

ELICITING IMMUNE RESPONSE BY USING NANOSTRUCTURES

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By
GÖKHAN GÜNAY

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By Gökhan Günay,

June, 2017

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Ayşe Begüm Tekinay (Advisor)

Michelle Marie Adams

Reyhan Neslihan Gürsoy

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan

Director of the Graduate School of Engineering and Science

ABSTRACT

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Gökhan GÜNAY

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Advisor: Ayşe Begüm Tekinay, PhD

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The ability of dendritic cells to coordinate innate and adaptive immune responses makes them essential targets for vaccination strategies. Presentation of specific antigens by dendritic cells is required for the activation of the immune system against many pathogens and cancer, and nanoscale materials can be functionalized for active targeting of dendritic cells. In this work, we integrated an immunogenic, carbohydrate melanoma-associated antigen-mimetic GM3-lactone molecule into mannosylated peptide amphiphile nanofibers to target dendritic cells through DC-SIGN receptor. Based on morphological and functional analyses, when dendritic cells were treated with peptide nanofiber carriers, they showed significant increase in antigen internalization and a corresponding increase in the surface expression of the activation and maturation markers CD86, CD83 and HLA-DR, in addition to exhibiting a general morphology consistent with dendritic cell maturation. These results indicate that mannosylated peptide amphiphile nanofiber carriers are promising candidates to target dendritic cells for antigen delivery. Overall these structures are proven to be effective in terms of dendritic cell activation and maturation and hold high potential to be used with a variety of antigens for different immunotherapy purposes.

KEYWORDS: Cancer immunotherapy, cancer antigens, dendritic cells, carbohydrate melanoma associated antigen, peptide amphiphiles



ÖZET

NANOYAPILAR KULLANILARAK İMMÜN YANIT TETİKLENMESİ

Gökhan GÜNAY

Nörobilim, Yüksek Lisans

Tez Danışmanı: Ayşe Begüm Tekinay

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Dendritik hücrelerin doğuştan gelen ve adaptif bağışıklık tepkilerini koordine etme kabiliyeti onları aşı stratejileri için önemli hedefler haline getirmektedir. Dendritik hücrelerin spesifik antijenler sunması, birçok patojen ve kansere karşı bağışıklık sisteminin aktivasyonu için gereklidir. Nano ölçekli materyaller, dendritik hücrelerin aktif hedeflenmesi için işlevselleştirilebilir. Bu çalışmada, DC-SIGN reseptörü vasıtasıyla dendritik hücreleri hedeflemek için immünojenik bir karbonhidrat olan melanoma ile ilişkili antijen-taklidi GM3-lakton molekülünü mannozile peptid amfifil nanofiberlere integre edilmiştir. Dendritik hücreler peptid nanofiber taşıyıcılarla muamele edildiğinde, antijenin hücre içine alımında önemli anlamlı bir artış gözlenmiştir ve aktiflik ve olgunlaşma belirteçleri olan CD86, CD83 ve HLA-DR'nin yüzey ekspresyonunda da buna eşdeğer bir artış görülmüştür. Ayrıca dendritik hücre olgunlaşması ile tutarlı genel bir hücre morfolojisi de gözlenmiştir. Bu sonuçlar, mannosillenmiş peptid amfifil nanofiber taşıyıcılarının, antijen sunumu için dendritik hücreleri hedeflemeye yönelik umut vaadeden adayları olduğunu göstermektedir. Genel olarak, bu yapıların dendritik hücre aktivasyonu ve

olgunlaşması açısından etkili olduğu kanıtlanmıştır ve farklı immünoterapi amaçları için çeşitli antijenler ile kullanılmak için yüksek bir potansiyel bulundurmaktadır

ANAHTAR SÖZCÜKLER: Kanser immünoterapisi, kanser antijenleri, dendritik hücreler, karbonhidrat melanoma ile ilişkili antijen, peptit amfifiller



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Abbreviations

ANOVA	Analysis of variance
CD	Circular dichroism
DCM	Dichloromethane
DIEA	<i>N,N</i> -diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	<i>N,N</i> -Dimethylformamide
ECM	Extracellular matrix
FBS	Fetal bovine serum
Fmoc	9-Fluorenylmethoxycarbonyl
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazole-1-yl) uronium hexafluorophosphate
HPLC	High pressure liquid chromatography
LC-MS	Liquid chromatography-mass spectroscopy
PA	Peptide amphiphile
PBS	Phosphate buffered saline
qRT-PCR	Quantitative real-time polymerase chain reaction
SEM	Scanning electron microscopy
TCP	Tissue culture plate
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TIS	Triisopropyl silane
UV	Ultraviolet

Chapter 1

1. Introduction

1.1. Nanomaterials for Biomedical Applications

Nanotechnology is an area which investigates nanomaterials and develops new functional materials for various applications. Improvements in nanotechnology have enabled the control of physical, chemical and biological properties of nanomaterials, eventually leading to the production of improved functionality for nanomaterials. Design and usage of nanomaterials provide important opportunities for biomedical applications, especially for the diagnosis and treatment of several disease conditions. Understanding the properties of natural materials at the nanoscale can facilitate the design of such materials and their usage in various industries such as medicine biotechnology, information technologies, textile etc. [1, 2]. These applications can vary from; construction of scaffolds for cellular studies and drug delivery, to growth factor vehicles, imaging or biosensors etc (Figure 1). Synthesized nanomaterials should carry out their biological functions but should not change the biological activity of unrelated molecules; they have to preserve their physical features upon chemical modification and they have to be nontoxic [3].

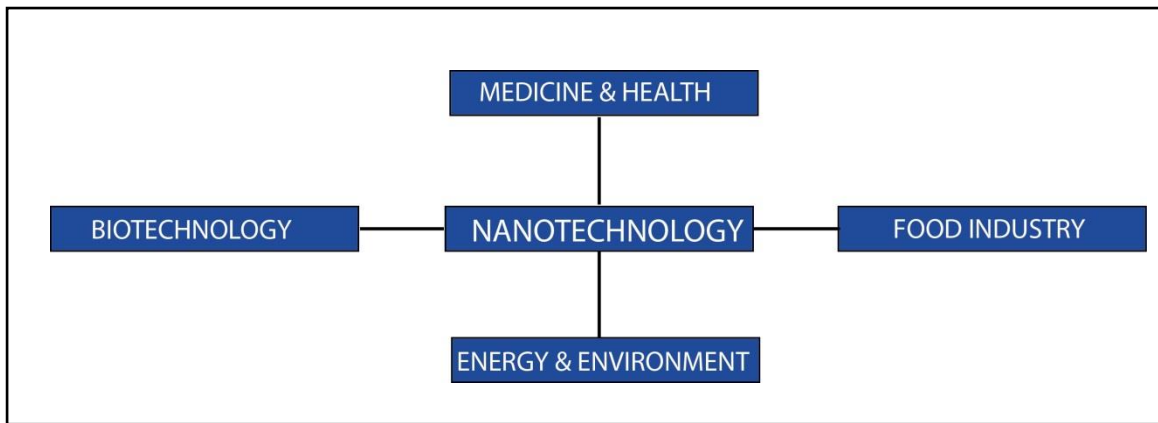


Figure 1 Utility of nanotechnology in various areas.

