups linked to the α-carbon of each amino acid: the carboxyl group of one amino reacts with the amino group of the other amino acid and forms a peptide bond (covalent bond) (Fig.1.4). In each linkage of two amino acids one water molecule is removed (Nelson et al., 2008; Sadava et al., 2011).

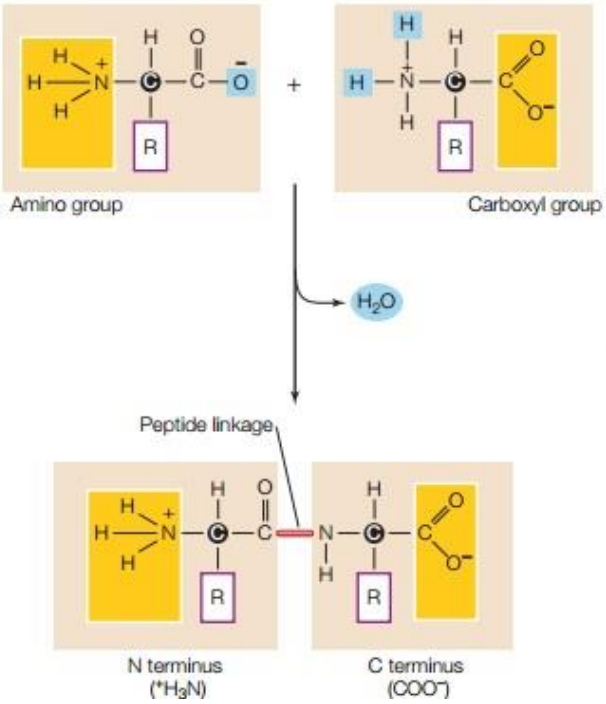


Figure 1.5: Formation of a peptide (Sadava et al., 2011)

Due to the good properties of peptides to self-assemble, its unique characteristics that allow them to work as building blocks, its ability to be carriers to provide diagnostic or therapeutic agents for target cells, to the fact that they can be used for structural studies mimicking cyclic peptides and due to its biocompatibility and biodegradability (Cui et al., 2010), many researchers have attempted to isolate these structures from natural sources, which proved to be very difficult. Therefore, peptides have been synthesized synthetically by using genetic engineering and chemical synthesis (Mendes et al., 2013).

There are two ways of making peptides by chemical synthesis: synthesis in solution or solid-phase synthesis. This second technique is used to synthesize short peptides (the ones that are composed from 2 to 30 amino acids) and it is simple to implement, which turns it the most used technique (Mendes et al., 2013). Solid-phase peptide synthesis (SPPS) technique was developed by Merrifield in 1963, Nobel Prize of Chemistry in 1984 (Coin et al., 2007; Merrifield, 1963). SPPS offers a wide variety of advantages compared to other synthetic methods (such as solution synthesis), like the fact that the steps are simple, being possible its automation which makes the process even faster; the coupling reactions can be performed more quickly and efficiently using excess reagents which are removed at the end of the reaction by washing procedures; and the peptides being built have high yields (Coin et al., 2007). The SPPS method consists in anchoring the peptide chain to a resin until the chain resin+peptide is complete forming the right sequence.

1.2.3- PEPTIDE SELF ASSEMBLY

During the last years, many peptide sequences have been designed for self-assembly based on the ability of certain amino acids to interact and form ordered aggregates with the desired properties (Gazit, 2010; Mendes et al., 2013). Some examples of these self-assembling peptides are α-helix forming peptides and β-sheet forming peptides, including glutamine-rich peptides, self-Complementary peptides and peptides amphiphiles (Mendes et al., 2013).

Under the present work, the concept of peptides amphiphiles (PAs) requires further description as their design was used as an inspiration to develop novel self-assembling peptides with binding ability to hyaluronan. Amphiphilic peptides are constituted by a hydrophobic and hydrophilic segments and include two classes of peptides: the surfactant-like peptides and peptides amphiphiles (Cui et al., 2010; Mendes et al., 2013).

PAs are peptides that are composed by the combination of a hydrophobic block (in most cases an alkyl chain, which means a tail constituted by carbons) positioned in the core of the assembly; a short hydrophilic sequence of a peptide located in the near the hydrophobic segment that is responsible to form intermolecular hydrogen bonding and consequently a β-sheet forming region; charged elements that improve the solubility in aqueous solutions; and a region with epitopes that can interact with cells or other molecules in order to show bioactive signals (Cui et al., 2010) (Fig.1.6) .

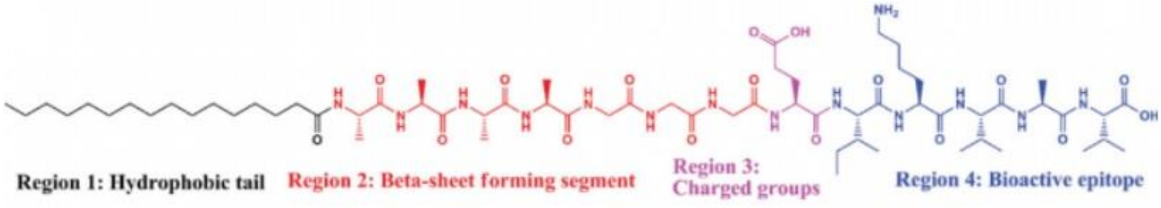


Figure 1.6: Chemical structure of a representative PA (Cui et al., 2010)

These PA molecules assemble into cylindrical nanostructures that have great interest for biomedical applications since they combine the structural characteristics of amphiphilic surfactants with the good properties of bioactive peptides (Cui et al., 2010).

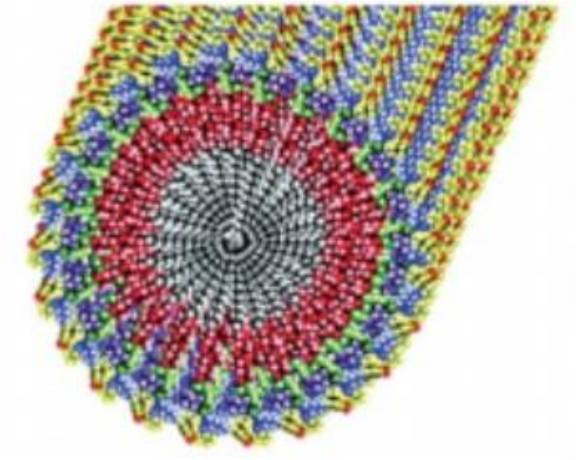


Figure 1.7: Example of how a PA molecule self-assemble into cylindrical micelles (Cui et al., 2010)

1.3- SELF-ASSEMBLY OF HYALURONIC ACID PEPTIDES

1.3.1- HYALURONAN (HA): STRUCTURE AND BIOLOGICAL ROLES

The extracellular matrix of tissues (ECM) is an organized, dynamic and complex system constituted mostly by proteins (like elastin, that confers elasticity, and collagen, responsible for providing structure and strength) and polysaccharides (such as glycosaminoglycan) that play an important role as a structural component of the cells , act as a scaffold for cell adhesion and differentiation (Alberts et al., 2002; Ferreira, Marques, et al., 2013). Cell adhesion is regulated by ECM molecules that bind to proteins located at the cell surface allowing communication between the cells and the environment (Ferreira, Marques, et al., 2013)

Hyaluronan (also known as hyaluronic acid, HA) is a large glycosaminoglycan that consists of the major component of the ECM (Ferreira, Marques, et al., 2013). This high molecular–mass polysaccharide was discovered by Meyer and Palmer in 1934 in the vitreous body of the eye (Meyer & Palmer, 1934). The molecule was called “hyaluronic acid“ remaining to the hyaloid appearance when it was put in water and the possible presence of hexuronic acid in it (Dicker et al., 2014).

Hyaluronic acid is a linear unbranched polymer that is constituted by N-acetyl-D-glucosamine and glucuronic acid disaccharide repeating units which does not have sulfate groups (such as keratin sulfate, heparin or dermatan sulfate) (Day & Prestwich, 2002; Ferreira, Marques, et al., 2013; Lapčík et al., 1998) (Fig.1.7).

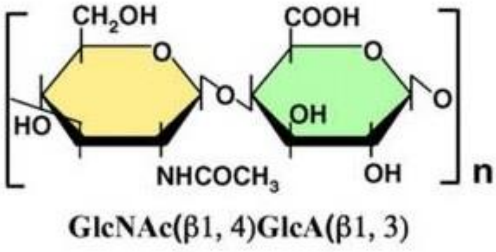


Figure 1.8: HA chemical structure : it N-acetyl-D-glucosamine (yellow) and glucuronic acid (green) (Barrett & Elmore, 1998).

