



**Márcio Gabriel Siva
Costa**

**Matriz enriquecida com RERMS da APP como
substrato de adesão para o crescimento de neurites**

**APP RERMS enriched matrix as an adhesion
substrate for neuritic outgrowth**



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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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Dedico este trabalho aos meus pais por tornarem todo este percurso possível,
Há minha avó por todos os valores que me passou. E aos meus amigos mais
chegados pelo apoio.

o júri

presidente

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palavras-chave

APP, RERMS, matriz de adesão, crescimento de neurites

resumo

Nas proteínas domínios específicos podem determinar as suas relações funcionais e estruturais. Na Proteína Precursora da Doença de Alzheimer (APP) já foram descritos vários domínios presentes em ambas as porções intra e extracelulares. Várias funções têm sido atribuídas à APP incluindo na adesão celular e no reconhecimento célula-célula. Isto coloca a APP em respostas biológicas chave como a transmissão sináptica. Tendo em conta estas funções, os domínios extracelulares merecem uma maior atenção. O domínio extracelular RERMS da APP é de facto um candidato provável para estar envolvidos nos processos fisiológicos acima referidos

Para investigar o papel do RERMS foi utilizada uma abordagem multidisciplinar. O péptido RERMS ligado ao PEG (polietileno glicol) através de uma reação de crosslinking, validada por FTIR (Fourier transform infrared spectrometry). A FTIR provou ser a técnica mais eficiente para a validação desta reação porque apenas necessita de uma gota de amostra, e providencia informação acerca das reações ocorridas numa mistura. O resultado obtido consiste num espectro de radiação infravermelha da amostra, onde as posições dos picos dão informação acerca da estrutura das moléculas, e a intensidade dos picos está relacionada com a concentração das moléculas.

Subsequentemente foram preparados substratos com PEG e RERMS, plaqueadas células SH-SY5Y (células de neuroblastoma humano) e induzida a sua diferenciação. Foram claramente evidentes várias alterações morfológicas. O péptido RERMS causou uma morfologia mais achatada e a arquitetura do citoesqueleto ficou alterada, com o aparecimento de fibras de stress, um indicador da reorganização da actina.

Dadas as adesões focais desempenharem um papel chave na determinação da estrutura celular, foram investigadas diretamente.

A Cinase de adesão focal (FAK) é uma das proteínas mais expressas no sistema nervoso central (CNS) durante o desenvolvimento. Esta proteína foi descrita como crucial para a migração radial dos neurónios. A FAK pode ser localizada nos cones de crescimento e medeia a resposta a sinais atrativos e repulsivos durante a migração. Um dos mecanismos através do qual a FAK fica ativa é a auto-fosforilação no resíduo tirosina 397.

Tornou-se evidente que na presença do péptido RERMS houve um aumento da marcação da pFAK (FAK fosforilada) nas adesões focais e mais adesões se tornaram evidentes. Além disso também aumentaram as estruturas arredondadas de pFAK no núcleo na presença do RERMS. Estes resultados indicam que o domínio RERMS da APP tem um papel crítico na determinação de respostas fisiológicas celulares.

Aqui é sugerido um modelo onde o domínio RERMS da APP é reconhecido pelas integrinas e medeia desta forma respostas intracelulares que envolvem a FAK, a talina, a vinculina e filamentos de actina. Este mecanismo é uma provavelmente é responsável pela mediação da adesão celular e crescimento de neurites em neurónios.

keywords

APP, RERMS, adhesion matrix, neurite outgrowth

abstract

Specific domains can determine protein structural functional relationships. For the Alzheimer's Amyloid Precursor Protein (APP) several domains have been described, both in its intracellular and extracellular fragments. Many functions have been attributed to APP including an important role in cell adhesion and cell to cell recognition. This places APP at key biological responses, including synaptic transmission. To fulfil these functions, extracellular domains take on added significance. The APP extracellular domain RERMS is in fact a likely candidate to be involved in the aforementioned physiological processes.

A multidisciplinary approach was employed to address the role of RERMS. The peptide RERMS was crosslinked to PEG (Polyethylene glycol) and the reaction validated by FTIR (Fourier transform infrared spectrometry). FTIR proved to be the most efficient at validating this reaction because it requires only a drop of sample, and it gives information about the reactions occurred in a mixture. The data obtained consist in an infrared spectra of the sample, where peaks positions give information about the structure of the molecules, and the intensity of peaks is related to the concentration of the molecules.

Subsequently substrates of PEG impregnated with RERMS were prepared and SH-SY5Y (human neuroblastoma cell line) cells were plated and differentiated on the latter. Several morphological alterations were clearly evident. The RERMS peptide provoked cells to take on a flatter appearance and the cytoskeletal architecture changed, with the appearance of stress fibres, a clear indicator of actin reorganization.

Given that focal adhesions play a key role in determining cellular structure the latter were directly investigated.

Focal adhesion kinase (FAK) is one of the most highly expressed proteins in the CNS (central nervous system) during development. It has been described to be crucial for radial migration of neurons. FAK can be localized in growth cones and mediated the response to attractive and repulsive cues during migration. One of the mechanisms by which FAK becomes active is by auto phosphorylation at tyrosine 397.

It became clearly evident that in the presence of the RERMS peptide pFAK staining at focal adhesions intensified and more focal adhesions became apparent. Furthermore speckled structures in the nucleus, putatively corresponding to increased expression activity, also increased with RERMS. Taken together these results indicate that the RERMS domain in APP plays a critical role in determining cellular physiological responses.

Here is suggested a model by which RERMS domain is recognized by integrins and mediate intracellular responses involving FAK, talin, actin filaments and vinculin. This mechanism probably is responsible for mediating cell adhesion and neurite outgrowth on neurons.

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III. Abbreviations

2D	Two dimensions
3D	Three dimensions
AICD	APP intracellular domain
AD	Alzheimer's disease
APLP1	APP-like proteins 1
APLP2	APP-like proteins 2
APP	Amyloid precursor protein
APP ₆₉₅	Amyloid precursor protein 695 isoform
APP ₇₅₁	Amyloid precursor protein 751 isoform
APP ₇₇₀	Amyloid precursor protein 770 isoform
APS	Ammonium persulfate
APTES	3-aminopropyltrimethoxysilane
ATR	Attenuated total reflection
A β	β amyloid
BSA	Bovine serum albumin
CAPPD	Central APP domain
CNS	Central nervous system
CuBD	Copper-binding domain
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FIR	Far-infrared radiation
Fn	Fibronectin
FTIR	Fourier transform infrared spectrometry
GFLD	Growth factor-like domain
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulfuric acid
HA	Hyaluronic acid
ICC	Immunocytochemistry
KOH	Potassium hydroxide
KPI	Kunitz type serine protease inhibitor
LB	Loading buffer
LGB	Lower gel buffer
MEM	Minimum essential medium
MIR	Mid-infrared radiation
NHS	N-hydroxysuccinimide esters
NIR	Near-infrared radiation
PBS	Phosphate buffered saline
PEG	Polyethylenoglycol
PEG-RERMS	Polyethylenoglycol-RERMS sequence crosslinker
PEG-RGDS	Polyethylenoglycol-RGDS sequence crosslinker
PFA	Paraformaldehyde
pFAK	Phosphorylated focal adhesion kinase
pFAK sY397	Phosphorylated focal adhesion kinase at tyrosine 397
RA	Retinoic acid
RERMS	RERMS sequence
RGD	RGD sequence

RGDS	RGDS sequence
sAPP	Secreted amyloid precursor protein
SCM	Succinimidyl
SDS	Sodium dodecylsulfate
SDS-Page	SDS-polyacrylamide gel electrophoresis
SNRs	Signal-to-noise ratios
UGB	Upper gel buffer
WB	Western blot

1.Introduction

1. Introduction

1.1. The human brain and neuronal cells

The human brain is the most complex human organ. It is responsible for the storage, processing, integration, and transmission of almost all the information generated by internal and external stimuli. It is also responsible for maintaining homeostasis.¹⁻³

It is believed that the human genome contains approximately 20 thousand genes, of which 14 thousand are expressed in the developing and/or the mature brain. From these 14 thousand genes, 8 thousand are also expressed in all cells and tissues. Taking this into consideration, 6 thousand genes are brain exclusive. Furthermore a great amount of brain-specific genetic information resides in the introns and regulatory sequences that control timing, quantity, quality, variability, and cellular specificity of gene expression.¹

Cells that compose the nervous system can be divided in two broad categories: nerve cells, also commonly called neurons, and supporting glial cells, also called neuroglia or glia. It is estimated that the human brain contains about 100 billion neuros and several times as many neuroglia. Nerve cells and glia share the same organelles, which are present in all cells. These include the endoplasmic reticulum, Golgi complex, mitochondria, and a variety of vesicular structures.¹

Nerve cells are specialized in fast transport of electrical signaling over long distances without loss or alteration of the initial signal. Nerve cells can be divided and classified with respect to their polarity (Figure 1), in other words, based on the number of extensions that arise from the cell body. They can be divided into unipolar or pseudounipolar, the axon and dendrite emerge from the same process; bipolar cells, have the axon and dendrites emerging from cell body on opposite sites; multipolar cells, have two or more dendrites and one axon emerging from the cell body; and finally in anaxonic cells, the axon cannot be distinguished from dendrites.^{1,3}

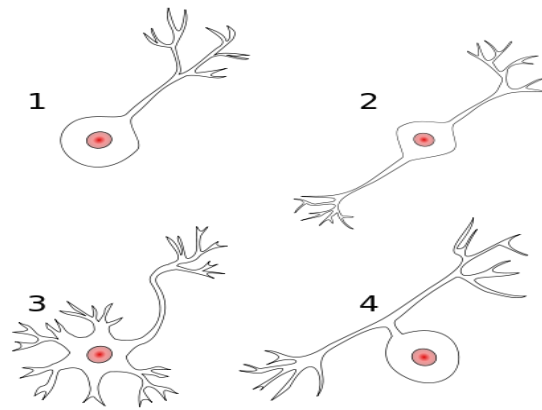


Figure 1. Morphology based classification of neurons by polarity.
 1 - unipolar neuron; 2 – bipolar neuron; 3 – multipolar neuron; 4 – pseudounipolar neuron.(taken from⁴)

Glia support rather than generate electrical signals. Glia are also essential to repair damage to the nervous system, acting as stem cells in specific brain regions, promoting regrowth of damaged neurons in regions where regeneration can usefully occur, and preventing regeneration in other regions where uncontrolled regrowth might do more harm than good.¹

In the mature nervous system three different types of glial cells are present; astrocytes, oligodendrocytes, and microglial cells.¹

Astrocytes, occur only in the central nervous system (CNS), which comprises the brain and the spinal cord. These cells have elaborate local processes that give them a star-like appearance. The major function of astrocytes is to maintain an appropriate chemical environment for neuronal signaling.¹

Oligodendrocytes, are also present only in the CNS, create a laminated, lipid-rich wrapping, called myelin, around some axons. This wrapping has important effects on the speed of the transmission of electrical signals, increasing the transmission speed.¹

Microglial cells are derived primarily from hematopoietic cells. This type of glia shares many properties with macrophages found in other tissues. They are primarily scavenger cells that remove cellular waste from injury sites of normal cell turnover. Moreover, microglia secrete signaling molecules, particularly cytokines, which can modulate local inflammation and influence cell survival or death.¹

1.2. Neuron morphology

The high level of differentiation for intercellular communication and moment-to-moment electrical signaling distinguishes neurons from other cell types. The most perceptible morphological aspect of neuronal specialization for communication is the extensive branching of neurons. This extensive branching is mainly due to the presence of an axon (Figure 2), an elongated structure that arises from the cell body and connects to the axon terminal arborization, the initial portion of the axon is called the axon hillock and is responsible for the generation of the electrical signal, also called telodendria. Additionally dendrites have an elaborate arborization that arises from the neuronal cell body in the form of dendritic branches. Dendrites are the primary target for the synaptic input from the axon terminals of other neurons, pre-synaptic neuron, and are distinguished by the high content of ribosomes and specific cytoskeletal proteins.¹

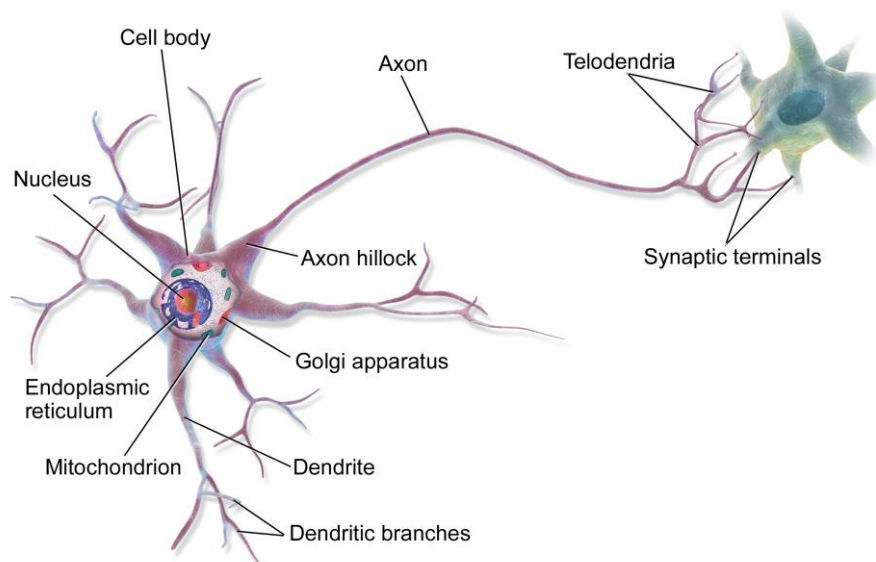


Figure 2. Nerve Cell.
(taken from⁴)