Nanoscale indicates a scale between 0.1 and 100 nm and there are 2 main techniques to synthesize nanoscale materials; top-down and bottom-up approaches (Figure 2) [4]. In top-down method, bulk materials are used for the production of nanoscale materials. This technique enables the production of nanomaterials with specific size and shape. Lithography techniques enable the tailoring of bulk materials into nanostructures [5]. On the other hand, bottom-up approach is based on obtaining nanoscale materials by using small molecules as building blocks through supramolecular interactions [2]. Even though there are certain drawbacks and benefits for both synthesis techniques, the advancements in technology can enable to overcome the current weaknesses.

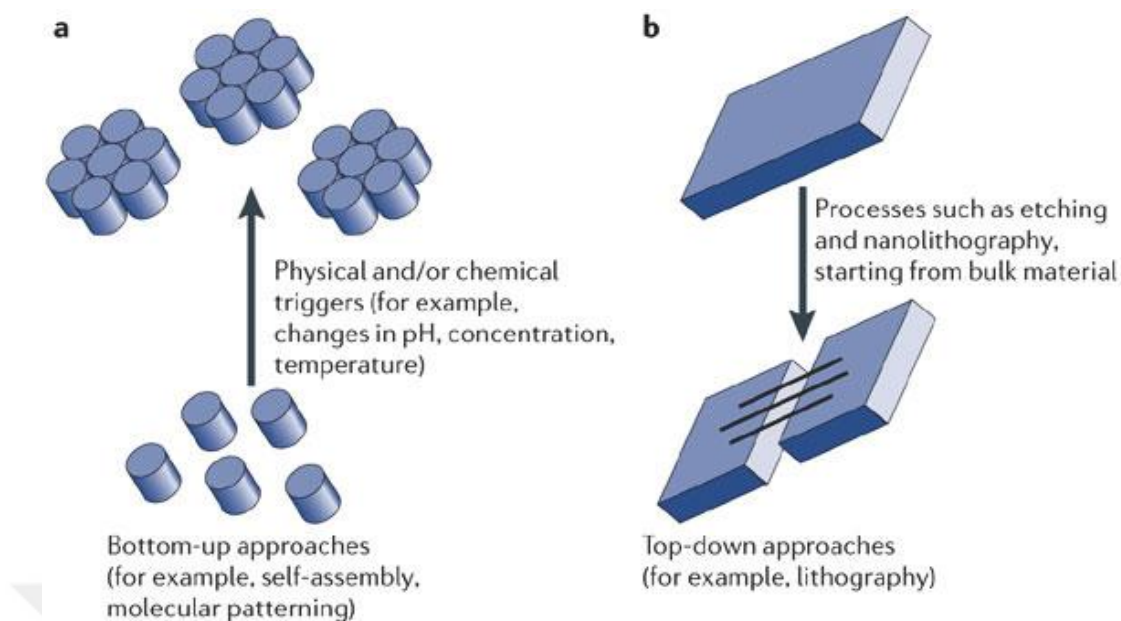


Figure 2 Bottom-up and top-down approaches. (Adapted from Ref. 4 with permission from Nature Publishing Group)

Recently there has been much advancement for the usage of nanotechnology for the cellular studies. Signals obtained from extracellular matrix, soluble signals or molecules participating in cell-cell interactions can be utilized through nanotechnology and enable to direct cellular differentiation, self renewal, migration etc. [6] (Figure 3). Stem cells hold high potential for the treatment of a variety of diseases such as degenerative diseases, aging etc. Therefore, nanomaterials conjugated with bioactive epitopes provide directed cellular behaviors, which then can be translated into the medicine.

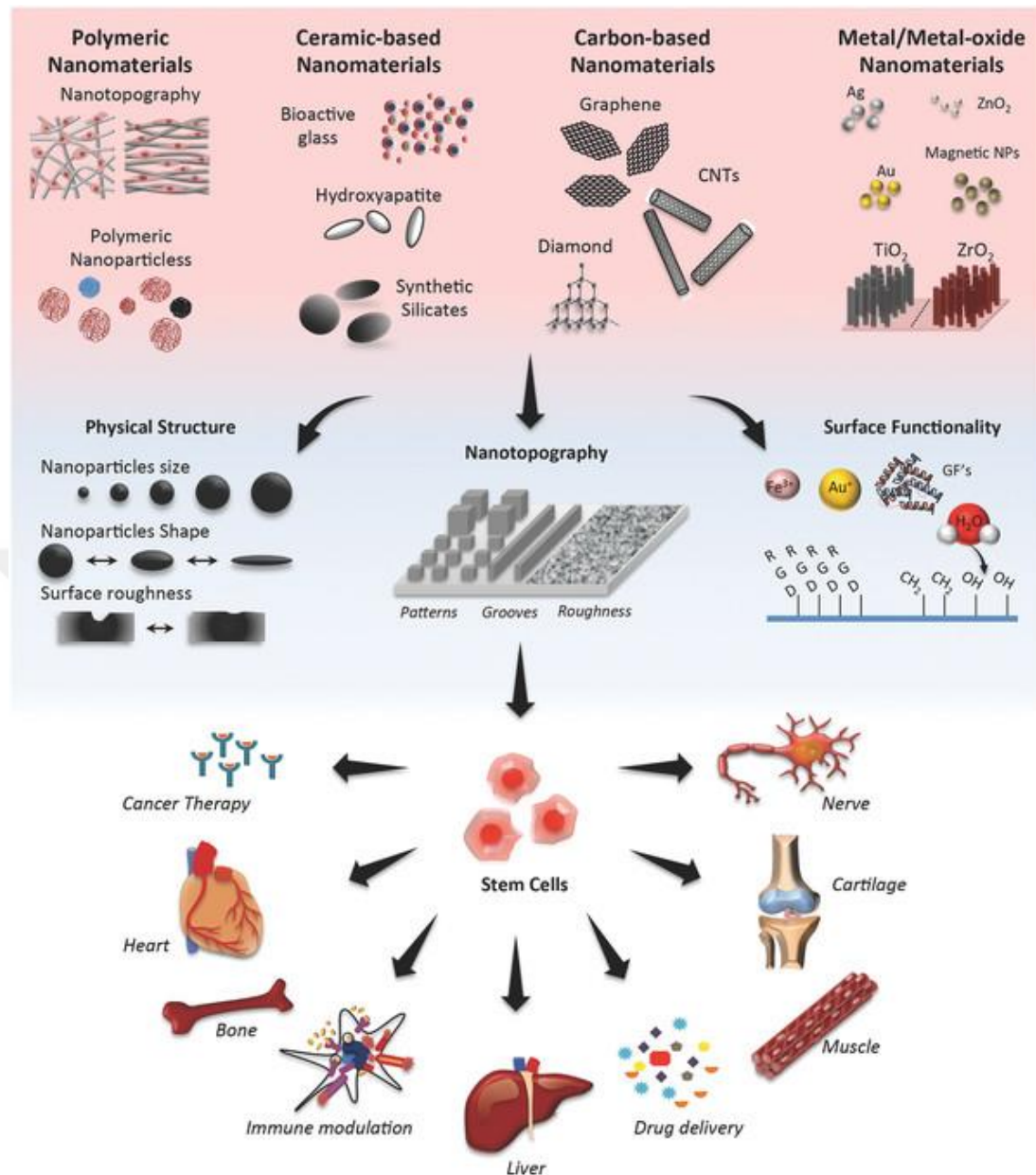


Figure 3 Nanotechnology approaches for cellular responses. (Adapted from Ref. 6 with the permission of Wiley)

1.2. Self-Assembling Nanomaterials

Molecular self-assembly is a process by which molecules organize together into more complex structures through non-covalent interactions. Peptides, proteins and

lipids are well known self-assembly monomers [7] and actin filaments, viral capsid proteins, and cell membrane's lipid bilayer are examples of self-assembly in nature [8]. Self-assembled nanoscale materials can be designed functionally through bottom-up fabrication techniques [9, 10] that can then be used for different purposes such as biomedical applications, drug delivery and regenerative medicine [11].