HA is crucial for the organization of the ECM playing an important role in the assembly of the proteins (Ferreira, Marques, et al., 2013).Therefore HA contributes to the structural, protective and good biomechanical characteristics of tissues (Dicker et al., 2014; Lapčík et al., 1998; Liao et al., 2005). HA also participates in the mediation of physiological functions through the signalization of the molecules by interacting with a variety of cell surface receptors to activate intracellular events to mediate cell functions such as adhesion and migration during morphogenesis, inflammation, and diseases (Dicker et al., 2014; Ferreira, Marques, et al., 2013; Lapčík et al., 1998).

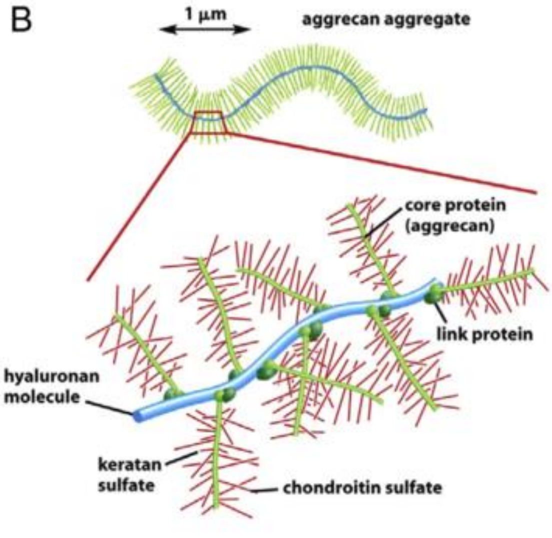


Figure 1.9: HA as a template directing the organization of proteins in the ECM (Dicker et al., 2014; Mummert et al., 2000).

HA bind to HA-binding proteins (the hyaladherins) that have specific functional and structural activities. This binding makes possible the arrangement of different conformations and their stabilization (Day & Carol, 2005; Ferreira, Marques, et al., 2013). Some examples of two known hyaladherins are: an inflammation-associated protein called TSG-6 and CD44, that is the major cell-surface receptor for HA (Day & Carol, 2005) (Fig.1.9).

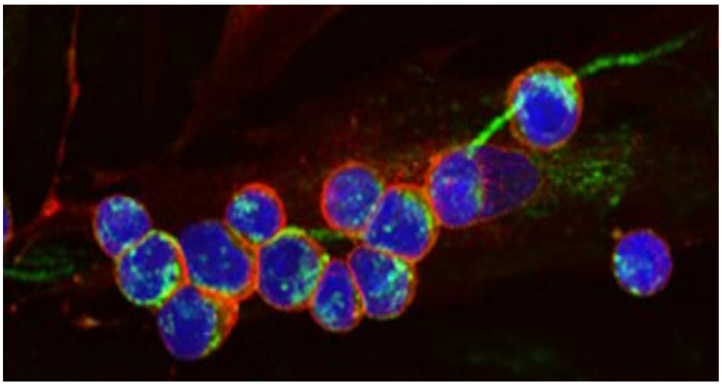


Figure1.10: Cells (blue) showing CD44 (red) binding to HA (green) (Day & Prestwich, 2002; Mummert et al., 2000)

HA can be isolated from animal sources, such as from synovial fluid, umbilical cord, skin; or bacteria sources by fermentation (Liao et al., 2005).

In the last decades, many studies were made and it was discovered that HA has an incredible viscoelasticity, a good biocompatibility and a conformational flexibility that makes it a polysaccharide of great interest to the scientists (Day & Carol, 2005; Liao et al., 2005). HA is a versatile molecule that alone, or through the modification produced by interaction between it and other materials, is able to form supramolecular assemblies that can result in promising biomaterials (Burdick & Prestwich, 2011; Dicker et al., 2014). Consequently, the properties of HA allow it to be used in various biomedical and clinical applications, such as the fact that it can be used as a drug delivery vehicle, as a tissue engineering scaffold, as a fabric filler, as a starting material for the manufacture of hydrogel matrices, among others (Burdick & Prestwich, 2011; Lapčík et al., 1998).

1.3.2- HA-BINDING PEPTIDE

Pep-1 [NH_2-GAHWQFNALTVR-NH_2] (Mummert et al., 2000) is a HA-binding peptide discovered by phage display, that has been used to track HA in tissues, for example (Mummert et al., 2000; Zmolik & Mummert, 2005). It is believed that interaction between Pep-1 and HA is mediated by hydrophobic and/or aliphatic residues of Pep-1 and polar, hydrophobic or charged groups of HA (Mummert et al., 2000).

This peptide shows specific binding to soluble, immobilized, and cell-associated forms of HA. Due to the fact that it also inhibits leucocyte adhesion to HA substrates almost completely, Pep-1 is an inhibitor of HA (Mummert et al., 2000).

1.4- AIM

In this work, new self-assembling peptides, based on HA binding peptide Pep-1 were exploited to investigate their ability to form supramolecular structures with HA.

The purpose of the present work is to attach Pep-1 to a fatty acid to form a peptide amphiphile that can self-assemble into a cylindrical micelle and form supramolecular assemblies with HA with utility as biomaterial.

The image below exemplifies this objective:

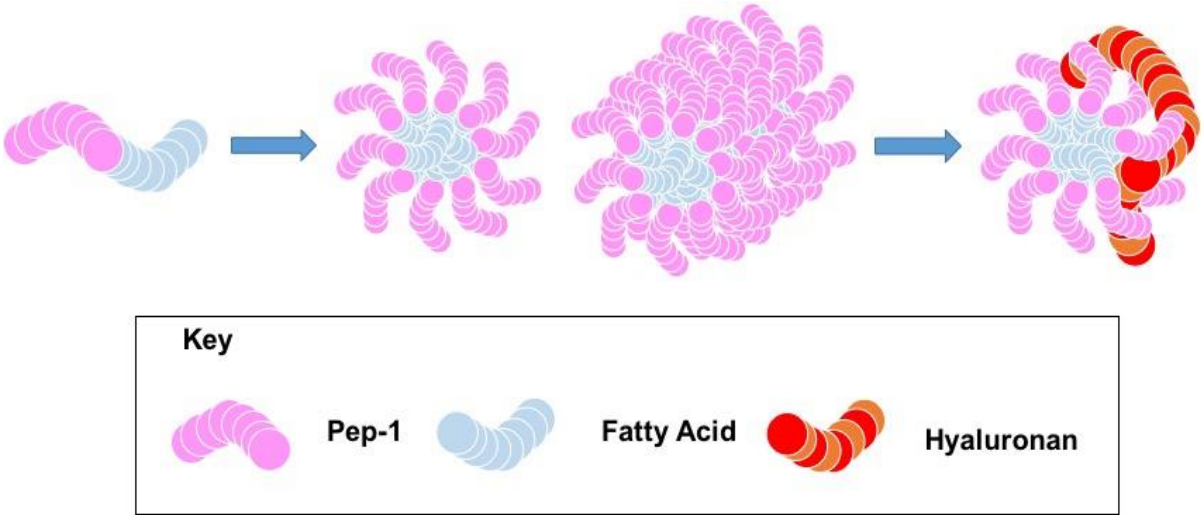


Figure 1.11: Representative schematic of the objective of this work

Chapter 2- MATERIALS AND METHODS

Chemicals. Fmoc-Ala-OH (Novabiochem®, UK), Fmoc-Arg(Pbf)-OH (Novabiochem®, UK), Fmoc-Asn(Trt)-OH (Novabiochem®, UK), Fmoc-Gln(Trt)-OH (Novabiochem®, UK), Fmoc-Gly-OH (Novabiochem®, UK), Fmoc-His(Trt)-OH (Novabiochem®, UK), Fmoc-Leu-OH (Novabiochem®, UK), Fmoc-Phe-OH (Novabiochem®, UK), Fmoc-Thr(tBu)-OH (Novabiochem®, UK), Fmoc-Trp(Boc)-OH (Novabiochem®, UK), Fmoc-Val-OH (Novabiochem®, UK) were used to synthesize Pep-1. Palmitic acid (Sigma-Aldrich, 99%, UK), Octanoic acid (Sigma-Aldrich, 99%, UK), Dodecanoic acid (Sigma-Aldrich, 99%, UK), Mercaptoethanol acid (Sigma-Aldrich, 99%, UK) were used to attach an alkyl tail at the N-terminal. Rink amide MBHA resin (Novabiochem®, UK) was used as a resin for the peptides chains. Piperidine (Sigma-Aldrich, 99%, USA) was used to perform the deprotection reactions. Dichloromethane (DCM, Fisher Scientific, UK), Dimethylformamide (DMF, Sigma-Aldrich, 99.9%, UK), 1-Hydroxybenzotriazole hydrate (HOBt, Carbosynth, UK), N,N-diisopropylcarbodimide (DIC, Alfa Aesar, 99%, UK) were used in the synthesis. Kaiser test used to check if the couplings between amino acids were made successfully, is from Fluka Analytical USA with three reagents: phenol solution (80% in ethanol), potassium cyanide solution and ninhydrin solution (6% ethanol). A mixture of Trifluoracetic acid (TFA, Sigma-Aldrich, 99%, UK), Triisopropylsilane (TIS, Alfa Aesar, 98%, UK), Water and a mixture of 1, 2-Ethanedithiol (Alfa Aesar, 98%, UK), Thioanisole (Sigma-Aldrich, 99%, UK), Anisole (Sigma-Aldrich, 99%, UK) were used to do the peptides cleavage. Diethyl ether (Sigma Aldrich, 99%, UK) was used to precipitate the peptides. Acetonitrile (ACN, Sigma-Aldrich, UK) was used in HPLC system.

2.1- PEPTIDES SYNTHESIS

2.1.1-GENERAL PROCEDURE FOR PEPTIDES SYNTHESIS

Peptides were synthesized using the solid-phase peptide synthesis (SPPS) technique. During the synthesis, protecting groups are used such as 9-Fluorenylmethoxy carbonyl (Fmoc) and t-Butoxycarbonylamino (Boc) to ensure that the right peptide sequence is being synthesized: Fmoc group protects the NH_2-groups and Boc groups protect the side R-groups, so the peptides in the present work were synthesized by using standard Fmoc based solid phase chemistry.

The SPPS method consists in anchoring the peptide chain to a resin which, initially, should be deprotected in order to remove the protective group Fmoc, thus the C-terminal of the first amino acid can be attached to the resin. After two – three hours, the first amino acid should be coupled to the resin and a Kaiser test is done to confirm it. A Kaiser test is used to verify if the couplings were effective: the ninhydrin from the Kaiser test reacts with the deprotected peptide´s N-terminal amine group producing a blue color, so if the test´s result is blue it means that the N-terminal amide is free, which means that there isn't a new amino acid coupled; so to verify the coupling the test shouldn't has a result a blue color. After the coupling, the excess of reactants is removed by washing processes. Then a deprotection is performed again and the cycle is repeated until the chain resin+peptide is complete forming the right sequence. The peptide is assembled from C-Terminal to the N-Terminal.

Some peptides were synthesized by manual coupling and others by microwave assisted synthesizer. Next it is explained how these two procedures were executed.

MANUAL PEPTIDE COUPLING

The rink amide resin was swelled in DMF (20 mL) for 30 minutes. The DMF was removed and the resin deprotected with 20% piperidine in DMF (2 x 20 mL) for 10 minutes. The resin was washed with DMF (2 x 20 mL) followed by DCM (2 x 20 mL). The resin was checked by Kaiser test for free amine. Once free amine was verified, the amino acid (x 4 molar excess) in DMF (20mL) along with HOBt (x 4molar excess) and DIC (x 4molar excess) was shacked for 3 hours, after which point the resin was drained ,washed with DMF (2x20mL) followed by DCM (2x20mL) and the Kaiser test was performed. Once coupling was confirmed, the cycle was repeated for the remaining amino acids and fatty acid chains.

MICROWAVE ASSISTED PEPTIDES SYNTHESIS

The peptides used in the present work were synthesized on a CEM Liberty Blue Peptide Synthesizer, UK automated peptide synthesizer.

The rink amide resin is loaded into the reaction vessel and placed under pressure with the peptide reagents of amino acids, HOBt and DIC (4 molar excess) are added in sequence by the machine using microwave irradiation to perform the couplings.

CLEAVAGE AND PRECIPITATION

To remove the protecting groups and the peptide from the resin a cleavage is performed. The completed peptide was cleaved from the resin by TFA, Water, TIS (95%, 2.5%, 2.5%). Peptides containing sulfur residues were cleaved using: TFA, thioanisole, anisole, and EDT (92.5%, 2.5%, 2.5%, and 2.5% respectively). The suspension was shacked for 3 hours after which point the solution was reduced under negative pressure. The peptide was precipitated in cold diethyl ether, with the precipitate being spun down on the centrifuge Heraeus Multifudge X1, Centrigue Theme Scientific, USA and the liquid decanted. The solid was allowed to fume to remove excess ether and then lyophilised to give a crude white powder. Finally, after this, the peptide is ready to be analyzed and purified.

2.1.2- PEPTIDES THAT WERE SYNTHESIZED IN THE PRESENT WORK

In this work, several peptides were synthesized:

- NH_2-GAHWQFNALTVR-$CONH_2$

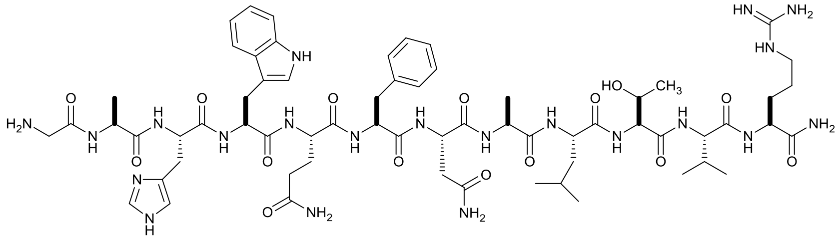


Figure 2.1: Pep-1 chemical structure

First, Pep-1 was synthesized to obtain the peptide to test the interaction between it and HA, since Pep-1 is an HA-binding peptide.

The synthesis reaction was run on a 0.5 mmol scale using the microwave assisted and manual coupling, The theoretical ESI-MS values are: 1398.60 $[M+H^+]^+$ and 700.30 $[M+2H]^{2+}$.

FIRST SERIES OF PEP-1 BASED ANALOGUES

To form self-assembling structures of Pep-1 (GAHWQFNALTVR) to interact with HA, two peptides constituted by Pep-1 attached to alkyl chains were designed to form structures of Pep-1: C_{16}-GAHWQFNALTVR-$CONH_2$ and C_{16}-$(VQ)_2$-GAHWQFNALTVR-$CONH_2$

(Fig.2.2). These peptides belong to the class of the peptides amphiphiles and they are the first series of Pep-1 based analogues.

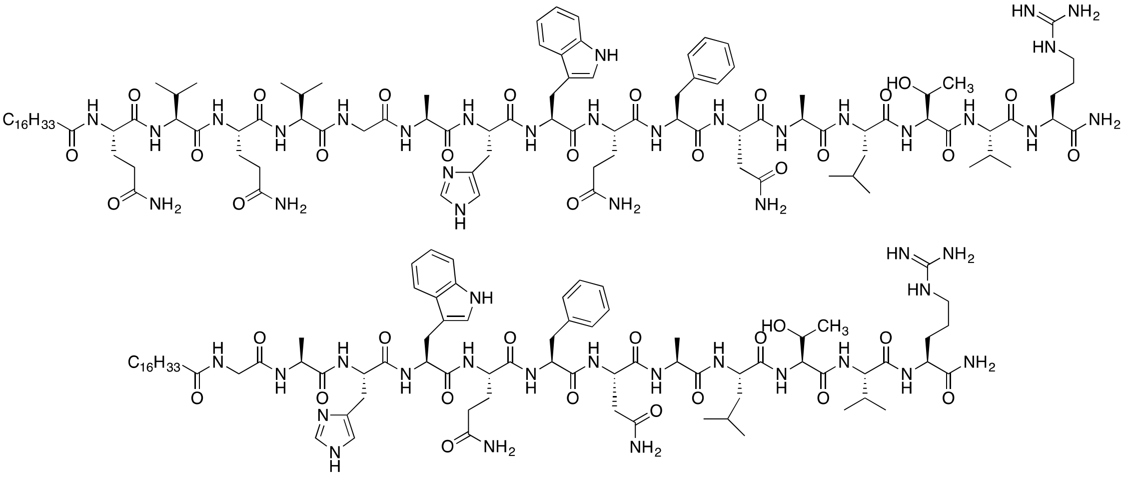


Figure 2.2: Peptides designed based on Pep-1 with N-terminus modification to induce self-assembly. C_{16}-$(VQ)_2$-GAHWQFNALTVR-CON_2 (Top) and C_{16}-GAHWQFNALTVR-$CONH_2$ (bottom).