1.3. Neurite outgrowth

During development, neuronal cells become organized into functional neuronal networks by developing dendrites and axons, collectively called neurites, which connect synaptically to other neurons. The structures responsible for this process are the growth cones, specialized structures at the tip of the outgrowing neurites, which integrate chemical and structural signals from the extracellular matrix, and control neurite extension. Growth cone migration

elongates or retracts the trailing neurite, whereas growth cone splitting creates two daughter branches. Through these growth cone activities, neurons gradually develop their characteristic, highly branched axonal and dendritic trees.⁵⁻⁷

The axon outgrowth process can be divided in three sequential stages: protrusion, corresponding to the extension of new membrane at the growth cone, driven by the polymerization of filamentous actin; engorgement which is the transportation of membranous organelles and vesicles, made by microtubules, into actin-dominated peripheral regions; and consolidation that is the contraction and stabilization of the proximal growth cone, which takes a cylindrical axon shaft. Completion of this process allows for the bidirectional movement of organelles and vesicles, made possible by microtubules^{7,8}

This outgrowth process involves traveling across long distances and pathfinding, which consists in a turn, of responses to extracellular signals.⁵⁻⁹

Focal adhesion kinase (FAK) is one of the most highly expressed proteins in the central nervous system during its development. It has been reported to be dispensable for tangential migration, but crucial for radial migration, because FAK regulates the formation of contact points formed by Connexin-26, between migrating cells and radial glial fibers.⁹⁻¹²

FAK can be localized in growth cones¹³ and mediates the response to attractive and repulsive cues during its migration.^{14,15} FAK has also been reported to be highly expressed in the hippocampus, and has an important role in long term memory storage.^{9,15-17}

Although FAK activity has been shown to be very important in the CNS formation, more precisely in neuronal growth and migration, the molecular mechanisms involved in these processes and regulation of FAK are still poorly characterized and require further study.

1.3.1. Growth cones architecture

Growth cones can be divided in three regions. These regions are not well defined, in other words, they are transient.^{5,8}

Peripheral regions of outgrowth cones are composed by Filopodia, tapered finger-like projections, and Lamellipodia, flat sheet-like protrusions, both together, forming a very dynamic region.^{5,8,18}

Surrounded by the peripheral region is a central region, that is more stable and exhibits less plasma membrane dynamics (Figure 3), but has substantial molecular motion, including

constant transport of vesicles and organelles along microtubules. More distally, this central region transitions into the cylindrical axon shaft.^{5,8,18}

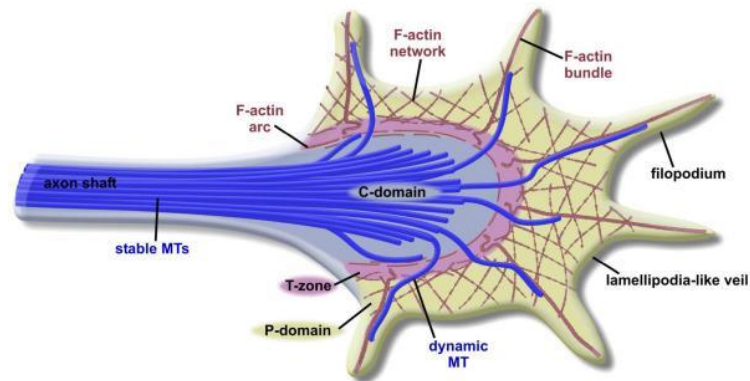


Figure 3. Growth cone architecture.

A peripheral region (P-domain) composed of Filopodia (F-actin bundles) and lamellipodia (F-actin network). A central region (C-domain) more stable, composed of a wide net of microtubules (blue) that send extensions to peripheral region, and connect to the peripheral region by a transition zone (T-zone). Also indicated the axon shaft region with microtubules (MTs). (Image taken from⁵)

1.4. β -Amyloid precursor protein (APP)

APP is a type I transmembrane glycoprotein expressed in a wide range of mammalian and non-mammalian cells¹⁹, and belongs to a small gene family of APP-like proteins, which includes APLP1 and APLP2.²⁰

The APP gene is found in chromosome 21 and contains 19 exons. By alternative splicing, APP generates 8 different isoforms, of which 3 are more common (Figure 4): The 695 amino acid form, expressed predominantly in the CNS, and the 751 and 770 amino acid forms, which are ubiquitously expressed.^{19,21,22}

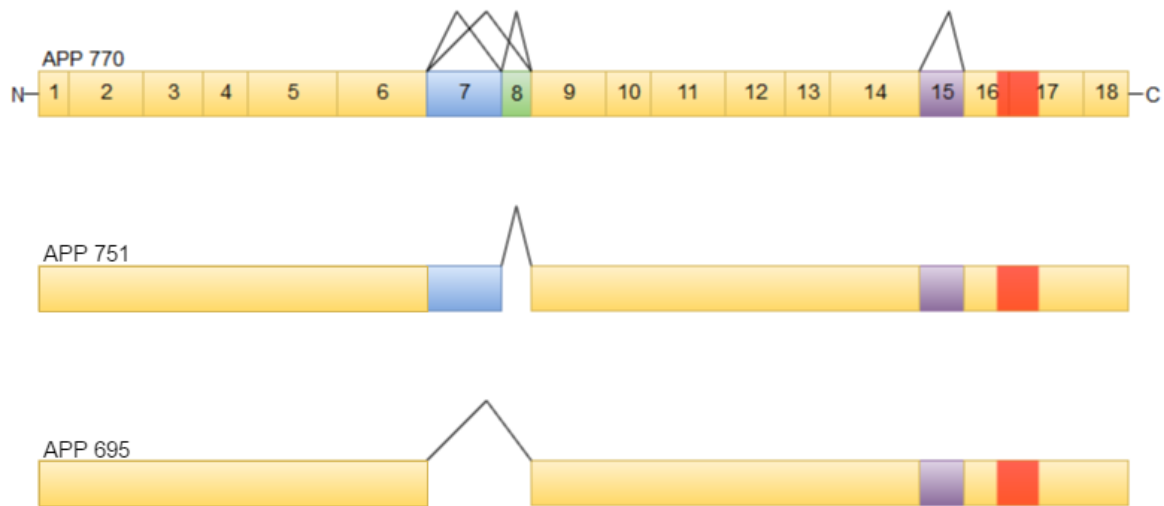


Figure 4. APP isoforms.
 Exon structure of full length APP transcript and the 3 most common isoforms APP770, APP751, and APP695. The alternative spliced exons are represented in blue (exon 7), green (exon 8) and purple (exon 15). The Aβ sequence is represented in red.

1.4.1. Proteolytic processing of APP

Two pathways can proteolytically process APP: the amyloidogenic, and the non-amyloidogenic (Figure 4). In the amyloidogenic pathway APP is first cleaved between amino acids 596 and 597 (APP 695) by β -secretase followed by γ -secretase that cleaves between 711-712 or 713-714 amino acids, resulting in the N-terminal sAPP (sAPP β), the intra cellular domain of APP (AICD), and the A β peptide. The latter can accumulate in neurons to form amyloid plaques, one of the hallmark of Alzheimer's disease (AD). In the non-amyloidogenic pathway APP is first cleaved by α -secretase between residues 596 and 597 (APP695) within the A β region, preventing the formation of the A β peptide, and then it is sequentially cleaved by γ -secretase.¹⁹⁻²⁵

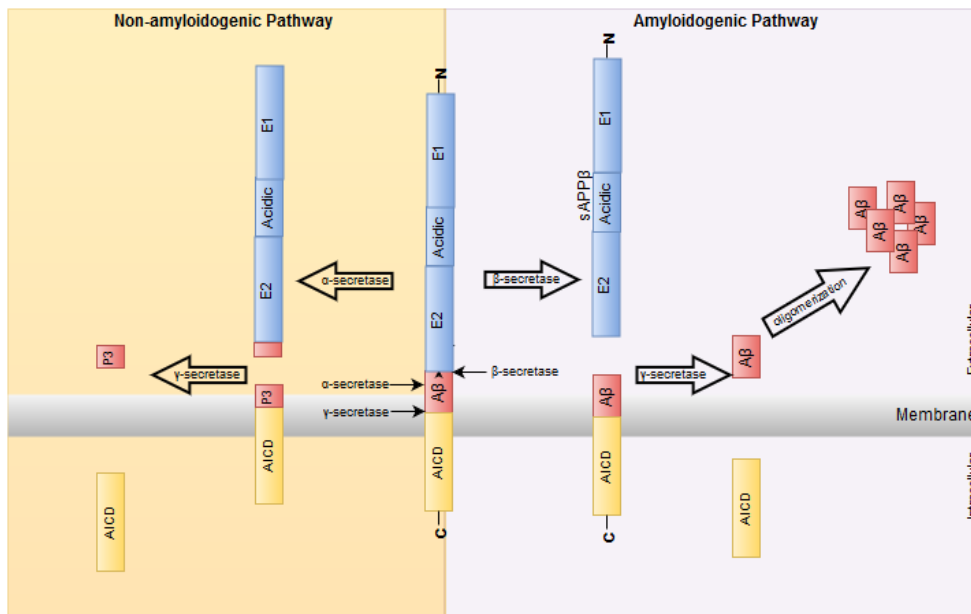


Figure 5. APP proteolytic cleavage.

In the amyloidogenic pathway APP is sequentially cleaved by β -secretase and γ -secretase leading to the formation of the A β peptide, sAPP β fragment and AICD fragment; and the non-amyloidogenic pathway where APP is sequentially cleaved by α -secretase and γ -secretase leading to the formation of sAPP α fragment, P3 fragment and AICD fragment.

1.4.2. APP domains and sub domains

Protein domains are critical for determining protein structure and function. Thus specific domains dictate structural functional relationships and specific isoforms can have different domains. For example, this is the case with the APP770 isoform that has the Kunitz Protease Inhibitor (KPI) domain (Figure 5, exon 8 and Figure 6), which is absent in APP751 and APP695. However, APP has other domains, critical for the protein's function.

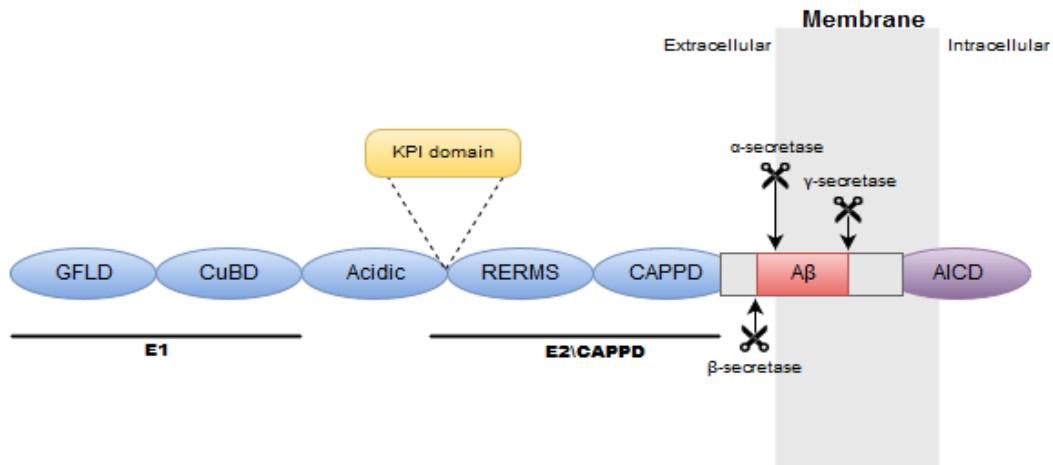


Figure 6. The domain structure of APP.

Showing E1 domain composed of the N-terminal growth factor-like domain (GFLD) and the copper-binding domain (CuBD). E1 domain is connected to the E2 domain, by the acidic domain. E2 domain contains the RERMS sequence and central APP domain (CAPPD). A β region and the APP intracellular domain (AICD) are also represented. Between the acidic domain and RERMS sequence there is the KPI domain. Secretases cutting sites are also represented.