Proteins are organic molecules in living organisms and participate in many processes inside the cell; these proteins constantly interact with other proteins or molecules and carry out their functions according to these interactions. Amino acids are the building blocks of proteins and each amino acid has different characteristics that distinguish them from each other with regards to their functional groups, which is essential for protein functionality and structure [12]. Self-assembled nanostructures can be composed of natural or synthetic amino acids which are able to self-assemble depending on their intrinsic chemical properties such as hydrophobicity, charge and size (Figure 4). These intrinsic properties can be utilized for self-assembling peptides. However, there are also external factors affecting the self assembly such as pH, temperature, ionic strength, light etc. [13].

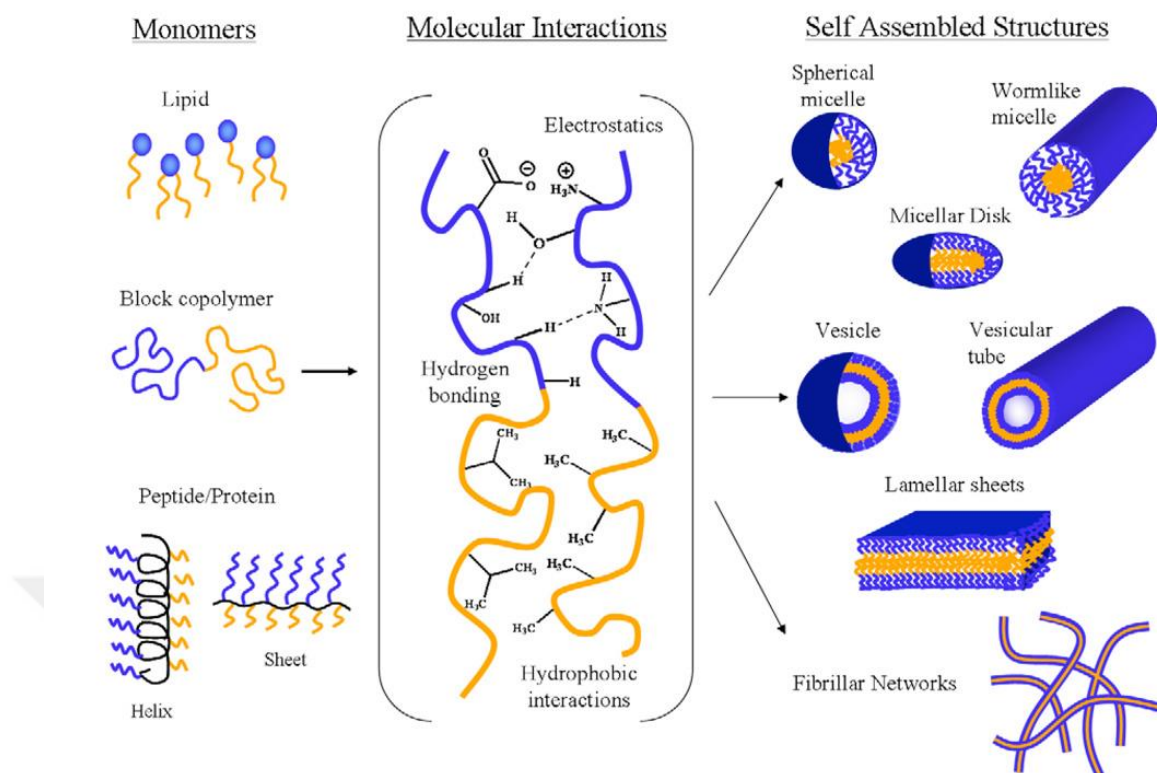


Figure 4 Self-assembling monomers and self-assembled structures. (Adapted from Ref. 6 with permission from Elsevier)

1.3. Self-assembling Peptide Amphiphiles

Peptide amphiphiles (PAs) are produced through solid phase peptide synthesis method, enabling accurate control on the peptide sequence. It provides the synthesis of peptides at high purities and without harsh chemical conditions [14]. Synthesis of the peptide starts with the conjugation of the first amino acid from its C-terminal to the solid support and subsequent attachment of amino acids to the N-terminal end of the alpha amino group [15]. Side reactions are prevented through the protection of amino acid's functional side chains. The end product, peptide amphiphile, can then organize into ordered structures through non-covalent interactions like electrostatic

interactions, hydrogen bonding and hydrophobic interactions and form supramolecular structures at different pH values [16]. Peptide amphiphile molecules contain hydrophobic fatty acids like lauric acid, they facilitate hydrophobic collapse and allow hydrophilic region to interact with water and eventually form micelle structures [17, 18]. Since there is hydrogen bonding interaction between these structures they are usually ordered into high ratio nanosystems such as nanofibers. Peptide amphiphiles have been extensively used for their biocompatibility and biodegradability [19]. In addition, these molecules can be designed to form nanofibers and micelles (Figure 5) and can be tailored with a variety of functional groups and residues for cellular targeting [20, 21]. Oppositely charged peptide amphiphile molecules can also be used for the formation of assembly through the coassembly of these peptide amphiphiles due to electrostatic interactions [22]. Additionally coassembly provide the dilution of bioactive epitope density through the differential mixing of oppositely charged PAs [23].

Self-assembled peptide nanofibers can be developed for 2D and 3D cell culture studies because they can be designed to mimic the microenvironment of cells due to their ability to support cell migration, differentiation and survival [19]. Since these structures are made up from natural amino acids, they are biocompatible and biodegradable, which makes them essential candidates for regenerative medicine purposes.