Longer hydrophobic alkyl chains allow PAs to form highly stable self-assembled nanostructures (Cui et al., 2010; Mendes et al., 2013). Frequently, the hydrophobic segment is constituted by the coupling of palmitic acid with the N-terminal amine of the peptide linked to the resin (Cui et al., 2010). After synthesized Pep-1, it was split in two in order to be possible to construct two different peptides. To each peptide two tails constituted by C_{16} were coupled.

It was added a different sequence that also influence the assembly of the structures, to one of the peptides , -$(VQ)_2$.The sequence -$(VQ)_2$ was added due to the fact that these amino acids have been shown to form β sheets (valine is an aliphatic residue that provide an hydrophobic environment and promote β sheet formation; glutamine is a neutral polar residue that can is involved in the formation of hydrogen bonding and induce β sheet formation) (Mendes et al., 2013). It was hoped that using these β sheet forming domains it would induce self -assembly into cylindrical nanostructures.

- C_{16}-GAHWQFNALTVR-$CONH_2$

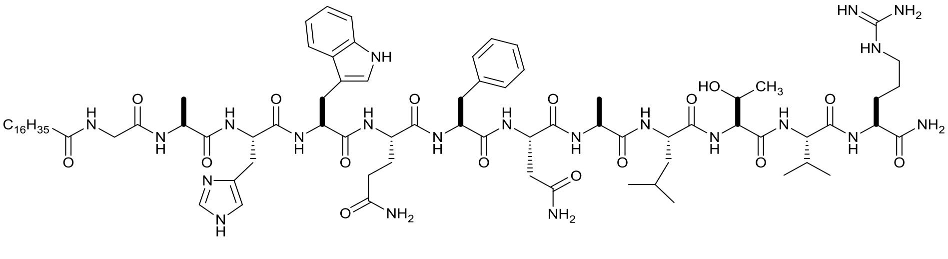


Figure 2.3: Chemical structure of C_{16}-GAHWQFNALTVR-$CONH_2$

The synthesis reaction was run on a 0.5 mmol scale using the microwave assisted synthesizer to obtain Pep-1. The resin was transferred to a vessel and palmitic acid was manual coupled. The theoretical ESI-MS values are: 1637.01[M+H$^+$]$^+$ and 819.51 [M+2H]$^+$.

- C_{16}-(VQ)$_2$-GAHWQFNALTVR-CONH$_2$

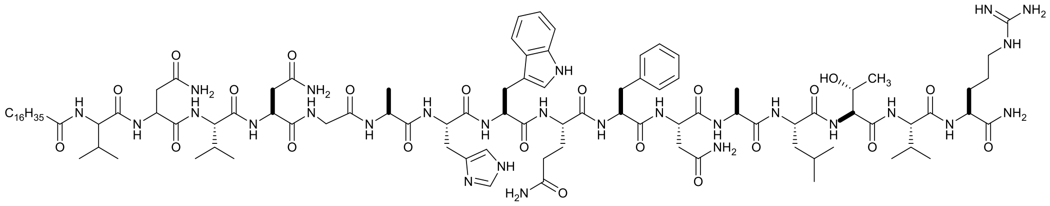


Figure 2.4: Chemical Structure of C_{16}-(VQ)$_2$-GAHWQFNALTVR-CONH$_2$

The synthesis reaction was run on a 0.5 mmol scale using the microwave assisted to synthesize the Pep-1. The resin was transferred to a vessel and the tail was manual coupled. The theoretical ESI-MS values are: 2091.54 [M+H$^+$]$^+$ and 1046.77 [M+2H]$^{2+}$.

After peptides synthesis, it was necessary to do solubility tests due to the fact that the peptides can only be analyzed and purified if they are soluble.

Solubility tests:

Each peptide (C_{16}-GAHWQFNALTVR-CONH$_2$ and C_{16}-(VQ)$_2$-GAHWQFNALTVR-CONH$_2$) was mixed in 1mg/mL water and 1mg/ml ACN in 4 different Eppendorfs.

Then, it was added 50 µL of water in each Eppendorf (3 times in each Eppendorf).

After, 50 µL of water 0.1% TFA was added to the fourth Eppendorfs (2 times in each Eppendorf).

After each time the solvents were added, the eppendorfs were vortexed and after sonicated in a Brandson 5800 Sonicator.

The peptides weren´t soluble in any solution.

SECOND SERIES OF PEP-1 BASED ANALOGUES

Other two new peptides were synthesized with smaller hydrophobic chains (C_8-GAHWQFNALTVR-CONH$_2$ and C_{12}-GAHWQFNALTVR-CONH$_2$, Fig 2.5). It was expected that these peptides were less hydrophobic since the alkyl chains are shorter.

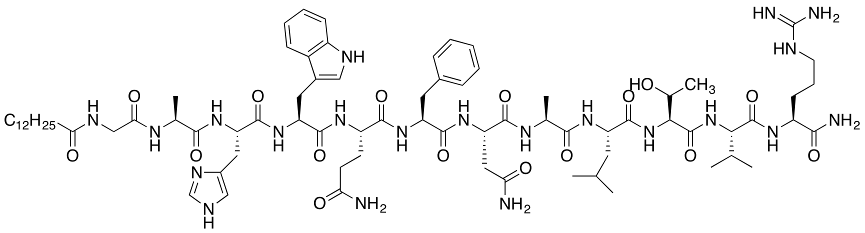
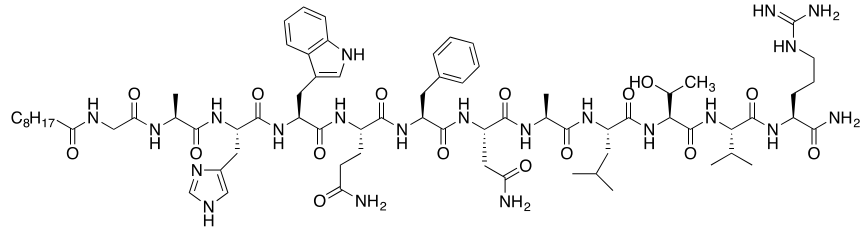



Figure 2.5: Second series of Pep-1 based analogues with shorter alkyl chains: C_{12}-GAHWQFNALTVR-CONH$_2$ (Top) and C_8-GAHWQFNALTVR-CONH$_2$ (Bottom).

- C_8 -GAHWQFNALTVR-CONH$_2$

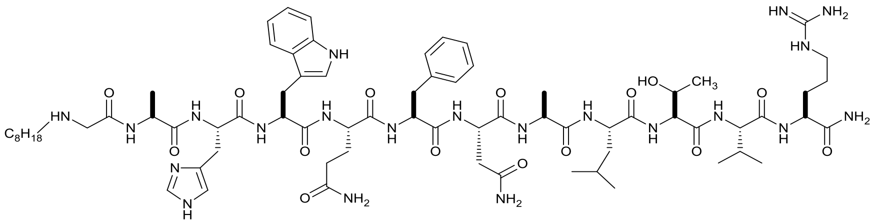


Figure 2.6: Chemical structure of C_8-GAHWQFNALTVR-CONH$_2$

The synthesis reaction was run on a 0.5 mmol scale using the microwave assisted to synthesize the Pep-1 analogues. The resin was transferred to a vessel and the octanoic acid was manually coupled. The theoretical ESI-MS values are: 1524.80 $[M+H^+]^+$ and 763.40$[M+2H]^{2+}$.

- C_{12}-GAHWQFNALTVR-CONH$_2$

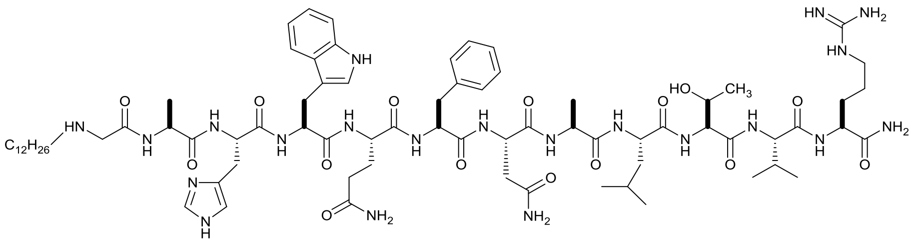


Figure 2.7: Chemical structure of C_{12}-GAHWQFNALTVR-CONH$_2$

The synthesis reaction was run on a 0.5 mmol scale using the microwave to synthesis the Pep-1 analogue. The resin was transferred to a vessel and the dodecanoic acid was manually coupled. The theoretical values from ESI-MS are: 1580.90 $[M+H^+]^+$ and 791.45$[M+2H]^{2+}$.