Members of the APP protein family have two extracellular conserved domains, E1 and E2. The E1 domain contains the N-terminal growth factor-like domain (GFLD) and the copper-binding domain (CuBD). The E1 domain is connected to the carbohydrate domain, namely the E2 domain, by the acidic domain (Figure 6). The E2 domain contains the RERMS sequence and central APP domain (CAPPD). Linked to the E2 domain is the A β region and the APP intracellular domain (AICD). AICD is the most conserved region. Between the acidic domain and RERMS sequence there is the KPI, already mentioned above.^{20,24–26}

1.4.3. APP functions

Several biological functions have been attributed to the extracellular, N-terminal region of APP during the last few years. Longer isoforms of APP (APP₇₅₁ and APP₇₇₀), containing the KPI domain have been implicated in the regulation of neurite extension, blood coagulation, and wound-healing. On the other hand APP₆₉₅ (lacking the KPI domain) plays a role in growth regulation and modulation of neural excitability, synaptic plasticity, and cell survival, showing that these functions are KPI-independent. Neuron excitability has been shown to be sAPP α specific.²⁶

RERMS

REMRS, is a five amino acid sequence (Arg₃₂₈-Glu₃₂₉-Arg₃₃₀-Met₃₃₁-Ser₃₃₂) located between the KPI and CAPPD domains (Figure 7), it has been reported to play role in neurite outgrowth on neurons: the N-terminal portion of sAPP containing RERMS sequence was proven to have growth-regulating activity in fibroblasts²⁷. The same sequence induced neurite outgrowth in a neuronal cell line from the rat central nervous system (B103 cell line)²⁸. RERMS-containing APP peptides were demonstrated to promote neuronal survival in primary rat cortical cells.²⁹ In a rabbit spinal cord model for ischemia, the ability of a 17-mer peptide containing the RERMS sequence to protect against neuronal loss was tested. This peptide largely increased the required ischemia time to produce paraplegia.³⁰ This same peptide when infused in elderly rats trained in a Morris water maze, led to increased memory retention and synaptic density in frontoparietal cortex.³¹

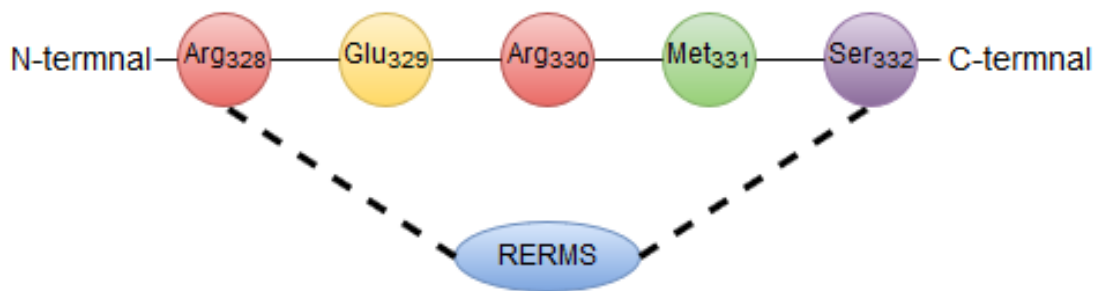


Figure 7. RERMS sequence in APP.

1.5. Extracellular Matrix

The extracellular matrix (ECM) comprises the non-cellular components of tissues, and water, proteins, and polysaccharides are important components. ECMs of different tissues, although they have the same basic components, differ in their composition and organization, leading to a wide diversity of ECMs over the whole body. ECMs are highly dynamic and undergo constant remodeling by adjacent cells through both enzymatic and non-enzymatic processes.³²

Fibrous ECM proteins such as collagen and fibronectin (Fn) as well as glycosaminoglycans such as hyaluronic acid (HA), can form complex three dimensional (3D) scaffolds that mechanically support cells and form the basis of tissue shape.³³⁻³⁵

The role of the ECM is not only for support, the ECM also provides a great number of biochemical cues to cells through the presentation of specific amino acid binding sequences. Cells bind to the ECM through ECM receptors such as syndecans, discoidin domain receptors, and the most important and common integrins.^{32,33,36,37}

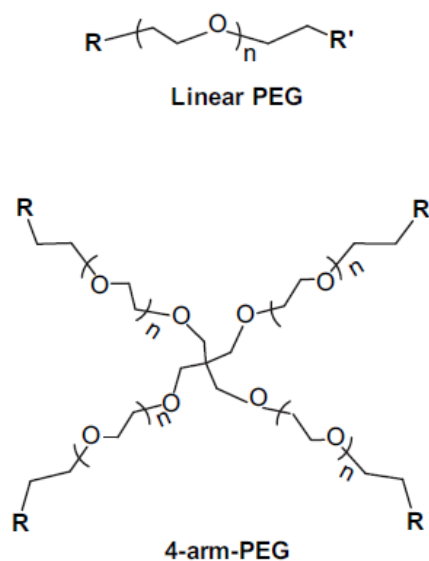
Fn has many binding sites for integrins, other ECM proteins, growth factors, and itself, mediating many cellular interactions with the ECM. Such interactions are crucial for many cell behaviors including adhesion, migration and differentiation, morphogenesis, and wound healing and morphogenesis.³⁸

One of the main, possibly the most pertinent, cell-binding site is the peptide sequence arginine-glycine-aspartic acid-serine (RGDS).^{32,36,37,39-46}

1.6. Polyethylenoglycol (PEG)

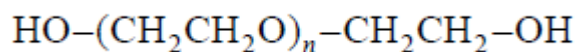
In order to better understand the function of the ECM, biomaterials are being pursued as attractive experimental models. Among biomaterials, PEG is particularly attractive. This fact is due mostly to its properties that include resistance to protein adsorption, it repels proteins without denaturing them, has high water solubility, good biocompatibility, is non-immunogenic, available in different molecular weights, and is fully transparent, facilitating microscopic analyses of cells. These properties have permitted PEG to be widely applied in biomedical sciences including surface modification, bioconjugation, drug delivery, and tissue engineering.⁴⁷⁻⁵¹

In the most common form, PEG can be linear or branched, and can have one or more branches terminated with hydroxyl groups, which can be converted into different functional groups, such as methoxyl, carboxyl, amine, thiol, azide, vinyl sulfone, azide, acetylene, and acrylate. In multi armed PEG, termination groups can be all the same, or they can be different within the same molecule (Figure 8).

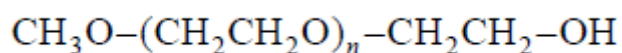


*Figure 8. PEG structure.
A linear and a 4-arm PEG are represented. (adapted from⁴⁷)*

PEG polymer is synthesized by anionic polymerization of ethylene oxides initiated by nucleophilic attack of hydroxide ion on the epoxy ring.^{47,48,51}



PEG can be modified in many ways by a wide range of processes, but the most useful polypeptide modification is monomethoxy PEG (mPEG), synthesized by anionic ring opening polymerization initiated with methoxide ions.^{48,51}



In order to conjugate PEG with biomolecules there are some factors that must be taken into consideration: the chemical structure, molecular weight, steric hindrance, and the reactivity of both PEG and biomolecules.^{48,49,51}

1.6.1. PEGylation and NHS-ester PEG reaction chemistry

PEGylation is the term used for the covalent attachment of PEG to peptides or proteins. This is a very advantageous process, making it possible to immobilize proteins or peptides in a matrix gel, in a 3D set up, or in a surface as a substrate for cells in a 2D manner. PEGylation has also been used as a drug delivery system because it enhances water solubility of hydrophobic drugs, prolongs the circulation time, and minimizes non-specific uptake.^{48,51-55} A significant amount of studies have been carried out with respect to PEGylation, thus the proof of concept is well established. Within the scope of this work particular attention will be dedicated to immobilizing RERMS sequence to a surface matrix suitable for cell culture. To achieve this NHS-ester PEG is an attractive model. For the work here described NHS-ester PEG will be employed, given that NHS-ester crosslinkers, such as NHS-ester PEG (Figure 9), reacts with primary amines in physiological to slightly alkaline conditions (pH=7,2-9). This reaction is covalent and thereby it is very stable.⁵⁶

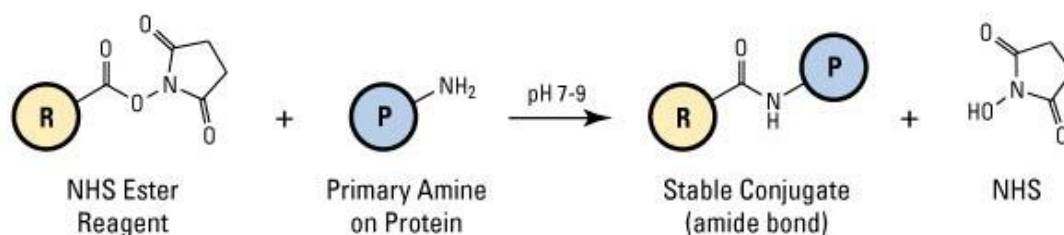


Figure 9. NHS ester reaction for chemical conjugation to a primary amine. (R) represents a labeling reagent or one end of a crosslinker having the NHS ester reactive group; (P) represents a protein or other molecule that contains the target functional group. (Taken from⁵⁶)

The major characteristics of this reaction are as follows:⁵⁶

- Half-life of hydrolysis for NHS-ester is 4 to 5 hours at pH 7,0 and 0°C;
- Half-life of hydrolysis for NHS-ester decreases to 10 minutes at pH 8,6 and 4°C;
- NHS, when free in solution, can be measured at 260 to 280nm, because it absorbs in that range.

In the present study, NHS-ester PEG will be used as a crosslinker for the RERMS sequence. This crosslinking reaction will be applied in pre-treated and amino-silanized coverslips, which will be used as a surface for cell culture. The model will be used to study the effect of the RERMS sequence with respect to cell adhesion, cell number and the effect of this surface on cell altering cell morphological, using immunocytochemistry, will also be tested.

2.Objectives of the study

2. Objectives

The major goal of this thesis is to investigate how the RERMS domain of APP can influence cell adhesion and neuritic outgrowth. The underlying hypothesis is that the RERMS domain, typically exposed extracellularly in APP, can influence cell morphology and neuritic outgrowth and is hence a critical domain in the developing CNS and in the maintenance of the neuronal network.

Thus the specific objectives of the present study are:

1. To optimize PEG crosslinking with the RERMS peptide;
2. To characterize the role of APP RERMS in cell adhesion;
3. To characterize the role of APP RERMS on cell morphology and neuritis outgrowth;
4. To identify potential molecular targets/mechanisms involved in RERMS mediated responses.

3. Materials and methods

3. Materials and methods

3.1. Culture, growth and maintenance of SH-SY5Y cell line

In neurosciences the use of mammalian neurons derived from embryonic the CNS tissue is very limited mainly because once these cells are terminally differentiated into mature neurons, they can no longer be propagated.

To overcome this limitation transformed neuron-like cell lines are commonly used. Among them, there is a very popular and well characterized one, the SH-SY5Y neuroblastoma cell line (ATCC® CRL-2266™).⁵⁷


The SH-SY5Y cell line was originally derived from a metastatic bone tumor biopsy, and a subline of the parental line SK-N-SH, which were subcloned three times: first to SH-SY, then to SH-SY5, and finally to SH-SY5Y.⁵⁷

In order to archive the objectives proposed for the present study, SH-SY5Y neuroblastoma cell line was chosen for the reasons described above, and because this cell line is a human cell line that can be easily differentiated into a more mature neuronal-like phenotype.

SH-SY5Y cells (ATCC® CRL-2266™) were grown and maintained in Minimal Essential Medium (MEM)/F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS), 0.5 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Cultures were maintained in a humidified chamber at 37°C under 5% CO₂.^{9,57} Cells were subcultured whenever 80-90% confluence was reached. The procedures are in routine use in the laboratory.

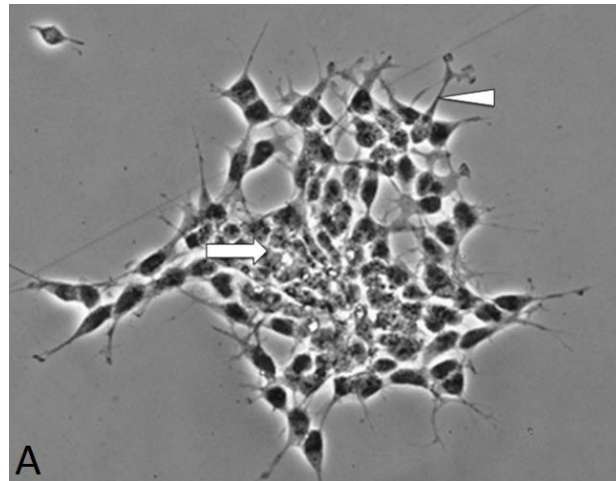
3.1.1. Differentiation of the SH-SY5Y neuroblastoma cell line

Differentiation of SH-SY5Y cells is relatively easy to achieve by manipulation of the culture medium. There are many reagents\compounds that can be added to culture medium in order to differentiate SH-SY5Y cells, but Retinoic Acid (RA) is the most commonly used.⁵⁷

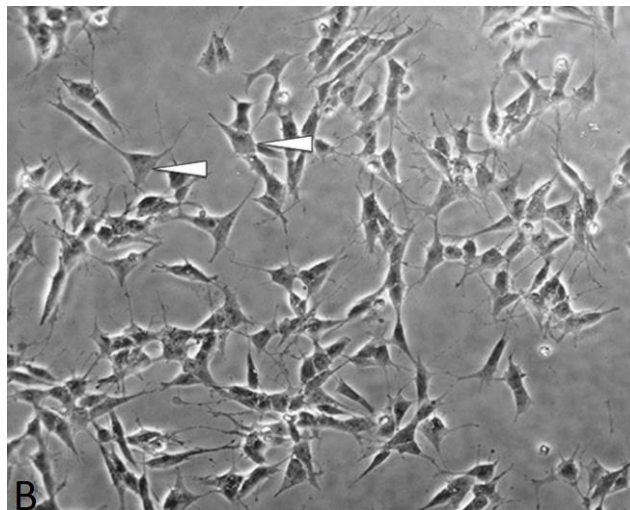
Cells were plated at a density of approximately 30 000 cells in 15 mm  plates (example - Figure 10). Cells were allowed to adapt for 24 hrs (day -1) and were then subjected to differentiation. In order to differentiate SH-SY5Y cells in this study, RA was added to the culture medium, at a

concentration of 10 μ M and FBS at 10%.⁵⁸ This culture medium was replaced every 48 hours until the time of 7 days was reached (example-figure 11).