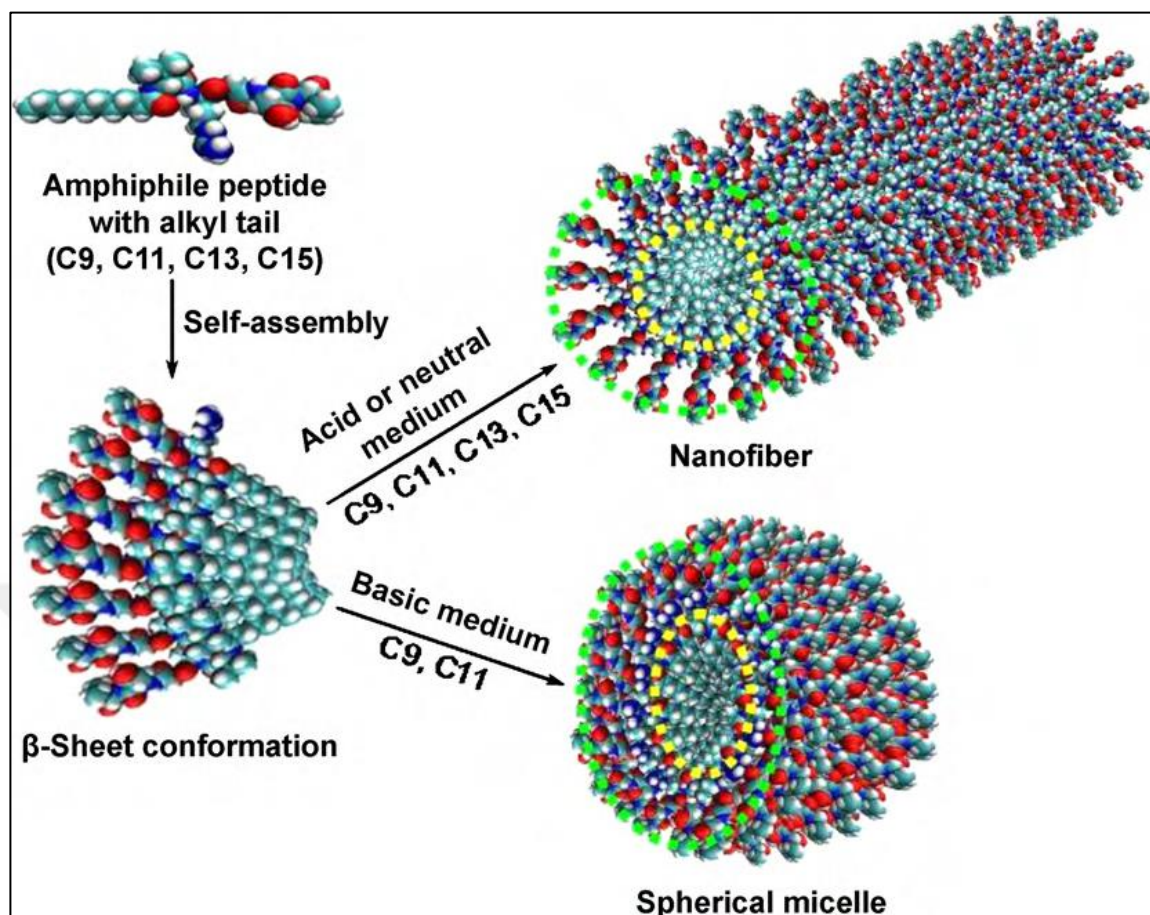


Figure 5 Schematics of Self-Assembly of Peptide Amphiphiles. (Adapted from Ref. 17 with permission from Elsevier.)

1.4. Regenerative Medicine

Regenerative medicine is one of the main areas in healthcare and advances in nanobiotechnology improve this field from different aspects. Bioinspired molecules are synthesized for many applications such as extracellular matrix mimicking, drug delivery, wound healing etc. Self-renewal and being able to differentiate into other cell types make stem cells suitable candidates for regenerative medicine applications. Therefore, scaffolds should be designed to support cellular viability and adhesion,

mimic the elastic properties of the target tissue and be nonimmunogenic to the host [24]. Peptide amphiphiles decorated with bioactive signals was shown to be effective in terms of supporting viability, adhesion and directing stem cell differentiation [25, 26].

Peptide amphiphiles can form nanofibers which can be used as scaffolds to mimic the ECM. They have high water content and form a porous network which is similar to the ECM. Mimicking ECM structure in terms of functionality and complexity holds high potential for regenerative medicine [27]. Peptide amphiphile nanofibers have been widely used in 2D and 3D cellular culture studies as scaffolds [28] and which make them suitable candidates for regenerative medicine applications due to their biocompatibility, biodegradability and incorporation of bioactive signals [29, 30] which enables the synthesis of scaffolds for different applications for guided differentiation; neural differentiation [31], adipogenesis [32], osteogenesis [33], chondrogenesis [26], wound healing [34]. Glycopeptides have many functional roles in tissue engineering and the bioactive region of the peptide amphiphile molecules can also be functionalized by using sugar moieties such as glucose. It has been shown by our group that these sugar conjugated peptides can modulate the differentiation of cells [35] (Figure 6) as well as can be used for targeted delivery [20].

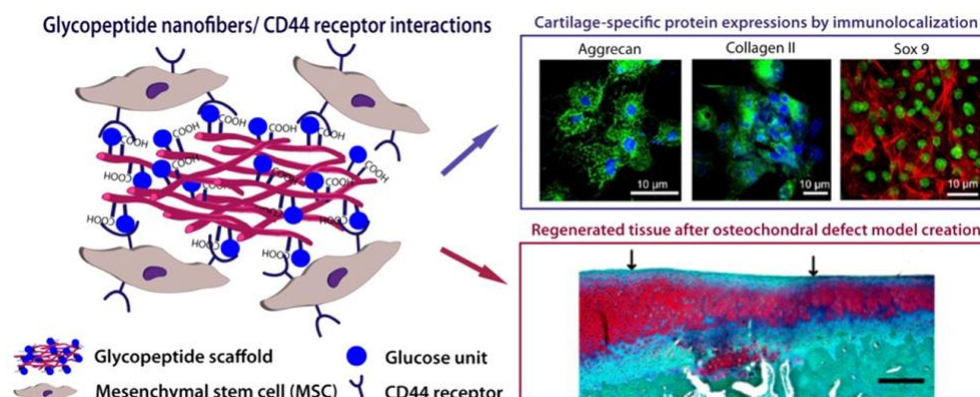


Figure 6 Supramolecular GAG-like self-assembled glycopeptide nanofibers induce chondrogenesis and cartilage regeneration. (Adapted from Ref. 37 with permission from American Chemical Society)

Peptide amphiphiles have also been shown to be effective in terms of neural regeneration and differentiation. Non-covalent functionalization of adamantine-conjugated IKVAV epitope with electrospun cyclodextrin nanofibers was shown to be inducing neural differentiation as well as guiding neurite extension. These nanofibers were biocompatible for the cells and also having adhesive properties which provided an ECM like environment for the cells. Presenting a bioactive epitope on the surface of electrospun nanofibers enhanced the directional neurite extension and neural differentiation of PC-12 cells [31].

Peptide amphiphiles having different bioactive moieties can also be utilized for cellular differentiation purposes. 3D construction of peptide amphiphiles into gel like structures provide a more *in vivo* like environment for the cells and enables directed differentiation through specific ECM mimetic signals [28] (Figure 7).