Solubility tests:

Test 1:

Table 2.1: C_8-GAHWQFNALTVR-$CONH_2$ and C_{12}-GAHWQFNALTVR-$CONH_2$ solubility tests

Peptide	Solvents	Soluble
C_8-GAHWQFNALTVR-$CONH_2$	1 mg/mL water in an Eppendorf	No
	1 mL of ACN in an Eppendorf	No
C_{12}-GAHWQFNALTVR-$CONH_2$	1 mg/mL of water in an Eppendorf	No
	1 mg/mL of ACN in an Eppendorf	No

Test 2:

Table 2.2: C_8-GAHWQFNALTVR-$CONH_2$ and C_{12}-GAHWQFNALTVR-$CONH_2$ solubility tests (Part 2)

Peptide	Solvents	Soluble
C_8-GAHWQFNALTVR-$CONH_2$	1 mg/mL water/ACN (50:50) in an Eppendorf	No
C_{12}-GAHWQFNALTVR-$CONH_2$	1 mg/mL water/ACN (50:50) in an Eppendorf	No

Test 3:

Table 2. 3: C_8-GAHWQFNALTVR-$CONH_2$ and C_{12}-GAHWQFNALTVR-CNH_2 solubility tests (Part 3)

Peptide solution	Solvents	Soluble	
1mg of C_8-GAHWQFNALTVR-$CONH_2$ + 500 µL water	50µL ACN in an Eppendorf (**solution 1**)	No	Solvents mixture ratio : 90:10
1mg C12-GAHWQFNALTVR-$CONH_2$+ 500µL water	50 µL ACN in an Eppendorf (**solution 2**)	No	
solution 1	50 µL ACN in an Eppendorf (**solution 3**)	Yes	Solvents mixture ratio : 80:20
solution 2	50 µL ACN in an Eppendorf (**solution 4**)	No	
solution 4	50 µL ACN in an Eppendorf (**solution 5**)	No	Solvents mixture ratio : 70:30
solution 5	50 µL ACN in an Eppendorf (**solution 6**)	Yes	Solvents mixture ratio : 50:50

After each time the solvents were added, the eppendorfs were vortexed and after sonicated.

THIRD SERIES OF PEP-1 BASED ANALOGUES

A new peptide was synthesized, HS-GAHWQFNALTVR-$CONH_2$. The SH group is a thiol (or mercaptan) which means that it is an organosulfur compound that contains a sulfur-hydrogen bond. Thiols bond to gold, silver and copper, due to the fact that their H-S end have the capability to be chemisorbed (is a kind of adsorption which involves a chemical reaction between surface and the adsorbate) on metal surfaces, forming very stable metal-sulfur bonds (Kudelski, 2003a, 2003b). Because of the facts presented, this peptide is expected to react with gold and displaying the Pep-1 free to interact with HA.

- HS- GAHWQFNALTVR-$CONH_2$

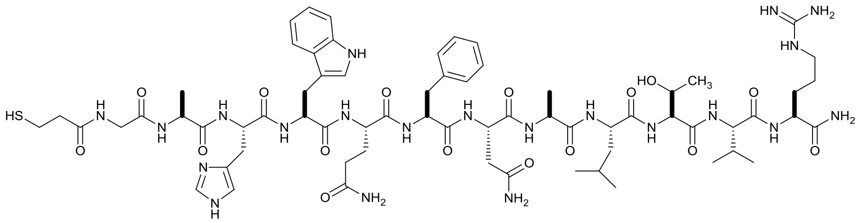


Figure 2.8: Chemical structure of HS-GAHWQFNALTVR-$CONH_2$

The synthesis reaction was run on a 0.5 mmol scale using the microwave assisted to synthesize Pep-1 analogue. The resin was transferred to a vessel and the Mercaptoethanol acid was manually coupled. The theoretical ESI-MS values are: 1486.72 $[M+H^+]^+$ and 744.36 $[M+2H]^{2+}$.

Solubility tests:

Test 1:

Table 2.4: HS-GAHWQFNALTVR-$CONH_2$ solubility tests

Peptide	**Solvents**	**Soluble**
HS-GAHWQFNALTVR-$CONH_2$	1 mg/mL water in an Eppendorf	No
HS-GAHWQFNALTVR-$CONH_2$	1 mg/mL ACN in an Eppendorf	No

Test 2:

Table 2.5: HS-GAHWQFNALTVR-$CONH_2$ solubility tests (Part 2)

Peptide solution	**Solvents**	**Soluble**	
1 mg HS-GAHWQFNALTVR-$CONH_2$ + 500 µL water	50 µL ACN in an Eppendorf (**solution 7**)	No	Solvents mixture ratio:90:10
solution 7	50 µL ACN in an Eppendorf (**solution 8**)	No	Solvents mixture ratio:80:20
Solution 8	50 µL ACN in an Eppendorf **(solution 9**)	No	Solvents mixture ratio:70:30

Solution 9	50 µL ACN in an Eppendorf(**solution 10**)	Yes	Solvents mixture ratio:50:50

After each time the solvents were added, the eppendorfs were vortexed and after sonicated.

Next, in table 2.6, is summarized the peptides that were synthesized and their specific properties.

Table 2.6: Summary of peptides properties

PEPTIDES	MOLECULAR WEIGHT	SOLUBILITY
NH_2-GAHWQFNALTVR-$CONH_2$	1398.60	Soluble in 80:20 H_2O:ACN
C_{16}-GAHWQFNALTVR-$CONH_2$	1637.01	Not soluble
C_{16}-$(VQ)_2$-GAHWQFNALTVR-$CONH_2$	2091.54	Not soluble
C_8-GAHWQFNALTVR-$CONH_2$	1524.80	Soluble in 80:20 H_2O:ACN
C_{12}-GAHWQFNALTVR-$CONH_2$	1580.90	Soluble in 50:50 H_2O:ACN
HS-GAHWQFNALTVR-$CONH_2$	1486.72	Soluble in 50:50 H_2O:ACN

2.2- PEPTIDE CHARACTERIZATION AND PURIFICATION

2.2.1-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) is one of the most powerful techniques in analytical chemistry by providing the ability to separate, identify and quantify, in a faster, reproducible and efficient way, the compounds that are present in a mixture that can be dissolve in a liquid(Fariss & Reed, 1987).

Molecule´s structure and reactivity are determined by the arrangement of the atoms and their bonds that constituted the molecule, more precisely the functional groups. The characteristics of the molecules influence the HPLC separations, such as the polarity of molecules that reflect the ability of the compounds to interact with each other: the compounds can interact through intermolecular forces, hydrogen bonding or weaker dispersion forces (Barnes, 1992). Molecules with similar chromatography polarity tend to be attracted to each other, and molecules with different polarity normally repel one another.

The base of chromatography separation system is to have a mobile phase (the solvent) and a stationary phase (column packing material) with different polarities. Compounds present in the sample (that is injected to the HPLC system) having a polarity similar to that of the mobile phase will be attracted to it and will move faster, compounds having a polarity similar to that of the stationary phase will be attracted to it and, consequently, will be delayed compared to other compounds (Barnes, 1992). Through this procedure based on different affinities and interactions between sample components and the column, is possible to separate them (Barnes, 1992).

In some cases, when the sample is constituted by mixtures of polar and non-polar compounds, the composition of the mobile phase can be constituted by a changing in the polarity of the solvent mixture during the separation, called gradient elution (Barnes, 1992; Harris, 2003). The HPLC system that was used during the experimental work was composed of a binary gradient which means that two solvents, which are mixed in a ratio that changes in a certain range of time (Currell, 2008).

The stationary phase column packaging material is generally composed of small porous silica particles which work with high efficiency because of ensuring uniformity of flow due to the resistance they provide (Barnes, 1992; Currell, 2008; Harris, 2003). Silica is a polar molecule but its silanol group (Si-OH) can be chemically modified by the attachment of different groups producing bonded phases that change the silica surface´s properties (Barnes, 1992). The HPLC system that was used contains a reverse-phase C_{18} – bonded silica column. The silica linked to a C_{18} become less polar which means that this linking turns the new structure to a non-polar one; this is the most popular linking used in a reverse-phase (Barnes, 1992). A reverse-phase system is constituted by a non-polar stationary phase and a polar mobile phase: the non-polar stationary phase used was made by C18–bonded silica and the polar mobile phase used was water with miscible a polar miscible solvent ACN.