This procedure was carried out on different substrates, some incorporating peptides like RERMS and RGDS as described below.



*Figure 10. Undifferentiated SH - SY5Y cells.
Cells tend to grow in clusters and may form clumps of rounded cells on top of one another (arrow). At the edges of the cluster, cells begin to extend short neurites (arrowhead). (taken from ⁵⁷)*



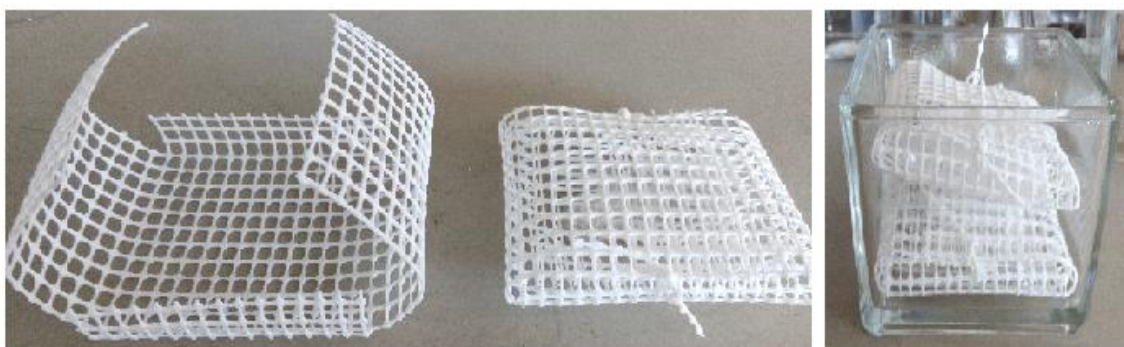
*Figure 11. Differentiated SH - SY5Y cells.
Cells do not cluster and have a more pyramidal shaped cell body (arrowhead). Neurites begin to extend, reminiscent of dendrites and/or axons. (taken from ⁵⁷)*

3.2. Surface cleaning and treatment of coverslips for cell culture

In order to remove all residues, organic and non-organic, a protocol from “*Journal of Visualized Experiments*” was followed, with some modifications. The same protocol was followed with few alterations for amino-silanization and PEG attachment to the surface.⁶⁶

3.2.1. Surface cleaning of coverslips

Before cleaning and treatment, coverslips were disposed in a homemade basket, made of Teflon net (bought in the local tools store). The selection of the material from which the basket was made, was of great importance and resolved many problems once the reagents used in this protocol are very strong and abrasive. The only materials resistant to all the reagents used are ceramic, glass and Teflon. The material easiest to get, and more affordable was the Teflon net.



*Figure 12. Packaging coverslips.
Teflon net basket (left). Baskets full with coverslips, contained in the glass staining jar (right).*

Coverslips were first rinsed with MilliQ water three times, and sonicated (a Branson 1510 sonicator was used) with MilliQ water for 10 minutes to remove dirt.

MilliQ water was replaced by acetone, and the coverslips were sonicated for 30 min. After this the acetone was disposed in a specific residue recipient, and replaced by MilliQ water. In order to remove all acetone residues from the coverslips, they were rinsed in MilliQ water three times.



*Figure 13. Preparing coverslips.
Basket with coverslips, inside a glass staining jar, placed in the immersible ultra sonicator Branson 1510.*

Then MilliQ water was replaced by potassium hydroxide (KOH) at a concentration of 1M, and sonicated for 30 minutes. After this, to remove all residues of KOH from the coverslips, they were rinsed three times in MilliQ water.

The final stage of coverslip cleaning was the piranha etching. This reaction was made in a 2 liter beaker, because it is a very exothermic and violent reaction. First the slide basket was placed in the bottom of the beaker, and to it were added 450 mL of sulfuric acid (H_2SO_4). Then 150 mL of hydrogen peroxide (H_2O_2) was added and all the solution was stirred. The reaction starts as soon as H_2O_2 was added and the basket was only taken out of the reaction when it stopped and the temperature decreased. Piranha solution was neutralized by slow addition of small volumes of water, and then discarded to appropriate residues. Once the basket was taken out from the piranha solution, it was rinsed three times in MilliQ water, in order to remove all possible piranha solution residues.

3.2.2. Amino-silanization of coverslips

In order to functionalize coverslips, MilliQ water from the previous step of cleaning was replaced by methanol. Then the amino-silanization solution was prepared by adding to a Pyrex flask 100 mL of methanol, 5 mL of acetic acid, and 3 mL of APTES (3-aminopropyl trimethoxysilane). This solution was gently mixed. In this solution, methanol functions as a solvent, and acetic acid functions as a catalyst for the amino-silanization reaction.

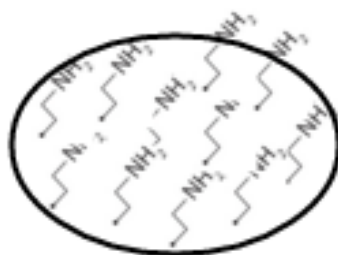


Figure 14. Representation of NH_2 groups attached after amino-silanization.

Methanol was replaced by the amino-silanization solution, and coverslips were incubated for 40 minutes, and sonicated once for one minute during this period.

Following this, amino-silanization solution was replaced with methanol and rinsed 3 times. Coverslips were left in methanol until the next steps, but it is not advisable for coverslips to stay more than 1 hour in methanol, because it can damage the amino-silanized coverslips.

3.2.3. Surface treatment with PEG and crosslinkers

The class of PEG chosen for this study, was and NHS ester PEG, more specifically 4-Arm PEG-SCM with a molecular weight of 20k (purchased from Creative PEGWorks). This is a multiarm PEG derivative with succinimidyl NHS ester groups at each terminal of the four arms connected to one pentaerythritol core.

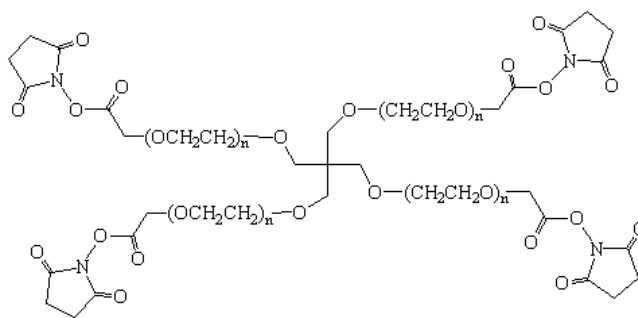


Figure 15. Structure of 4-arm NHS-ester PEG (20 kDa) used in this study.

3.2.4. Crosslinking reaction

In order to attach PEG and the crosslinkers to NH_2 groups present at the surface of the previous amino-silanized coverslips, first PEG has to be reconstituted in a buffer with a pH between 7,5 and 8,9. For this experiment, a buffer with pH=8,5 was chosen, because this was consistent with the buffer used in the previous procedures.

For the buffer preparation 84 mg of sodium bicarbonate (Thermo scientific) were dissolved in 10 ml of MilliQ water.

PEG was then weight and dissolved in a concentration of 20 mg of PEG in 1 mL of sodium bicarbonate, buffer was prepared previously. This concentration was chosen, because it a thicker PEG gel was desired, if the concentration was increased it was very difficult to dissolve all the PEG.

Following this step, the peptides were added to the PEG solution. Theoretically 4-Arm PEG-SCM is capable of linking to 4 peptides, but NHS which is released into solution competes with NH₂ from the N-terminus of each peptide for the position at the end of PEG arms. Furthermore, RERMS and RGDS peptides are very reactive. Thus, it was decided to use a higher ratio between PEG and peptides than that theoretical calculated. For the present experiments a ratio of 1/10 of PEG/peptide (number of molecules/number of molecules) was used. In this case peptides were reconstituted in deionized water at a concentration of 5 mg/mL. Concerning the molecular weight of peptides, the following was used; 10 µl of peptide solution for 100 µL of PEG solution.

Table 1. Table showing the concentration of stock solutions of PEG, RGDS, and RERMS, and the volumes used in the crosslinking reactions.

Stock solution			Volume used in crosslinking reaction			Proportion (molecule/molecule) 1 PEG/10 peptide
PEG	RGDS	RERMS	PEG	RGDS	RERMS	
20 mg/mL	5 mg/mL	5 mg/mL	100 µL	10 µL	10 µL	

3.3. Evaluating the crosslinking reaction

3.3.1. Western blot

Western blot (WB), also known as immunoblot, is one of the most common techniques for detection and analysis of proteins. This technique is extremely well established and involves several steps. Firstly, the sample has to be prepared, then the proteins in the sample are separated on an electrophoresis gel and finally transferred onto a nitrocellulose membrane.

Following transfer, membranes are incubated with antibodies, and then the complex of antibody:protein can be detected and analyzed with one of several detection methods.⁵⁹

In the present work electrophoresis gels used were 8% SDS-PAGE, a denaturing gel, which allows for separation of proteins solely on the basis of their molecular weight. It consists of two gels, a resolving (bottom) gel, with higher concentration of polyacrylamide to separate proteins, and a stacking (top) gel, with lower concentration of polyacrylamide.

Prior to loading the samples, a loading buffer is added, which consists of glycerol, SDS, a reducing agent (β -mercaptoethanol) and a dye (bromophenol blue). Glycerol increases the density of the samples and helps anchor the sample in the wells; SDS masks any inherent charge of the proteins; the reducing agent breaks any disulfide bonds, disrupting quaternary and tertiary protein structures; and the dye enables tracking the progression of the sample in the gel.⁵⁹

Electrical current is then applied to the gel, enabling the separation of proteins. After separation, the proteins are transferred electrophoretically onto a nitrocellulose membrane. This immobilized all proteins, that were initially loaded into the gel and separated, at their respective relative migration positions at the time point when the electric current of the gel run was stopped.⁵⁹

After this, membranes were stained with Ponceau S to confirm whether the proteins were transferred. This permits assessing if all the wells were equally loaded. Membranes are blocked with non-fat dry milk or BSA to block any non-specific binding sites of the primary antibody. After blocking, membranes are incubated with a specific primary antibody and the appropriate secondary antibody. Following incubation, proteins can be detected through chemiluminescence, a method that incorporates the ability of the horseradish peroxidase conjugated on the secondary antibody to catalyze the oxidation of luminol, resulting in the emission of light. This light signal can then be detected on X-ray film which can be analyzed using a densitometer.⁵⁹

In the present work, samples of crosslinking reactions were prepared taking into account the proportions/concentrations of table 1. The running 8% gel was prepared and allowed to polymerize for 45 minutes at room temperature. Subsequently, the stacking gel solution was prepared and loaded on the top of the resolving gel. A comb was inserted into the stacking gel and left to polymerize for 30 minutes at room temperature. Prior to loading, Loading Buffer (LB) 4x (form composition see appendix 1) was added to the samples (1/4 of the final volume), boiled for 10 minutes at 90°C and spun down.

Samples were gently loaded into the wells, as well as a molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad) and gels were run at 100 V for approximately 2 hours.

The samples were: the sodium bicarbonate buffer pH =8,5, PEG, RGDS, RERMS, PEG-RGDS, and PEG-RERMS.

25 ul of each sample were loaded and once the gel run was stopped no further steps were carried out. This was due to an unexpected problem, which made impossible to use this technique to identify and prove the crosslinking reaction. The problem will be discussed in section 4.