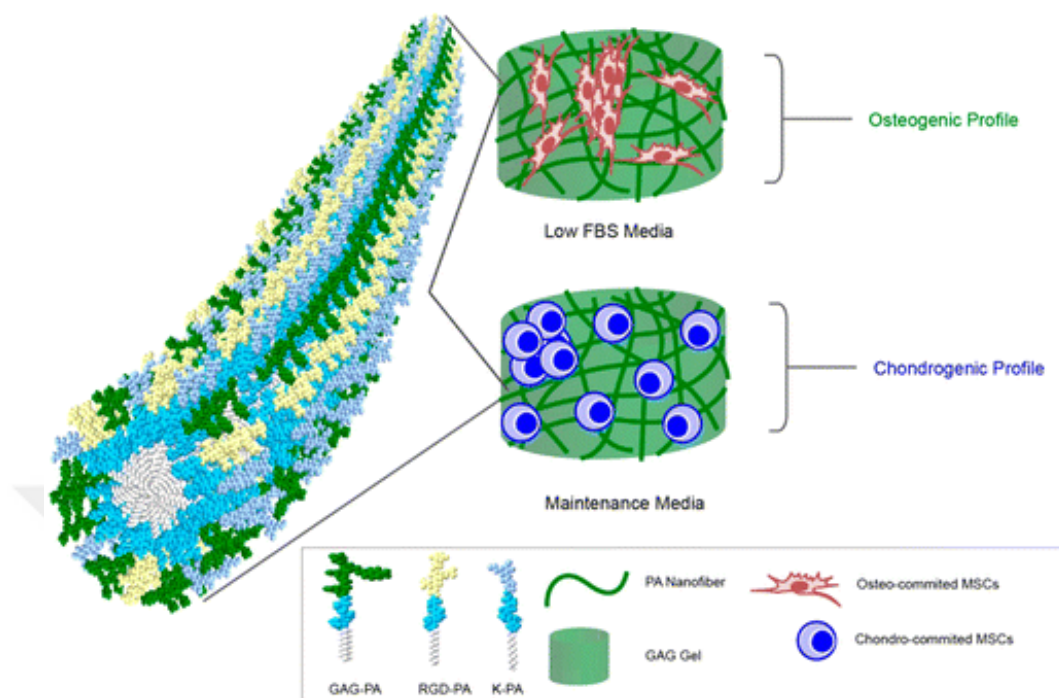


Figure 7 Glycosaminoglycan-Mimetic signals direct the Osteo/Chondrogenic differentiation of mesenchymal stem cells in a three-dimensional peptide nanofiber extracellular matrix mimetic environment. (Adapted from Ref. 30 with permission from American Chemical Society)

1.5. Drug Delivery

Peptide amphiphiles are potential candidates to be used as delivery agents, through the modification of the bioactive epitope region, peptide amphiphiles can be synthesized to target cells and deliver agents into the cells [20, 36]. Their internalization ability into cells also makes them essential candidates for drug delivery applications. Covalent conjugation of drugs to peptide amphiphile molecules also enables the preservation of the stability of the drug in blood stream

and increase the effectivity of the drug molecule [37]. Additionally these molecules can form supramolecular nanofiber gels and can be used for local delivery of drugs [38] (Figure 8).

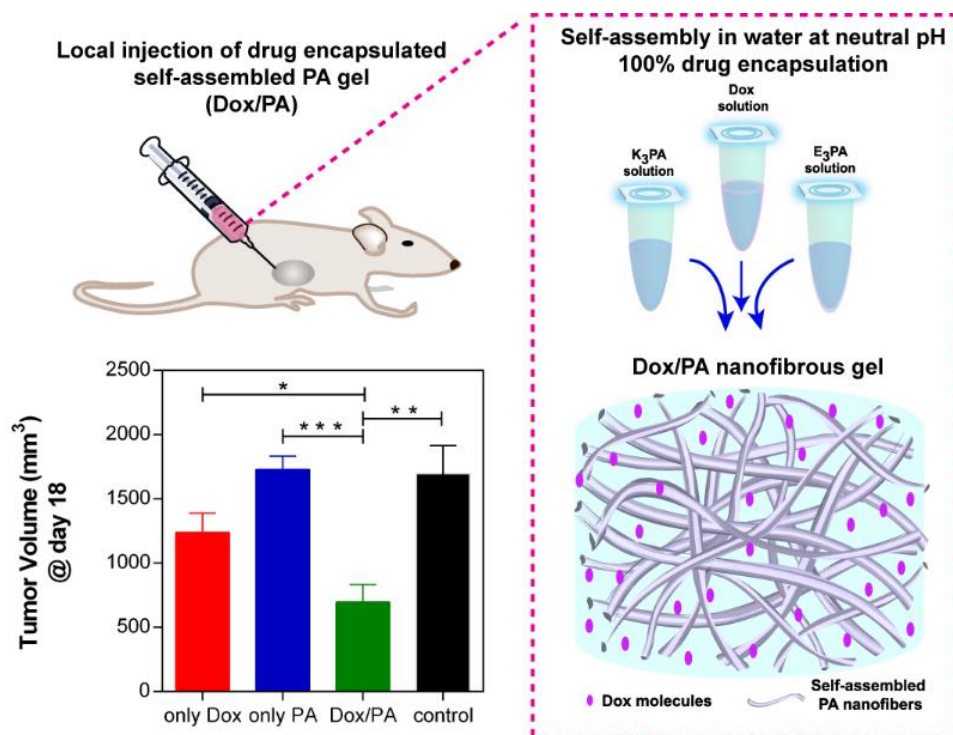


Figure 8 Local delivery of anti-cancer drug doxorubicin in a mice breast cancer model. (Adapted from Ref. 29 with permission from Royal Society of Chemistry)

Overall nanomaterials offer various improvements in many different areas. Although there are many types of nanomaterials developed for various biological applications, their clinical translations are limited. In the production of nanomaterials there are certain aspects that need to be covered in more detail such as the stability of the nanomaterial should be high in different acidic and basic conditions, side effects and

side products should be eliminated, accumulation of the nanoparticle should be limited and biodegradable molecules should be designed for therapeutic purposes in order to prevent metabolic problems in organs. In this thesis work I have utilized biomaterials for two different purposes: for the activation of dendritic cells and as a 3D scaffold for neural tissue engineering.

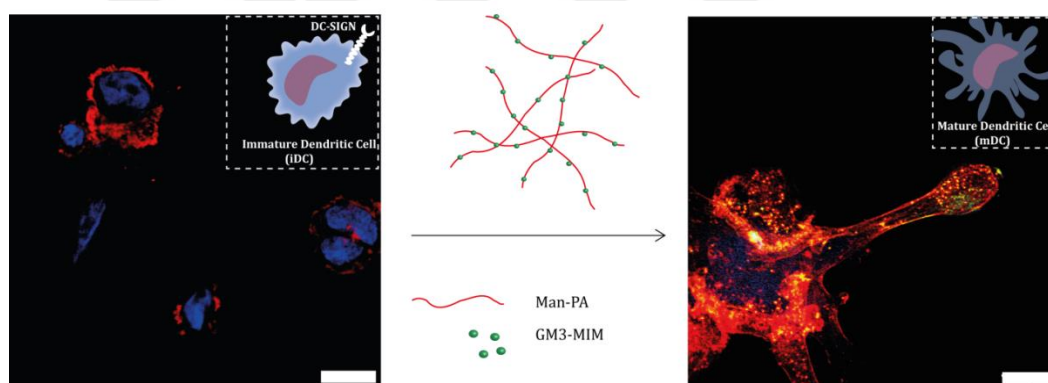


Chapter 2

2. Objectives

This work is partially described in the following publication[39]:

G. Gunay, M. Sardan Ekiz, X. Ferhati, B. Richichi, C. Nativi, A. B. Tekinay, *et al.*,
"Antigenic GM3 Lactone Mimetic Molecule Integrated Mannosylated
Glycopeptide Nanofibers for the Activation and Maturation of Dendritic
Cells," *ACS Applied Materials & Interfaces*, 2017.



The ability of dendritic cells to coordinate innate and adaptive immune responses makes them essential targets for vaccination strategies. Presentation of specific antigens by dendritic cells is required for the activation of the immune system against many pathogens, and nanoscale materials can be designed for active targeting of dendritic cells. In this work, we integrated an immunogenic, carbohydrate melanoma-associated antigen-mimetic GM3-lactone molecule into mannosylated peptide amphiphile nanofibers to target dendritic cells through DC-SIGN receptor, as it is well known that the DC-SIGN receptor has affinity to highly mannosylated structures and facilitates their uptake. Human monocytic THP-1 cell line was

cultured in IL-4 and GM-CSF and differentiated into immature dendritic, previous studies indicated the expression of DC-SIGN in these cells. Overall aim was to activate and mature immature dendritic cells into mature dendritic cells through the delivery of GM3-MIM into cells by using mannosylated PA molecules.