The detector presented on the system collect the information from the eluted compound and generates signals that are saved by a computer. The computer displays the signals graphically where it is shown the absorbance of the sample compound and it retention time to a pre-established wavelength (Barnes, 1992).

HPLC system has the capability to separate the mixtures but sometimes it does not allow the identification of the separated compounds since this system does not provide the details necessary to do that. Because of this fact, in the last years this type of system is constituted by the combination of a HPLC and a mass spectrometry (Barnes, 1992).

Before the purification of the peptides, it was used analytical HPLC to find out the elution times of the peptides, and also to optimize the eluent gradient to be used for preparative HPLC. After this, it was used the preparative HPLC to purify the peptides

In the present work, the peptides were analyzed by analytical Alliance HPLC System (Waters, UK)with X-Bridge column $C_{18,}$ 3.5 µm, 4.6 x 150mm at a flow rate of 1 mL/min with water/ACN (0.1%TFA) gradient; the absorbance was monitored with a wavelength of 220nm in a waters 2489 UV/visible detector (Waters, USA). The peptides were purify by Waters 2489 Binary HPLC system, Waters UK, which contains a reverse-phase C18 column X-Bridge Prep OBD, 5µm, 30 x 150 nm, Waters UK at a flow rate of 20 mL7min with water/ACN (0.1%TFA) gradient; the absorbance was monitored with a wavelength of 220nm in a Waters 2489 UV/visible detector (Waters, USA) .

Table 2.7: HPLC procedures

Peptide	Samples´ preparation	Method used
NH_2-GAHWQFNALTVR-$CONH_2$	Sample: 80:20 H_2O:ACN	Method 1 (see Appendix 6.1)

C_8 -GAHWQFNALTVR-$CONH_2$	Sample: 80:20 H_2O:ACN	Method 1 (see Appendix 6.1)
C_{12}-GAHWQFNALTVR-$CONH_2$	Sample: 50:50 H_2O:ACN	Method 2 (see Appendix 6.1)
HS-GAHWQFNALTVR-$CONH_2$	Solubility: 50:50 H_2O:ACN	Method 1 (see Appendix 6.1)

2.2.2- ELECTROSPRAY IONIZATION MASS SPECTROMETRY

Electrospray ionization mass spectrometry (ESI-MS) is an analytical technique that offer a qualitative (structure) and quantitative (molecular mass) information of the analyte molecules after their conversion to ions (Ho et al., 2003; Kujawinski et al., 2002; Xiao et al., 2000).

The sample is introduced and pass through a stainless steel or quartz silica capillary from the ionization source of the system (ESI) to be ionized and acquire positive or negative charges (Ho et al., 2003). Then, the ions go to a quadrupole mass analyzer (MS) which is a system consisting of 4 parallel metal rods held at the same distance electrically connected with a DC voltage and radio frequency applied to the diagonal pair of rods that result in a field which ensures the movement of the ions in the z-direction with oscillatory motion on the xy-axis, the amplitude of the oscillatory waves is related to the ratio m/z and can be controlled by the DC and the radio frequencies so that the ions can go along the direction and pass through the detector. In mass analyzer, the ions move through a magnetic or electric field and arrive at different parts of the detector according to the ratio m/z (mass / charge), which means that the ions are classified and separated according to their mass and charge. The separated ions are measured and the results generate different signals that are displayed on a graph appearing in the computer system (Ho et al., 2003; Sauer & Kliem, 2010).

The peaks present in the mass specification correspond to the ionisable states of each peptide (in the case of the present work) depending on the number of charges of each peptide.

In the latest years, ESI-MS has coupled with HPLC in order to have the subdivision of the molecules before the MS analysis, and together became a powerful technique for the analysis of molecules with a variety of polarities.

Before put the peptides in the MS, it was necessary to prepare de solutions that are described in table 2.8:

Table 2.8: ESI-MS procedures

Peptide	Samples ‘preparation
NH_2-GAHWQFNALTVR-$CONH_2$	1 mg/mL methanol in an Eppendorf (solution 11)
C_8 -GAHWQFNALTVR-$CONH_2$	1 mg/mL methanol in an Eppendorf (solution 12)
C_8 -GAHWQFNALTVR-$CONH_2$	1 mg/mL DCM in an Eppendorf 25 (solution 13)
C_{12}-GAHWQFNALTVR-$CONH_2$	1 mg/mL methanol in an Eppendorf 26 (solution 14)
C_{12}- GAHWQFNALTVR-$CONH_2$	1 mg/mL DCM in an Eppendorf 27 (solution 15)
HS- GAHWQFNALTVR-$CONH_2$	1 mg/mL DCM/methanol (0.5mL DCM + 0.5 mL methanol in an Eppendorf (solution 16)

The solutions that have been put in the MS were solution 11, solution 13 and solution 16.

2.3- INTERACTION BETWEEN HA AND PEP-1

General Procedure for testing the interaction between HA and Pep-1 1% solution HA in water was made: the HA used had various sizes (5, 60, 200, 700 kDa) and it was tested against various concentrations of Pep-1 of 1 and 2%; the mixtures were left at room temperature during one week.

Figure 2.9 shows the plate where these solutions were combined in the plate: in the first line it is presented Pep-1 1% and the different sizes of HA that were tested for this concentration; in the second line it is presented Pep-1 2% and the different sizes of HA that were tested for this concentration;

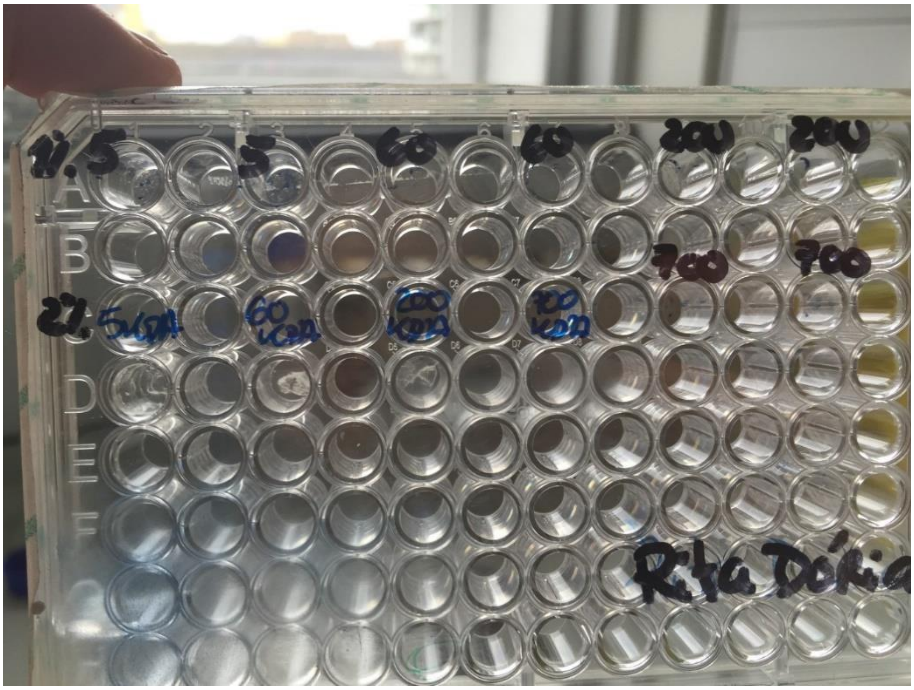


Figure 2.9: Combinations of: Pep-1 1% - different sizes of HA combinations; Pep-1 2%-different sizes of HA

Chapter 3-RESULTS AND DISCUSSION

3.1- PEP-1

NH_2-GAHWQFNALTVR-$CONH_2$ was synthesized successfully.

To be sure that the right peptide was synthesized, the values obtained from MS should be compared to the theoretical values.

The theoretical m/z values from NH_2-GAHWQFNALTVR-$CONH_2$ are:

ESI-MS: 1398.60 $[M+H^+]^+$ and 700.30 $[M+2H]^{2+}$.

As it is shown (Fig 3.1), these two peaks are present in the ESI-MS graphs, which means that the correct peptide was synthesized. The other peaks that is identified in the graph correspond to impurities of the Mass Spec machine.