3.3.2. General considerations on infrared spectroscopy

Infrared spectroscopy

Spectroscopy comprises a technique based on the interaction between sample and electromagnetic radiation, in order to analyze chemical compounds including biomolecules such as proteins, nucleic acids, and metabolites. In fact spectroscopy contributes with detailed information concerning the structure and mechanism of action of such molecules.⁶⁰

Infrared radiation of electromagnetic spectrum is located in the invisible area of the spectrum, and its wave length ranges from 14 000 to 4 cm⁻¹ (Figure 12). These energy frequencies are responsible for molecular vibrations, energy frequencies higher than 14 000 cm⁻¹, comprising ultraviolet and visible light, cause electrical transitions, and energy frequencies lower than 4 cm⁻¹, microwaves, cause molecular rotation. Furthermore, infrared spectrum can be subdivided in three categories: Near-infrared region (NIR), wave length range between 14 000 and 4000 cm⁻¹; Mid-infrared region (MIR), wave length range between 4000 and 400 cm⁻¹; and Far-infrared region (FIR), wave length range between 400 and 4 cm⁻¹.⁶⁰

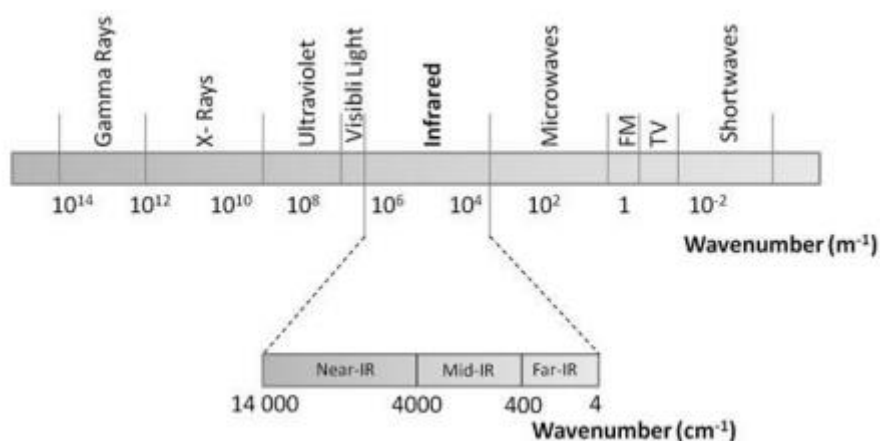


Figure 16. The electromagnetic spectrum, with IR region magnified.(taken from ⁶⁰)

Fourier transform infrared spectrometry

Fourier transform infrared spectrometry (FTIR) is a widely used technique for the large number of molecules that have strong absorbance in the MIR region. Its high versatility is due to the many types of samples it can analyze, including solids, liquids, gases, pastes, and gels. Furthermore, in order to obtain a spectrum only a very small sample is required.^{60,61}

In the infrared spectra obtained, peaks positions give information about the structure of the molecules in the sample, the intensity of peaks is related to the concentration of the molecules, and peak widths are sensitive to the chemical matrix of the sample, taking into account pH and hydrogen bonds.^{60,61}

The further advantage that comes from the use of FTIR, rather than other types of spectrometers, is the ability to acquire spectra with high signal-to-noise ratios (SNRs). This is due to some features of the spectrometer like throughput, multiplex and wavenumber precision. Throughput comes from the high-intensity of infrared beam that arrives at the detector, increasing the signal level. Multiplex is related to the possibility of co-adding scans, improving the SNR of a spectrum. Finally the high precision comes from the laser that acts as an internal wavenumber standard. All these features increase SNRs, which lead to an increase in the sensitivity of the instrument, and allows for quantitative accuracy, increasing its applications.^{60,61}

FTIR equipment

A light source, an optical system, a detector and a sample holder compose a spectrophotometer. The radiant energy is directed from the source to the sample, and the detector measures the intensity of the emergent beam.^{60,61}

FTIR spectrophotometers use an interferometer, the most common is the four arms Michelson interferometer (Figure 13). In this interferometer the top arm contains the infrared source and a collimating mirror that collects the light from the source and makes its rays parallel. The bottom arm contains a stationary mirror, the right arm contains a moving mirror, and the left arm contains the sample holder and the detector. At the center of the interferometer there is a beam splitter.^{60,61}

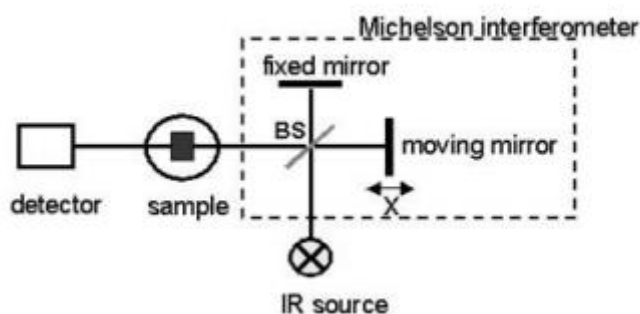


Figure 17. Schematic representation of a FTIR spectrometer.
The major compartments - BS:beamsplitter; X: relative position variation of moving mirror. (taken from ⁶⁰)

Functioning

At the source, IR energy is emitted from a glowing black-body source, this beam passes through an aperture which controls the energy presented to the sample and, ultimately, to the detector. The beam enters the interferometer and promotes the spectral encoding, this generates an interferogram signal with information of every IR frequency that comes from the source. The interferometer divides radiant beams, generates an optical path difference between beams and then recombines them in order to produce repetitive interferences signals measured as a function of optical path difference by the detector. At the sample holder, the beam is reflected off or transmitted through the sample surface. The specific energy frequencies characteristic of the sample are then absorbed. In the detector the last measurement of the interferogram occurs. Finally the signal is sent to the computer where the Fourier transform takes place, resulting in a final FTIR spectrum that can be interpreted and further manipulated.^{60,61}

Golden Gate™ Diamond ATR

Presently IR-measurements are mainly performed in Attenuated Total Reflection (ATR) mode because this technique is simpler and more versatile than the conventional transmission mode. The latter implies that IR beam cross the whole sample, thus being necessary to control the sample thickness and opacity. With the transmission mode it is possible to analyze liquid samples, but extremely difficult.⁶²⁻⁶⁴

ATR allows a quick analysis of solids, liquids, powders or pastes. In ATR the sample is in direct contact with a crystal. The IR beam passes through the crystal, within it is totally internally reflected beyond its surface into the sample. The sample, placed on the crystal, absorbs energy, so the beam will be attenuated or altered. This attenuated beam reaches the detector in the IR

spectrometer and the information is converted into spectra. The modification, absorption or attenuation, of the IR beam is proportional to the chemical concentration of the sample, making it possible to extract quantitative data from the sample.⁶²⁻⁶⁴

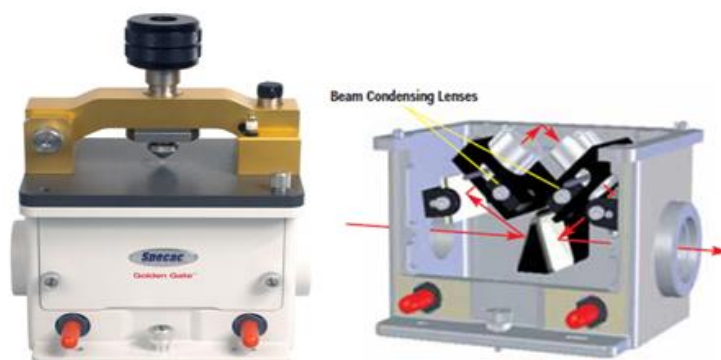


Figure 18. Golden Gate™ Diamond ATR.
(adapted from⁶⁵)

The Golden Gate™ Diamond ATR (Figure 14) is one of the most sensitive, robust and versatile infrared accessories designed for all the high performance FTIR spectrometers. The most advantageous features of this accessory are the diamond surface to place the sample, and its rugged construction. Being made of diamond it is very resistant to chemical attack and it allows for the analysis of corrosive liquids, and resists high loads applied against a solid sample. Furthermore, due to its resistance it can be cleaned quite aggressively, removing all the residues of the samples analyzed.^{63,64}

3.3.3. MIR spectral acquisition

In order to collect the spectra, PEG, RGDS (a control test peptide), and RERMS (APP derived peptide) were kept in ice until the moment of reaction, because of their high reactivity. 10 mg of PEG powder were dissolved in 1ml of sodium bicarbonate buffer pH=8,5, and then crosslinking reactions were prepared using the concentrations mentioned in Table 1.

Spectra were acquired immediately after the solution was prepared with a Perkin-Elmer Spectrum BX FTIR™ spectrometer in the range of 4000-900 cm^{-1} , at a resolution of 8 cm^{-1} with 64 co-added scans. During acquisition, room temperature and humidity were kept at $\pm 25^\circ\text{C}$ and $\pm 37\%$ respectively.

For spectra acquisition, by drying kinetics, it was performed the acquisition of background single beam against air (with the empty crystal). Then a drop of the PEG solution or the crosslinking

reactions was placed on the ATR crystal. The drying time was achieved and established after approximately 30 minutes, which corresponds to the acquisition of 16 consecutive spectra.

For the arithmetic subtraction of the buffer spectra it was necessary to acquire a spectrum of buffer, and of all crosslinking reactions.

At the end, a spectra of the air background was obtained, as well as spectra for the sodium bicarbonate buffer, PEG, and PEG crosslinking reactions with RGDS and RERMS.

3.4. Cell Plating

3.4.1. Cell counting and viability assay

In order to plate cells an initial confluency of approximately 30%, 10 000 cells per well of 24 well plates was used. To confirm, a dye exclusion assay was employed, in which living cells with an intact cytoplasmic membrane exclude the reagent, while dead cells stain blue due to dye incorporation through permeabilized damaged cytoplasmic membranes. To an aliquot (90 μ L) of cells suspension, 10 μ L of 0,4% Trypan Blue were added and incubated for 1 minute at room temperature. The unstained (viable) cells were counted in a hemacytometer, and cell concentration calculated for further cell plating.

3.4.2. Cell plating on treated coverslips

After coverslips were treated, they were placed in a well, 30 000 cells in 1 mL culture medium was added. Cell plating scheme is describe In table 2.

Table 2. Organization of the 24 well plates used in cell culture. These two plates were replicated for 48 hours, 7 days, and 7 days with 10 μ M of RA.

Glass	Glass	Glass	Glass	Glass	Glass	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS
Glass	Glass	Glass	Glass	Glass	Glass	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS	PEG-RGDS
PEG	PEG	PEG	PEG	PEG	PEG	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS
PEG	PEG	PEG	PEG	PEG	PEG	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS	PEG-RERMS

These conditions were performed at two different times. A time point at 48 hours in order to study the number of cells that adhere to the different surfaces treatments was chosen, and 7 days in order to study the morphology and neurite outgrowth in the different surfaces treatments. In the time of 7 days a set of plates were incubated with non-differentiating medium, and another set was incubated with medium containing 10 μ M of RA to permit cell differentiation.

In the 7 days assays, cell medium was replaced with new medium every 48 hours. In the 7 days assay, with retinoic acid, cells were plated for one day with medium without retinoic acid, corresponding to a -1 day, only at day 0 (zero) was medium replaced with new medium, containing retinoic acid at a concentration of 10 μ M. This medium was renewed every 48 hours until the time of 7 days was reached.

When the time of 48 hours or 7 days, depending the assay, was achieved, culture medium was removed, coverslips washed once with PBS pH=7,4 and then the Immunocytochemistry protocol was applied followed by sample fixation.

3.5. Immunocytochemistry

Immunocytochemistry consists of a technique whereby cells, are fixed in paraformaldehyde (PFA), in order to permit the visualization and subsequent determination of the location of a given protein or peptide by the use of specific antibodies.⁶⁷

This technique involves several and crucial steps in order to obtain good final preparations. First samples are prepared by fixing with a fixation agent, this agent helps to stabilize and preserve cells as close to life-like as possible by binding to reactive groups on proteins and lipids and holding them in the same position as if the cells were still alive. The second step is the permeabilization of the cells achieved by the addition of detergents, which can solubilize membranes without destroying the protein-protein interactions. After this cells are blocked, incubated with the chosen antibody and corresponding fluorescent secondary antibody and mounted in a media with anti-fading and anti-photobleaching properties. Finally the preparation can be visualized through a microscope, and images analyzed.⁶⁷

In the present work, cell culture was performed as described in the previous section in 24-well plates (Corning) with glass coverslips of 15mm diameter. Cells were then washed with PBS 1x

pH=7,4 and 300 μ L of 4% PFA was added for 30 minutes. Following fixation, cells were washed three times with PBS 1x pH=7,4 and 300 μ L of 0.2% Triton-X was added for 10 minutes. Cells were then incubated for 2 hours with the specific antibodies, as indicated in Table 3, followed by three washes with PBS 1x pH=7,4 and incubation for 1 hour with the appropriate fluorescent antibodies (Table 4). After this cells were washed five times with PBS 1x pH=7,4 in order to remove all non-ligand antibody. Samples were then mounted onto a microscope slide with VECTASHIELD® Mounting Media with DAPI (Vector Laboratories) and visualized using a LSM510-Meta confocal microscope (Zeiss) and a 63x/1.4 oil immersion objective. The argon laser lines of 405 nm, 488 nm, and a 561 nm DPSS laser were used. Profiles were acquired using the Zeiss LSM 510 4.0 software.

Table 3. Primary antibodies used in immunocytochemistry.