2.1. Introduction

2.1.1.Dendritic Cells and Adaptive Immunity

Dendritic cells (DCs) were discovered in 1973 [40], and are part of the mammalian immune system. Dendritic cells are professional antigen-presenting cells; they have ability to sense and capture microorganisms, antigens and present these processed antigens to T & B cells through MHC molecules [41]. This presentation initiates the adaptive immune response through the activation of T cells to specific antigens. The activation of dendritic cells and their capability to induce other cells depend on the state of their maturation, which is induced through the up-regulation of certain co-stimulatory molecules [42].

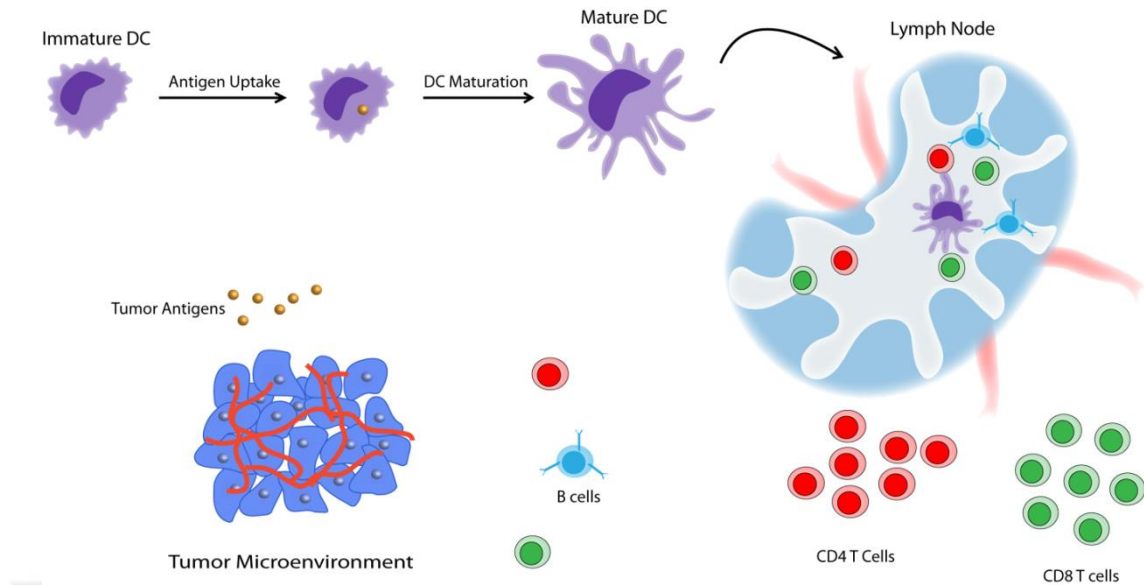


Figure 9 Generation of cellular immune response against tumor antigens.

In their immature state, DCs sample antigens in their native environment. Upon antigen sensing and uptake, they start to mature, migrate to lymph nodes, and present the antigenic peptides for the induction of an adaptive immune response [43] (Figure 9). Maturation of dendritic cells is vital because they cannot initiate an immune response in their immature state [44]. In order for a dendritic cell to activate T cells properly, T cells antigen receptors (TCRs) must recognize the antigens which is presented on the surface of antigen-presenting cells like dendritic cells. These antigens (which may be peptides, nucleic acids or glycans) are typically presented in association with major histocompatibility complex (MHC) molecules [45]. MHC class I molecules present antigens from intracellular sources, while MHC class II molecules present peptides from exogenous sources; their targets are CD8⁺ and CD4⁺ T cells, respectively [46]. However, the signal between MHC complex and TCRs is not by itself enough for a proper T cell activation and subsequent

proliferation, as CD80 or CD86 molecules on the surface of dendritic cells should also interact with the CD28 molecule on T cells to initiate the activation process [47, 48]. Another marker that is very important for dendritic cell maturation is CD83, which is expressed on mature human dendritic cells and upregulated during the transition from the immature state to mature state [49-51] (Figure 10).

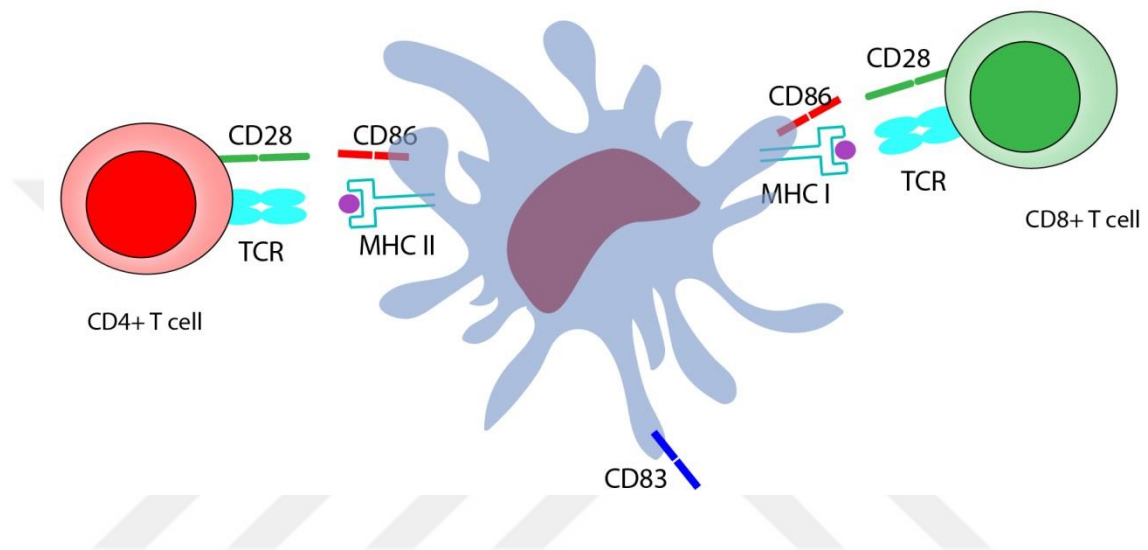


Figure 10 Interaction of dendritic cells with CD4 and CD8 T cells.

In terms of activating dendritic cells, all of these co-stimulatory molecule expressions are significant for complete activation and the ability of these cells to initiate immune response makes them vital for antigen-specific immunotherapy applications [52]. DCs have immunostimulatory functions, which have been confirmed both in *in vivo* and *in vitro* conditions [53], DCs pulsed with proteins [54] or with synthetic peptides [55] have been shown to induce antigen-specific effector T cells.

2.1.2. C-Type Lectins

Host recognition by dendritic cells occurs through several receptor families such as C-type lectin receptors (CLRs), which are mainly responsible for carbohydrate recognition [56]. These receptors are generally expressed on the immature state and down-regulated upon activation. The specificity of carbohydrate recognition has made CLRs very good vaccine targets for improved antigen presentation [57]. Among CLRs, dendritic cell intracellular adhesion molecule 3 (ICAM-3) grabbing non-integrin (DC-SIGN) exhibits considerable affinity to mannose residues and is mainly expressed on immature dendritic cells [58, 59] (Figure 11). It modulates the uptake of specific pathogens through its interactions with mannosylated structures [58, 60, 61]. Ligands that are internalized through DC-SIGN receptor are processed and presented to CD4⁺ T cells following their binding to MHC-II molecules [62].