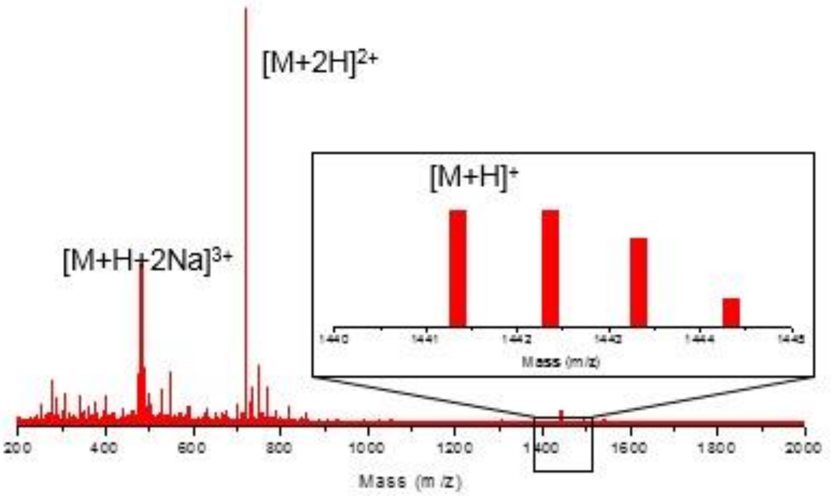


Figure 3.1: ESI-MS Result from Pep-1

Relatively to HPLC results, NH_2-GAHWQFNALTVR-$CONH_2$ was eluted at 6 minutes. (Fig.3.2)

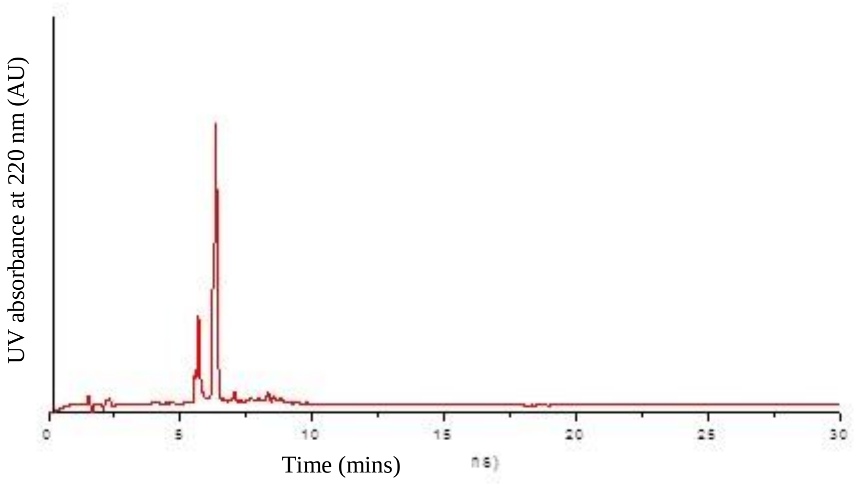


Figure 3.2: HPLC graph from Pep-1

This peptide is hydrophobic due to its residues with hydrophobic side chains, but the solubility was worked up and the peptide became soluble at 80:20 water:ACN which explains the faster elution time since the polarity of the peptide has become similar to polarity of the mobile phase.

The other peak that appear first represents the same compound since the mass spec for both peaks is the same. This fact occurs due to the racemization (the process in which one enantiomer of a compound, such as an L-amino acid, converts to the other enantiomer(Reist et al., 1995)) of the arginine at the C-terminus that causes the second peak in the HPLC (Fig.3.3).

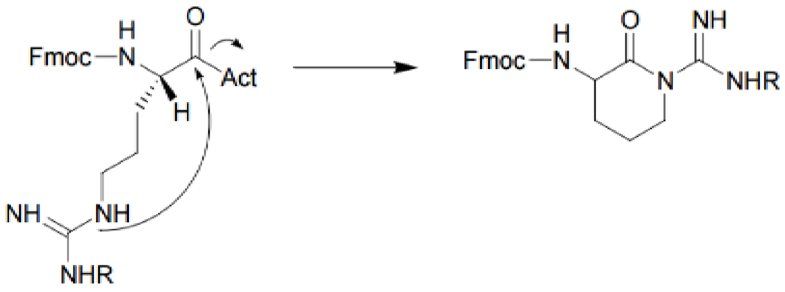


Figure 3.3: Racemization of the arginine

After the Pep-1 characterization, the mix between different % of Pep-1 and HA was analysed (Table 3.1).

Table 3.1: Pep-1-HA tested combinations

	HA /kDa							
	5		60		200		700	
Experiment	1	2	1	2	1	2	1	2
Pep-1 1%	x	x	x	x	x	x	x	x
Pep-1 2%	x	x	x	x	✓	✓	x	x

✓ interaction observed

x nothing was observed

The results from Table 3.1 shows that 1% solutions were unable to form any gels which means that with this % of Pep-1 with none different size of HA is possible to form any self-assemble structure.

An interaction between 2% Pep-1 and HA was observed. By increasing the concentration of Pep-1, and mixed it with an amount of HA with a higher molecular weight, the two compounds can self-assemble and form a structure similar to a gel.

3.2- FIRST SERIES OF PEP-1 BASED ANALOGUES

The peptides C_{16}-GAHWQFNALTVR-$CONH_2$ and C_{16}-$(VQ)_2$-GAHWQFNALTVR-$CONH_2$ were synthesized; however, the peptides were unable to be solubilized. The solubility issue arises as Pep-1 contains more hydrophobic side chains (alanine, tryptophan, phenylalanine, leucine and valine) than hydrophilic chains making the molecule slightly hydrophobic. Furthermore, by adding C_{16} /C_{16}-$(VQ)_2$- that are two hydrophobic residues, to an already hydrophobic molecule, causes the molecule to become insoluble .

From these results, it was necessary to make other new peptides, the second series of Pep-1 based analogues with shorter hydrophobic chains.

3.3- SECOND SERIES OF PEP-1 BASED ANALOGUES

The peptides C_8-GAHWQFNALTVR-$CONH_2$ and C_{12}-GAHWQFNALTVR-$CONH_2$ were less hydrophobic due to the shorter alkyl chains.

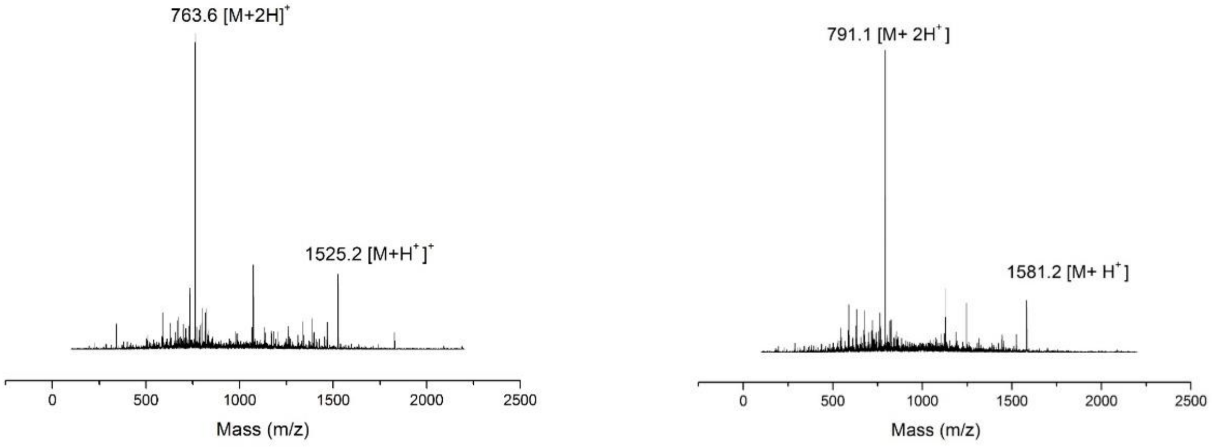


Figure 3.4: ESI-MS of C_8-GAHWQFNALTVR-$CONH_2$ ESI-MS (left) and C_{12}-GAHWQFNALTVR-$CONH_2$ (right) peptides

Relative to the peptide C_8-GAHWQFNALTVR-$CONH_2$, the calculated theoretical mass values are: ESI-MS: 1524.80 $[M+H^+]^+$ and 763.40 $[M+2H]^{2+}$.

The graph presented in the table shows these two peaks (1525.2$[M+H^+]^+$ and 763.6 $[M+2H]^{2+}$) demonstrating that the correct peptide was synthesized. The other minor peaks represent the impurities that the sample could have.