Antibody	Antibody type	Target	Dilution
Anti-FAK (34Q36) (Thermo scientific)	Mouse, monoclonal	Focal adhesion kinase	1:200
Anti-FAK [pY397] ABfinity™ (Invitrogen™)	Rabbit, monoclonal	Focal adhesion kinase pSer397	1:300

Table 4. Secondary antibodies used in immunocytochemistry.

Antibody	Excitation maximum (nm)	Emission maximum (nm)	Observed colour	Dilution
Alexa Fluor 488 goat anti-mouse IgG (Invitrogen)	495	519	Green	1:300
Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)	495	519	Green	1:300
Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen)	590	617	Red	1:300
Alexa Fluor 568 Phalloidin (life technologies)	578	600	Red	1:500

Each condition described in section 2.3.2 contained 9 coverslips, for each set:

- 3 were incubated with DAPI, Phalloidin, and anti-FAK antibody;
- 3 were incubated with DAPI, Phalloidin, and anti-FAK pY397 antibody;
- 3 were incubated with DAPI, anti-FAK antibody, and anti-FAK pY397 antibody.

3.6. Data collection and analysis

In order to collect data from cell staining, 5 fields from each coverslip were photographed. To analyze the data for cell adhesion studies, the ImageJ program from Fiji was used to count cell number per coverslip, and then the GraphPad Prism program was used to carry out statistical analysis with a P value of $<0,05$. Data are presented as mean \pm SEM (standard error of the mean). Statistical significance analysis was conducted by one way analysis of variance (ANOVA) followed by the Tukey's test. To analyze data from cellular morphology a qualitative analysis was also carried out.

4. Results and Discussion

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4.1. Crosslinking Reaction evaluation

4.1.1. *Western blot*

In order to evaluate if crosslinking reaction, between PEG and peptides, the western blot technique was performed, as described in section 3.2.1. Molecular weight of PEG used was 20 kD, and the molecular weight of peptides was about 0,5 kD. Taking into consideration that only proteins can be identified in this technique, and that the marker used ranges only from 250 kD to 37 kD, bands would only be observed if the reaction between PEG and peptides had occurred, once PEG is a polymer and the peptides are too small to alone 'be caught' in the gel.

After about 30 min, when the samples entered the running gel, it was observed that the running front was not linear, and it seemed that some wells were destroyed/deformed.

When running was finally stopped, it was evident that the gel wells were deformed, and that the sample didn't run in a straight line downwards, loading buffer was found throughout the running gel. The most deformed wells were those loaded with samples containing PEG. Due to this result, no further steps were executed in the western blot, and no data was collected from this technique.

This technique was attempted twice, and in both assays the same occurred, all wells containing PEG were totally deformed, and the running buffer was dispersed throughout the running gel. The only explanation plausible for this event is that 4-Arm PEG-SCM have quite big arms, and a considerable molecular weight. PEG's structure is not altered with the loading buffer, in other words, it can't be denatured like proteins. Because of this, PEG's long arms do not easily pass through running gel pores, and because the electrical pulse pulls samples down the gel, the gel pores start to breakdown. Because of this, western blot analysis was excluded as a technique to evaluate the crosslinking reaction between PEG and peptides.

4.1.2. FTIR

As an alternative the FTIR technique described in section 3.2.2.2 was used. Although the methodology is simple, handling and interpretation of data is very complex and requires a great deal of experience to perform a detailed and correct analysis.

Samples were prepared, and the data handling and interpretations was carried out with the support of Prof. Alexandra Nunes, who has great expertise in this area.

In order for PEG to react with the peptide, it has to lose the NHS-ester group to the NH-terminal group of the peptide covalently bound to PEG. The peak of spectra found in the literature and tables of wavelengths that correspond to the loss of NHS-ester bonds, is around 1650 cm^{-1} .

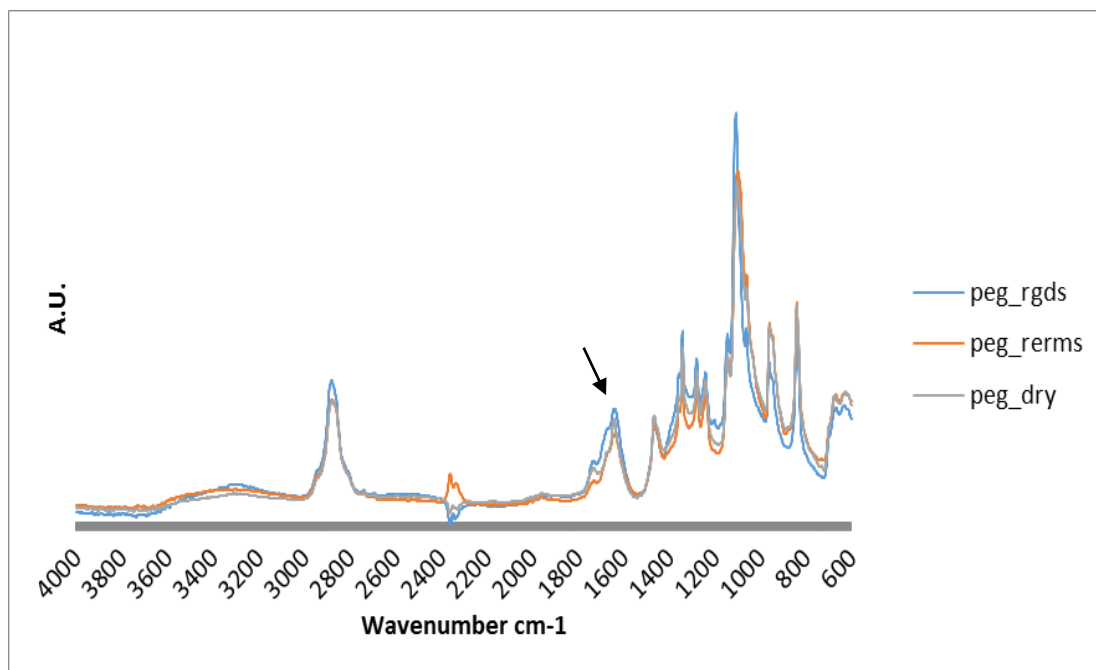


Figure 19. Loss of NHS-ester bonds general spectra.
The black arrow points the peak corresponding to the NHS-ester bond. (Resolution 8 cm^{-1})

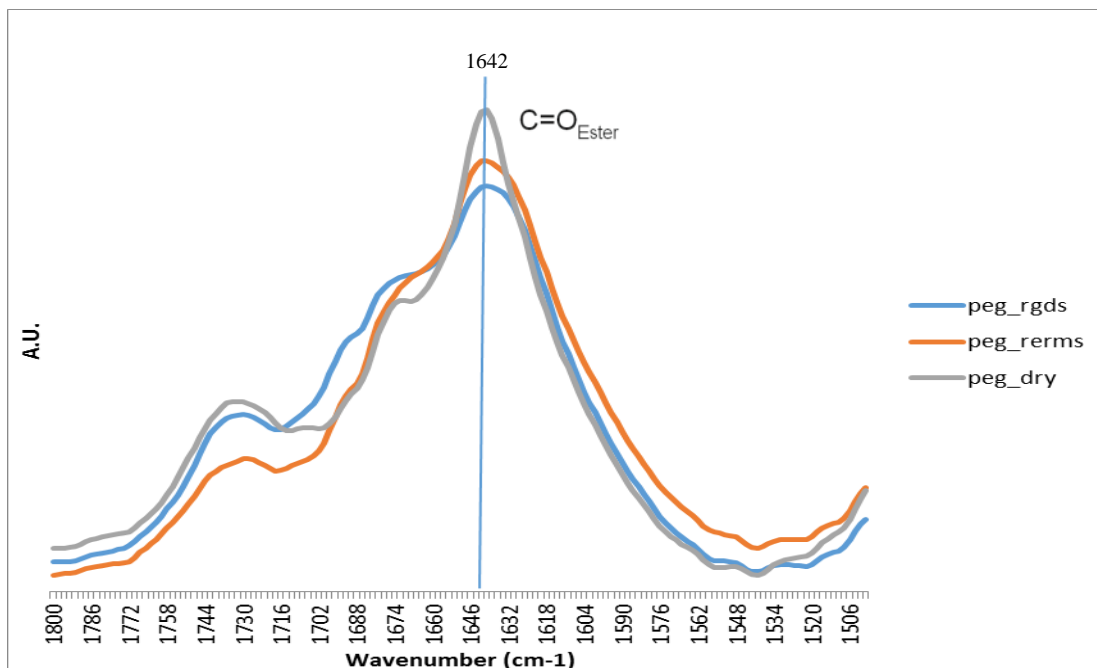


Figure 20. Loss of NHS-ester bonds detailed spectra. (Resolution 8 cm⁻¹)

As a first examination of data in Figure 19, all spectra looked generally the same, showing no apparent relevant differences. But after a thorough examination of the data, alterations between spectra in the peak of 1642 cm⁻¹ were found (Figures 19 and 20).

Being that the resolution of the FTIR spectrometer 8 cm⁻¹, the peak of 1642 cm⁻¹ can be related to the peak of 1650 cm⁻¹, described in the literature, the latter corresponds to the loss of NHS-ester bonds.⁶⁸

This result suggest that NHS-ester bond at the end of each PEG arm has been loss, leaving space for the N-terminal of the peptides to form covalent bonds with PEG arms.

4.2. Effect of the APP RERMS enriched matrix on SH-SY5Y cell adhesion

This experiment was performed in order to evaluate the effect of PEG, and the effect of the peptides RGDS, as a positive control, and RERMS as the peptide of study. Peptides were covalent linked to PEG by a crosslinking reaction that was validated as described above By carrying this

crosslinking reaction, peptides can be immobilized in a matrix that serves as substrate for SH-SY5Y to grow on.

After 48 hours of culture, cells were fixed and immunocytochemistry was performed in order to count cell number. The results obtained are shown below (Figure 21):

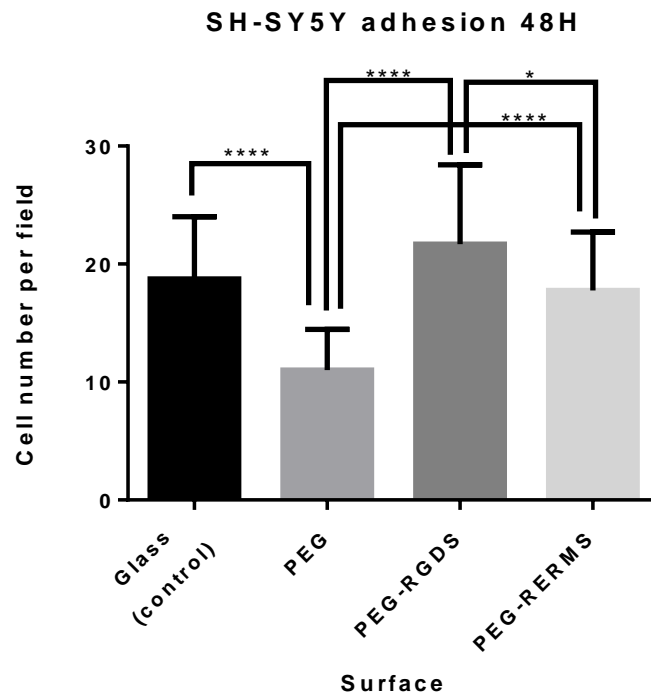


Figure 21. Cell adhesion to different substrates. **** p -value $< 0,0001$; * p -value $< 0,05$. $n=3$

From data analysis it can be observed that PEG coated coverslips reduced significantly cell adhesion, in comparison to glass control coverslips. Further, in glass coverslips coated with PEG-RGDS crosslinker (positive control) the adhesion was significantly higher than PEG coated coverslips, and significantly higher than PEG-RERMS coated coverslips (Figure 21). PEG-RERMS coated coverslips showed an increase in cell adhesion compared to PEG coated coverslips.

In the absence of matrix interactions, cells undergo an apoptotic process. Indeed RGDS has been well described to increase cell adhesion, viability, survival and proliferation. One of the most important extracellular proteins that is responsible for adhesion between cells, but also between cells and extracellular matrix (ECM) is fibronectin.³²

It follows that for the work here described, RGDS was chosen to be the positive control because it has been widely studied and used in cellular adhesion and proliferation.^{32-46,69} The RGDS sequence present in fibronectin is recognized by integrins and mediates cellular adhesion.