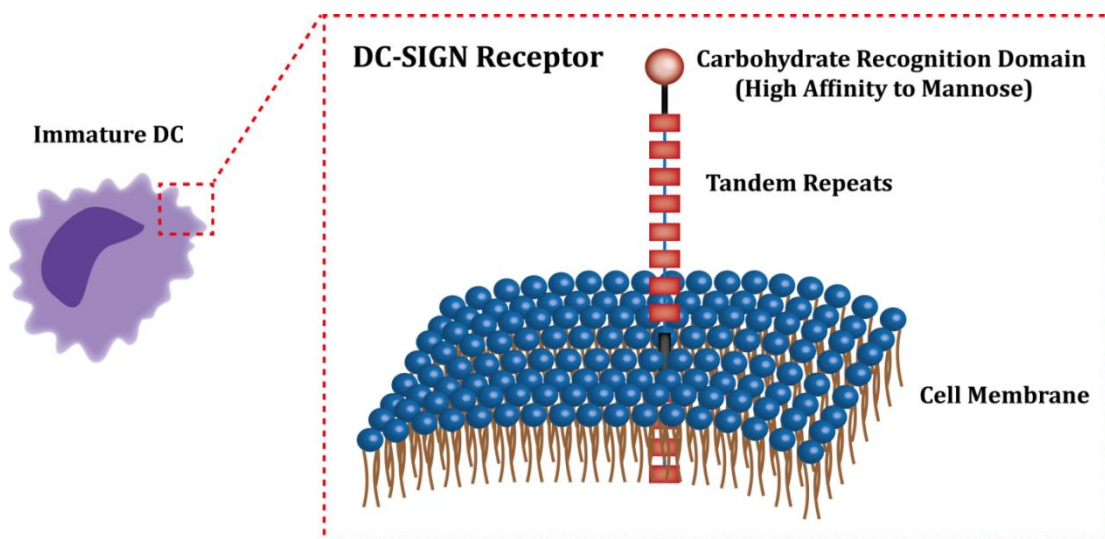


Figure 11 DC-SIGN receptor on immature DCs.

2.1.3. Carbohydrate Associated Antigens

Discovery of human cancer antigens enabled scientists to develop new immunotherapy strategies [63, 64]. Melanoma, as a major type of cancer, is widely appreciated to be a promising target for immunotherapy applications [65]. GM3 ganglioside is a major glycosphingolipid that is overexpressed in metastatic melanoma cells, and there is an abundance of GM3 ganglioside studies that focus on its potential to be used as a carbohydrate-based, melanoma-associated antigen for cancer diagnosis [66]. A metabolite of GM3 ganglioside, called GM3 lactone, is also present in melanoma cells; however, due to its low amounts it is below the recognition threshold and does not induce an immune response. However, GM3 lactone is more immunogenic than GM3 ganglioside and can induce stronger immune responses at equal concentrations. A previous study had shown that permannosylated dendrons containing an antigenic GM3 lactone mimetic is able to induce dendritic cell activation through DC-SIGN internalization [67].

Recently, antigens delivered through nanoscale materials have been shown to provide considerable advantages over soluble antigen administration, due to the stimulation of antigen uptake and cellular activation by surface-functionalized delivery vectors. Gold and PLGA nanoparticles, dendrimers and liposomes have previously been functionalized for *in vitro* dendritic cell targeting purposes [67-69].

In this project we targeted dendritic cells by using glycopeptides and delivered a mimetic of GM3-lactone (GM3-MIM) and analyzed the activation and maturation levels of immature dendritic cells.

2.2. Experimental Section

2.2.1. Materials

9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids, [4-[α -(2',4'-dimethoxyphenyl) Fmoc aminomethyl] phenoxy] acetamidonorleucyl-MBHA resin (Rink amide MBHA resin) and 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem. Fmoc-Ser[β -Glc(OAc)₄]-OH was purchased from AAPPTec. Lauric acid and N,N-Diisopropylethylamine (DIEA) were purchased from Merck. Other chemicals were purchased from Alfa Aesar or Sigma-Aldrich and used without any purification. Deionized water (resistance of 18 M Ω .cm) was used during the experiments.

2.2.2. Synthesis of Amphiphilic Glycopeptides

Protected glycopeptides (Man-PA and Glc-PA) were constructed on MHBA Rink Amide resin. All amino acid couplings were performed with 2 equivalents of Fmoc protected amino acid, 1.95 equivalents of HBTU and 3 equivalents of N,N-diisopropylethylamine (DIEA) in DMF for 3 h. Fmoc deprotections were performed with 20% piperidine/ dimethyl formamide (DMF) solution for 20 min. Cleavage of the peptides from the resin and deprotection of acid labile protecting groups were carried out with a mixture of trifluoroacetic acid (TFA) : triisopropylsilane (TIS) : water in the ratio of 95 : 2.5 : 2.5 for 2 h. Excess TFA was removed by rotary evaporation. The remaining residue was triturated with ice-cold diethyl ether and the resulting white pellet was freeze-dried. Deacetylation reaction was carried out in solution. For the cleavage of acetyl groups, 210 mg of protected glycopeptide (1 eq.)

was dissolved in 105 ml of anhydrous methanol. 2M of NaOMe (4.4 eq.) was dissolved in methanol and poured into the solution. After adjusting pH to 8-8.5, the reaction was carried out at room temperature for 2-3 h. The solution was neutralized with a few drops of acetic acid in order to terminate the reaction. The solvent was removed by vacuo. After water addition, it was frozen at -80°C and freeze-dried. Before and after the deacetylation reaction, glycopeptides were identified and analyzed by reverse phase HPLC on an Agilent 6530 accurate-Mass Q-TOF LC/MS equipped with an Agilent 1200 HPLC. An phenomenex Luna 3 μ C8 100A (50 x 3.00 mm) column as stationary phase and water/acetonitrile gradient with 0.1% volume of formic acid as mobile phase were used to identify peptide amphiphile. They were purified on Agilent 1200 by using a Zorbax prepHT 300CB-C8 column with a water–acetonitrile (0.1% TFA) gradient.

2.2.3.Synthesis of Fluorescent GM3-MIM Derivatives (GM3-MIM-Bodipy)

The alkyl derivative of GM3-MIM was prepared and provided by Prof. Cristina Nativi according to the previously reported procedure [70].

2.2.4.Preparation of GM3-MIM Integrated Glycopeptide Nanofibers

1 mM of Man-PA and Glc-PA (2 ml) were dissolved in water and heated upto 50°C during sonication. 1.33 mM of GM3-MIM was dissolved in DCM:MeOH mixture (7:1 v/v %, 610 μ l) and added into each glycopeptide solution. They were sonicated and vortexed for an hour at 50°C. While the mixture was initially turbid, it became transparent as the solvents evaporated. Final GM3-MIM concentration to PA concentration ratio was adjusted to 1:2.5.