The theoretical values from C_{12}-GAHWQFNALTVR-$CONH_2$ are: ESI-MS: 1580.90 $[M+H^+]^+$ and 791.45$[M+2H]^{2+}$

This peptide was sucessfully synthesized, as we can see by comparing the theoretical values and the ESI-MS data (1581.2 $[M+H^+]^+$ and 791.1 $[M+2H]^{2+}$).

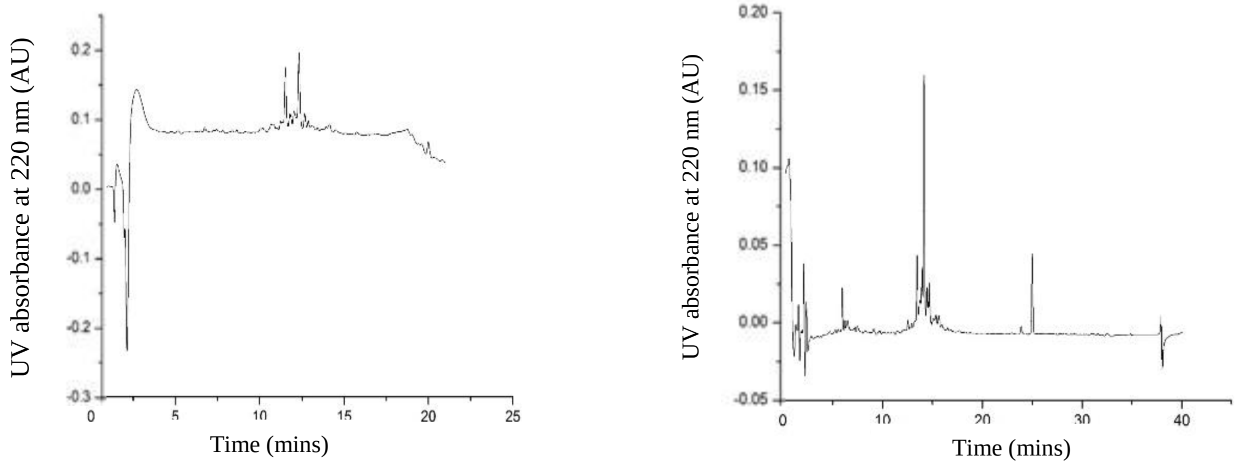


Figure 3. 5 HPLC graphs for C_8-GAHWQFNALTVR-$CONH_2$ (left) and C_{12}-GAHWQFNALTVR-$CONH_2$ (right)

C_8-GAHWQFNALTVR-$CONH_2$ eluted at 13 minutes. The sample injected into the HPLC system was constituted by the peptide and an 80:20 ratio of water and ACN. The polarity of the sample is similar to that of the mobile phase having the major compound, water, leading to the rapid elution of the analyte. In the other hand, the elution time is at 13 minutes because this time is when the percentage of the organic solvent, ACN, increases (the % of ACN changes from 20% to 50%). This point is explained due to the fact that the eluent strength increases as the % of the ACN increases and all the compounds are eluted in a faster way (Harris, 2003).

After the purification, it is necessary to calculate the yield, which gives the amount of product obtained. The yield's calculation corresponds to obtained mass divided by the theoretical mass:

$$\frac{Obtained\ mass}{Theoretical\ mass} \times 100\% \qquad \text{(3.1)yield Calculus}$$

The peptide's volume left was so low that was not possible to calculate the yield of the peptide.

C_{12}-GAHWQFNALTVR-$CONH_2$ was eluted at 15 minutes. The sample injected in the HPLC was constituted by a 50:50 water: ACN ratio, which explains that the sample has a polarity similar to the mobile phase being eluted in a faster period of time.

The other considered peaks (the one that was eluted at 6 minutes and the other that was eluted at 25 minutes can be consider part of the impurities that the samples can had or little parts of the peptide that had eluted first or after the whole peptide, since the mobile phase has the same constitution during the first 30 minutes (80:20 water: ACN).

The peptide's volume left was very low that was not possible to calculate the yield of the peptide.

After purification, the volume left from the two peptides was very low because the lower solubility did not allow the peptide to interact with the column.

So the third series of Pep-1 based analogues was done: HS-GAHWQFNALTVR-$CONH_2$.

3.4 - THIRD SERIES OF PEP-1 BASED ANALOGUES

HS-GAHWQFNALTVR-$CONH_2$ has also poor solubility that had to be worked out in order to allow the purification of the peptide by HPLC.

The peptide was eluted at 3 minutes. This peptide was also hydrophobic, so its solubility had to be worked out with the purpose of trying to purify it in the HPLC. The ratio that makes it possible (50:50 water: ACN) get the peptide with a polarity so similar to the mobile phase that it was eluted faster than it would be advisable to ensure good separation of the compounds in the sample. The solubility should be improved to get a lower elution time.

The theoretical/expected mass is 798.8 mg and after weighing out the obtained peptide that was purified by HPLC, the mass was 34.9 mg. So, the percentage of the yield is 4.93 %.

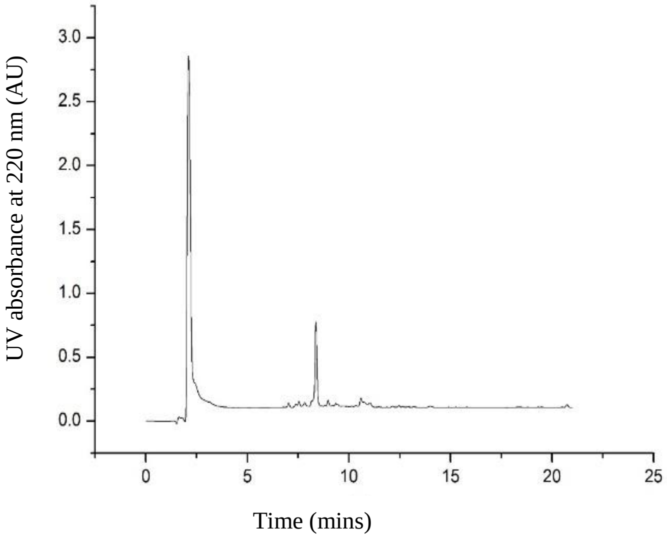


Figure 3.6: HS-GAHWQFNALTVR-$CONH_2$ HPLC graph

Chapter 4- CONCLUSION

From the peptide synthesis work, it can be concluded that peptides having a long alkyl chain are very hydrophobic to be purified and consequently it is impossible to study their interactions with HA.

When the alkyl chains are shorter, the peptides become less hydrophobic and its purification was possible. However, its lower solubility does not allow a considerable amount of peptide to be collected following HPLC procedures. Therefore, the alkyl chains used should be shorter than those presented in this work but the problem is that the solubility may improve but the peptides won´t self-assemble. It is possible to conclude that the interaction between HA and peptides constituted by alkyl chains is problematic to test, since the peptides are very hydrophobic, consequently insoluble and difficult to purify. Furthermore if the chains become shorter the peptides won´t have the properties that allow the self-assembly process, so may have less hydrophobicity but won´t interact with HA after all.

On the other hand, the peptide synthesized with SH group also demonstrated a lower solubility but this peptide had to be work so that it may be possible to try to purify it and its interaction with HA can, thus, be tested.

Finally, the interaction between Pep-1 and HA was observed and it is conclusive that by increasing the percentage of Pep-1 and with an HA with significant molecular weight occurs the formation of a structure similar to a gel. The gel formation was not immediate what supports the idea that Pep-1 to interact with HA, should be linked to other structures that could induce the self-assembly process in a faster way.

Chapter 5-FUTURE WORK

In future work:

- Test the interaction between HS-GAHWQFNALTVR-CONH$_2$ and HA and after characterize and analyze it by using Transmission electron microscopy (TEM) that is a technique with a higher resolution when compared to optical and scanning electron microscopy (SEM) which allows the examination in detail of the structure; and Quartz crystal microbalance (QCM) that is efficient at determining the affinity of molecules.
- The interaction between HA and 3% Pep-1 should be tested in order to know if by increasing further the percentage of Pep-1, the gel formation would be faster and more visible.

Chapter 6-APPENDIX

6.1- HPLC METHODS

METHOD 1:

The method used for the purification takes 21 minutes. The mobile phase was constituted by the following solvents:

A: water 0.1%TFA

B: ACN 0.1%TFA.

Time	%A	%B
0	80	20
15	50	50
17	0	100
19	0	100
21	80	20

METHOD 2:

The method used for the purification takes 40 minutes. The mobile phase was constituted by the following solvents:

A: Water 0.1%TFA

B: ACN 0.1%TFA

Time	%A	%B
0	80	20
30	0	100
36	0	100
37	80	20
40	80	20

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