Integrins are transmembrane cell surface receptors. These heterodimeric proteins are composed of an α -subunit and a β -subunit.^{32, 36,37} The α - and β -subunits can be combined and form at least 24 unique heterodimers. Some integrins can bind to more than one ECM protein, and some ECM proteins can interact with several different integrin heterodimers.^{32,70,71}

Ligand specificity is provided by the active site pocket located between the α - and the β -subunits, where ECM proteins bind.^{32,36,37}

The three amino acid sequence arginine-glycine-aspartic acid (RGD), present on the 10th type III repeat of Fn, is the major cell binding site for many integrins. Integrins that engage with RGD peptides include α IIb β 3, α V β 3, α V β 6, α V β 1, α 5 β 1, and α 8 β 1.^{32,36,37}

RGDS in fact has been widely used to enable and promote cell adhesion by recognizing activated integrins which, bind to RGD sequence (Figure 22) and initiate an intracellular response, via talin, thus forming an important adhesion complex.^{32,36,37,72}

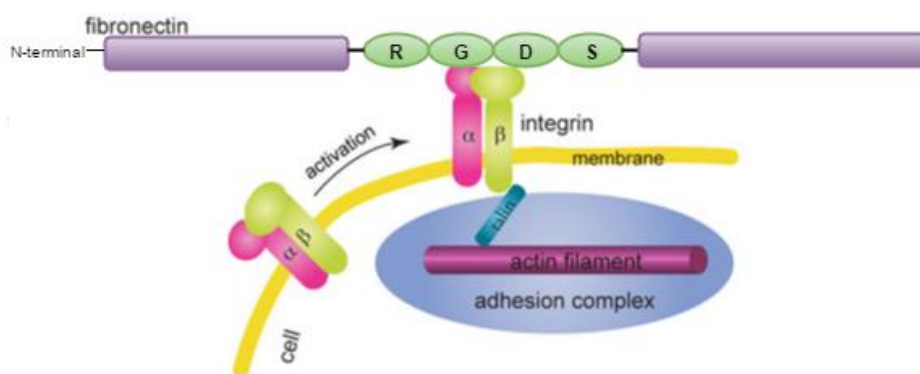


Figure 22. Adhesion complex. Showing RGDS tethering to the integrin heterodimer, and further formation of the adhesion complex with talin and actin filaments. (Adapted from ⁷³)

The model presented in Figure 22 is consistent with the data obtained. It also eliminates a doubt left upon analyzing the FTIR spectra (Figures 19 and 20) that is, if the N-terminal of the peptides, was effectively bond to PEG arms terminal.

Treated coverslips with crosslinking reactions were N₂ air dried after its final cleanings before cell plating, leaving no liquids residues at the coverslips surface. Thus, if cells are binding significantly more in PEG-RGDS and PEG-RERMS treated surfaces than in glass or PEG treated coverslips, it

means that all the peptides (RGDS or RERMS) promoting cell adhesion had to be covalently bond to PEG, which by itself had to be covalently bond to the amino-silanized glass coverslips.

4.3. Effect of the APP RERMS enriched matrix on SH-SY5Y cells morphology and neurite outgrowth

In order to study the effect of RERMS on cell morphology and neurite outgrowth, 7 days differentiated cells were chosen for ICC. Cells incubated with Phalloidin (red), pFAK sY397 (green), and mounted with VECTASHIELD® Mounting Media with DAPI (blue) will be presented in this section.

During the ICC data collection it was noticed that in coverslips incubated with FAK and pFAK sY397 antibodies FAK was dispersed all over the cells, and at the nucleus and membrane there were small round-like agglomerates of FAK. It was also noticed that on this coverslips wasn't no sign of pFAK sY397 antibody.

When pFAK antibody was incubated in coverslips without FAK antibody, the same round-like agglomerates appeared marked by pFAK sY395. This data let us to the conclusion that FAK antibody was detecting all phosphorylated and non-phosphorylated FAK, and that the most pertinent data to analyze would be from coverslips marked only with pFAK sY397 (figure 23).

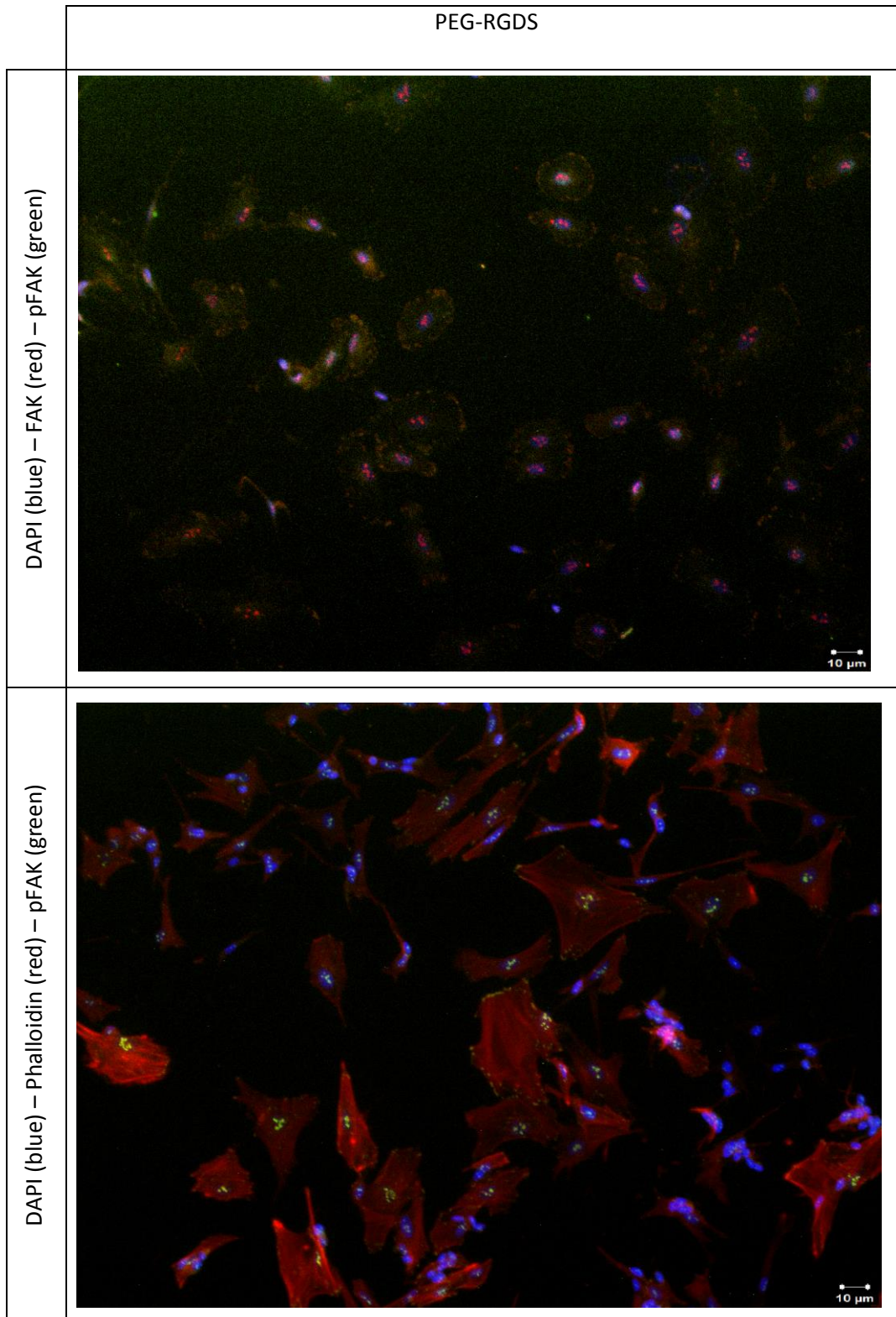


Figure 23. Immunocytochemistry of SH-SY5Y cells. Upper –cells stained with anti-FAK antibody (red) and anti-pFAK sY397 antibody (green), mounted with VECTASHIELD® Mounting Media with DAPI (blue). Lower – Cells stained with phalloidin (red) and anti-pFAK antibody (green) and mounted with VECTASHIELD® Mounting Media with DAPI (blue).

4.3.1. RGDS and RERMS affect general morphology

Close observation of all images revealed several differences, most evident was the appearance of stress fibres (phalloidin red staining), which is recognizing actin filaments. Figure 24, column on the right, clearly shows an increase in these stress fibres for both PEG-RGDS and PEG-RERMS. Glass and PEG coated coverslips had actin filaments but less well defined than coverslips coated with PEG-RGDS or PEG-RERMS. It is also noteworthy that the nuclei are far more evident in the plasma membrane focal plane for coverslips coated with PEG-RGDS in particular. It is reasonable to deduce that this is a consequence of a flatter cell, which results from the increase in stress fibres. To validate this finding quantitative studies need to be pursued in future.

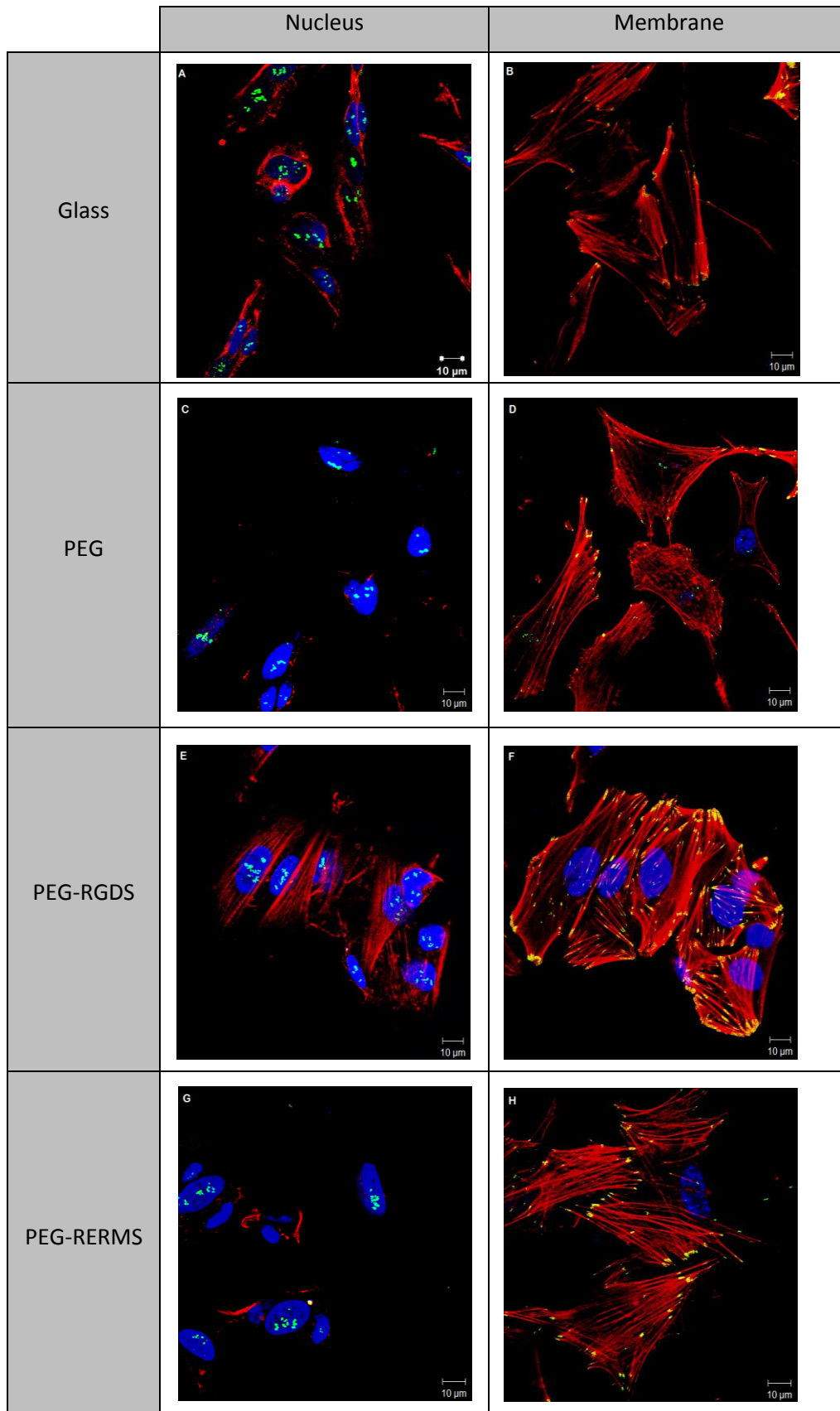


Figure 24 SH-SY5Y morphology on different substrates.
 Immunocytochemistry: SH-SY5Y cells stained with Phalloidin (red), anti-pFAK sY397 antibody (green), and mounted with VECTASHIELD® Mounting Media with DAPI (blue).

4.4. RGDS and RERMS promote increases in focal adhesion phosphoFAK

The antibody pFAK sY397 stained the plasma membrane in a pattern consistent with focal adhesions. This staining pattern was significantly more abundant in surfaces coated with PEG-RGDS and PEG-RERMS (Figure 23, column on the right). On PEG only coated coverslips there was much less pFAK sY397 at the end of the f-actin filaments (red staining) on the cell membrane than in coverslips coated with PEG-RGDS and PEG-RERMS respectively. Cover slips coated with PEG-RGDS showed the most pFAK sY397 at specific plasma membrane points, and all of this pFAK was co-localized at the tip of actin filaments.

In fact FAK has been described to play an important role in cell adhesion and proliferation, its major phosphorylation site is at serine 397, and it is more phosphorylated in adherent cells that have $\alpha 1\beta 5$ integrins.⁷⁴ The interaction between RGDS present in the ECM and the integrin heterodimer $\alpha 5\beta 1$ has been described to induce FAK phosphorylation or to induce the formation of phosphorylated complexes.⁷⁵⁻⁷⁹

A process that has already been described to explain FAK presence at the tip of f-actin is the following: initial clusters of integrin are activated by talin which binds to integrins. This recruits FAK, Src family kinases (SFKs) and paxillin, which leads to initial polymerization of actin forming new filaments of actin. These filaments tether to talin, and the cluster starts to get pushed away and then pulled closer by myosin contractions. This forces and causes cycles of transient talin stretching and vinculin binding until talin-actin bond become stable.⁷⁵⁻⁷⁹

This mechanism has been used to explain the relationship between RGD sequence, integrins, FAK and f-actin. On PEG-RERMS coated coverslips there was also observed an increase of pFAK sY397 at the tip of actin filaments, and these filaments were more prominent than those in glass and PEG coated coverslips. Although this effect was observed to a lesser degree than PEG-RGDS coated coverslips, the morphology of cells on PEG-RERMS coated coverslips was very similar to those in PEG-RGDS coated coverslips.

Thus the underlying mechanisms in response to RERMS are likely to be similar to those initiated by the RGDS sequence. Taking this into consideration, and the results here presented, it is possible to deduce that RERMS sequence present at the ECM (due to APP) can be recognized by the same integrins that recognize RGDS sequence. Further, these integrins can potentially tether

to RERMS, initiating the same signals as seen with RGDS, parallels are likely given that in both instances pFAK sY397 is present at the tip of the actin filaments.

4.5. RGDS and RERMS increase nuclear staining

Surfaces coated with PEG-RGDS and PEG-RERMS also showed a greater speckled staining in the nucleus for pFAK sY397 (Figure 24, column on the left). The increase in green nuclear specks reflects that more pFAK is being localized; the staining showed a tendency to form well-defined rounded agglomerates. Although further experimentation is necessary these structures are consistent with transcription factories. The fact that these increase with the presence of RERMS and more so with RGDS supports that these peptides have the capacity to induce cellular physiological responses not only at the site where they are localized but can also influence the expression of other genes.

Taken together coverslips coated with PEG-REMRS were identical to those coated with PEG-RGDS, but the “concentration” of pFAK sY379 at the membrane, in the f-actin tips, and in nucleus was marginally lower but markedly higher when compared to glass or in particular PEG alone. This places the RERMS peptide of APP as a key in mediating cellular signal and this area of research should be further explored in the future.

5. Conclusion

5. Conclusion

This thesis aimed to evaluate whether RERMS sequence could induce cellular responses, in particular cell adhesion and neurite outgrowth of neuronal cells. RERMS sequence of APP has been described over the last few years to play role in cell adhesion and neurite outgrowth in neurons, although the mechanism have yet to be elucidated.

Results from cell adhesion assay revealed a significant increase in cell adhesion for the PEG-RERMS treated coverslips. A plausible hypothesis is that RERMS is recognized by integrins, as is RGDS, and when they are tethered, integrins initiate signaling transduction that leads to a stable focal adhesion.

Immunocytochemistry here performed revealed differences at the actin filaments and stress fibres, and at the localization of phosphorylated FAK at tyrosine 397. It was evident that on coverslips treated with PEG-RGDS pFAK sY397 was much more co-localized at the tip of actin filaments near the cell membrane, than glass and PEG coated coverslips. PEG-RERMS coated coverslips revealed that pFAK sY397 likewise co-localized at the tip of actin filaments more than in glass and PEG coated coverslips, but less so than PEG-RGDS coverslips. Actin filaments were much longer and pronounced in PEG-RGDS and PEG-RERMS.

At the nucleus there was evidence of round-like agglomerates of pFAK sY397 in PEG-RGDS and PEG-RERMS coated coverslips, more than in glass and PEG coated coverslips. PEG coverslips were the ones with lowest pFAK sY397 at the nucleus, and at the membrane. This pFAK sY397 at the nucleus can be located to possible transcription factories, where pFAK could be mediating the transcription of genes that encode adhesion proteins necessary for the adhesion and cell proliferation.

Taking all these results, and the hypotheses that exist at this moment, one can propose a role for RERMS in cell adhesion on neurite outgrowth (Figure 25).

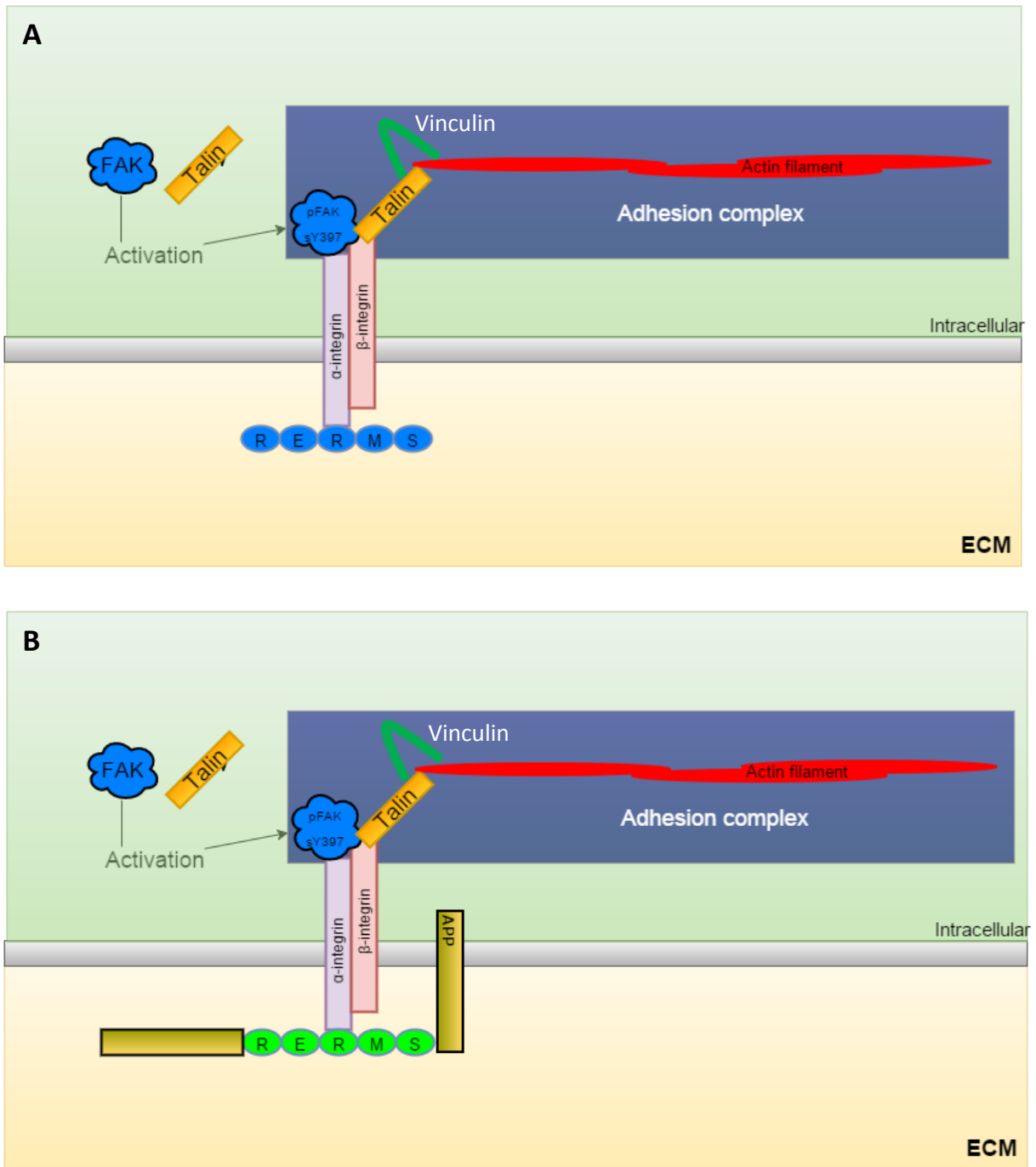


Figure 25. Functional model of RERMS domain in APP in focal adhesions.

Panel A represents the experimental model showing RERMS sequence as a matrix substrate for adhesion. Panel B represents APP-RERMS sequence exposed and recognized by integrins. When RERMS sequence tether to α-integrin it recruits talin to β-integrin. FAK is activated by auto-phosphorylation at tyrosine 397. Actin filaments start to polymerize and bond to talin, leading to formation of the adhesion complex. Vinculin binds to talin and actin filaments until their bond becomes stable.

To summarize, the top panel represents the experimental model of PEG-RERMS and the bottom panel, APP-RERMS as part of a protein complex in the plasma membrane. The complex has the potential to be particularly relevant in focal adhesions, the ECM, synaptic architecture

and ultimately synaptic transmission. The RERMS domain of APP has the capacity to bind to α -integrin with the same complex or to a similar complex in a nearby cell. Thus this work provides important preliminary evidence with respect to APP and its function in adhesion and cell to cell contacts.

In closing, the work in this thesis has provided novel research avenues with respect to pursuing APP function, its association in focal adhesion and potentially a role in mediating the synaptic signal. Abnormal synaptic transmission is one of the first hallmarks in AD preceding other anomalies such as deposition of protein aggregates in the brain.

6. References

6. References

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7.Appendix

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Cell Culture Solutions

▪ PBS (1x)

For a final volume of 500 mL, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionized H₂O. Final composition:

- 8 mM Sodium Phosphate
- 2 mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10 mM Potassium Chloride

Sterilize by filtering through a 0.2 µm filter and store at 4°C.

▪ 10% FBS MEM:F12 (1:1)

- 4.805 g MEM
- 5.315 g F12
- 1.5 g NaHCO₃
- 0.055 g Sodium Pyruvate
- 10 mL Streptomycin/Penicillin/Amphotericin solution
- 100 mL 10% FBS
- 2.5 mL L-glutamine (200 mM stock solution)

Dissolve in deionized H₂O. Adjust the pH to 7.2-7.3. Adjust the volume to 1000 mL with deionized H₂O.

For other combinations of FBS, replace 100 mL FBS with 30 mL (3% FBS MEM:F12), 10 mL (1% FBS MEM:F12) or remove FBS (serum-free MEM:F12).

Western Blot Solutions

▪ LGB (lower gel buffer) (4x)

To 900 mL of deionized H₂O add:

- 181.65 g of Tris
- 4 g of SDS

Mix until the solutes have dissolved. Adjust pH to 8.9 and adjust the volume to 1 L with deionized H₂O.

▪ UGB (Upper gel buffer) (4x)

To 900 mL of deionized H₂O add:

- 75.69 g of Tris

Mix until the solute has dissolved. Adjust the pH to 6.8 and adjust the volume to 1 L with deionized H₂O.

▪ **10% APS (ammonium persulfate)**

In 10 mL of deionized H₂O dissolve 1 g of APS. Note: prepare fresh before use.

▪ **10% SDS (sodium dodecylsulfate)**

In 10 mL of deionized H₂O dissolve 1 g of SDS.

▪ **Loading Gel Buffer (4x)**

- 2.5 mL 1M Tris solution (pH 6.8) 2.5 mL (250 mM)
- 0.8 g SDS (8%)
- 4 mL Glycerol (40%)
- 2 mL β-mercaptoethanol (2%)
- 1 mg Bromofenol blue (0.01%)

Adjust the volume to 10 mL with deionized H₂O. Store in darkness at room temperature.

▪ **1 M Tris (pH 6.8) solution**

To 150 mL of deionized H₂O add:

- 30.3 g Tris base

Adjust the pH to 6.8 and adjust the final volume to 250 mL with deionized H₂O.

▪ **10x Running Buffer**

- 30.3 g Tris (250 mM)
- 144.2 g Glycine (2.5 M)
- 10 g SDS (1%)

Dissolve in deionized H₂O, adjust the pH to 8.3 and adjust the volume to 1 L.

▪ **Stacking and resolving gel**

Type of Gel	Stacking	Resolving
Polyacrylamide percentage	6%	8 %
H₂O	2,75 mL	5,5 mL
Acrylamide stock mixture	0,75 mL	2,0 mL
UGB (5x)	1,25 mL	n/a
LGB (4x)	n/a	2,5 mL
10% SDS	50 µL	100 mL
10% APS	100 µL	100 µL
TEMED	4 µL	5 µL

Immunofluorescence Solutions

- **Blocking solution**

To 10 mL of PBS 1x add 0.3 g of Bovine Serum Albumine (BSA).

- **4% Paraformaldehyde**

For a final volume of 100 mL, add 4 g of paraformaldehyde to 25 mL deionized H₂O. Dissolve by heating the mixture at 58°C while stirring. Add 1-2 drops of 1 M NaOH to clarify the solution and filter through a 0.2 µm filter. Add 50 mL of PBS 2x and adjust the volume to 100 mL with deionized H₂O.

Coverslips cleaning and amino-silanization solutions:

- **Piranha etching:**

To a final volume of 600 mL add 450 mL of H₂SO₄ (pure) and 150 mL of H₂O² (30%) Having a proportion of 3 by 1 of H₂SO₄ and H₂O₂.

- **Amino-silanization solution:**

Pour 100 mL of methanol (pure) into a flask. Add 5 mL of acetic acid (pure), and 3 ml of APTES. Gently mix the solution.