2.2.5.Characterization of Fluorescent GM3-MIM Integrated Glycopeptide Nanofibers

Fluorescence spectra of GM3-MIM and GM3-MIM integrated glycopeptide nanofibers were recorded by a Varian Cary Eclipse spectrophotometer with an excitation wavelength of 503 nm. Varian Cary 100 UV-VIS spectrophotometer was used to record absorption spectra of GM3-MIM-BodiPy and GM3-MIM integrated glycopeptide nanofibers. For UV and fluorescence measurements, DCM:MeOH mixture (7:1, v/v %) was used to dissolve GM3-MIM-BodiPy, and water was used as the solvent for GM3-MIM integrated glycopeptide nanofibers. A Jasco J-815 CD spectrophotometer was used to investigate the secondary structures of PA molecules and GM3-MIM integrated PA nanofibers. All samples were measured at physiological pH at 0.25 mM concentration. 300 μ L of the sample was transferred into a 1 mm quartz cuvette and spectra were obtained at room temperature from 300 nm to 190 nm with a data interval of 1 nm and a scanning speed of 100 nm/min. TEM images were obtained with a FEI Tecnai G2 F30 TEM at 200 kV. A high-angle annular dark field (HAADF) detector was used for images taken in STEM mode. 1 mM of Glc-PA, Man-PA and GM3-MIM integrated glycopeptide nanofiber systems were first diluted to 150 μ M and then dropped on 300-mesh carbon TEM grids. Samples were allowed to stay at room temperature for 3 min, stained by 2 wt % uranyl-acetate staining for another 1-2 min and air dried prior to STEM imaging. Zeta potential of the nanofibers was measured by ZetaSizer. A Malvern Nanosizer/ZetaSizer Nano-ZS ZEN 3600 (Malvern Instruments, USA) instrument was used for the analysis. Measurements were performed in quartz cuvettes and

repeated at least three times. Samples were prepared by dissolving each component in water at a concentration of 0.25 mM.

2.2.6. Cellular Viability

THP-1 human monocytes were kindly provided by Prof. E. Erbay of Bilkent University. Cells were seeded onto 96 well plates at a density of 5000 cells/well within media containing Man-PA and Glc-PA. Viability was assessed at 150 μ M and 250 μ M for both peptide molecules. At the end of 24 h, cells were washed with 1X PBS and were stained with Calcein-AM and ethidium homodimer for 30 min at room temperature in dark. Then, images of the cells were taken and quantified by using Image J program.

2.2.7. Differentiation of THP-1 Human Monocytes into Immature Dendritic Cells

THP-1 human monocyte cell line was differentiated into immature dendritic cells in RPMI medium containing 10% FBS, rhIL-4 (100 ng/mL) and rhGM-CSF (100 ng/mL) for 5 days. Medium was exchanged with fresh cytokines after 2 days.

2.2.8. Internalization of Fluorescent GM3-MIM Integrated Glycopeptide Nanofibers

13 mm glass coverslips were placed in 24-well plates and 5×10^4 THP-1 cells were seeded and differentiated into immature dendritic cells as previously stated. After 5 days, the PA molecules were administered and cells were incubated for 24 h. After incubation, cells were washed with PBS two times, fixed with 4% paraformaldehyde, and permeabilized with 3% Triton-X and stained with phalloidin and TO-PRO-3.

Cells were visualized by using Laser Scanning Confocal Microscope (LSM 510, Zeiss).

2.2.9. Surface Marker Analyses of Dendritic Cell Activation

iDCs were obtained from THP-1 monocytes as mentioned above. At the end of 5 days, cells were treated with; Man-PA, Glc-PA, Man-PA/GM3-MIM, Glc-PA/GM3-MIM, and only GM3-MIM for 24 h. Total peptide concentration for each group was 150 μ M and GM3-MIM amount was 40 μ g. At the end of 24 h, media were discarded, cells were centrifuged at 2500 rpm for 5 min and washed with 1X PBS and then centrifuged again. Then, pellet was dissolved in 1X PBS and cells were stained with CD86 (PerCP), CD83 (Phycoerythrin) and HLA-DR (MHC-II) (PE / Cy5) antibodies for 15 min at room temperature. 10.000 cells were recorded for each condition during flow cytometry analyses.

2.2.10. Scanning Electron Microscopy (SEM) Imaging

SEM imaging was carried out in order to investigate the morphological changes during dendritic cell maturation. 5×10^4 THP-1 cells were seeded onto coverslips in 24 well plates and differentiated into immature DCs as previously stated. Treatment was carried out for 24 h and at the end of 24 h cells were washed once with 1X PBS, fixed with 2% glutaraldehyde/PBS and 1 wt % OsO_4 for 1 h each. After fixation, cells were washed with ddH₂O and dehydrated by using increasing ethanol concentrations; 20%, 40%, 60%, 80% and 100% v/v. Critical point dryer (Autosamdri 815B equipment from Tousimis) was used afterwards and coverslips were coated with 4 nm Au/Pd. SEM (FEI Quanta 200 FEG) images were taken through using Everhart–Thornley Detector (ETD) at high vacuum mode at 5 keV beam energy.

2.3. Results and Discussion

2.3.1. Design, Synthesis and Characterization of Glycopeptide Conjugates

In this study, mannose-PA (Man-PA) and glucose-PA (Glc-PA) were used for the conjugation of a GM3-lactone mimetic molecule (GM3-MIM) and targeting of immature dendritic cells (Figure 12). The purity of the synthesized peptides was assessed through liquid chromatography-mass spectrometry (LC-MS) (Figure 13). Purity of synthesized peptides was higher than 90%, and GM3-MIM or GM3-MIM Bodipy was integrated into glycopeptides following the confirmation of peptide purity.

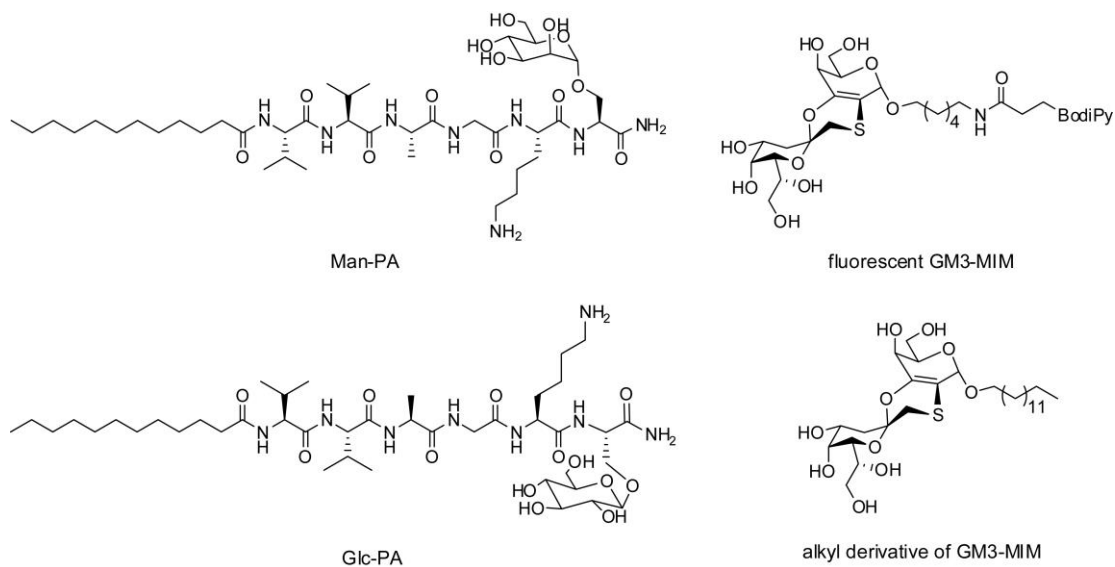


Figure 12 Chemical structures of molecules used in the study.

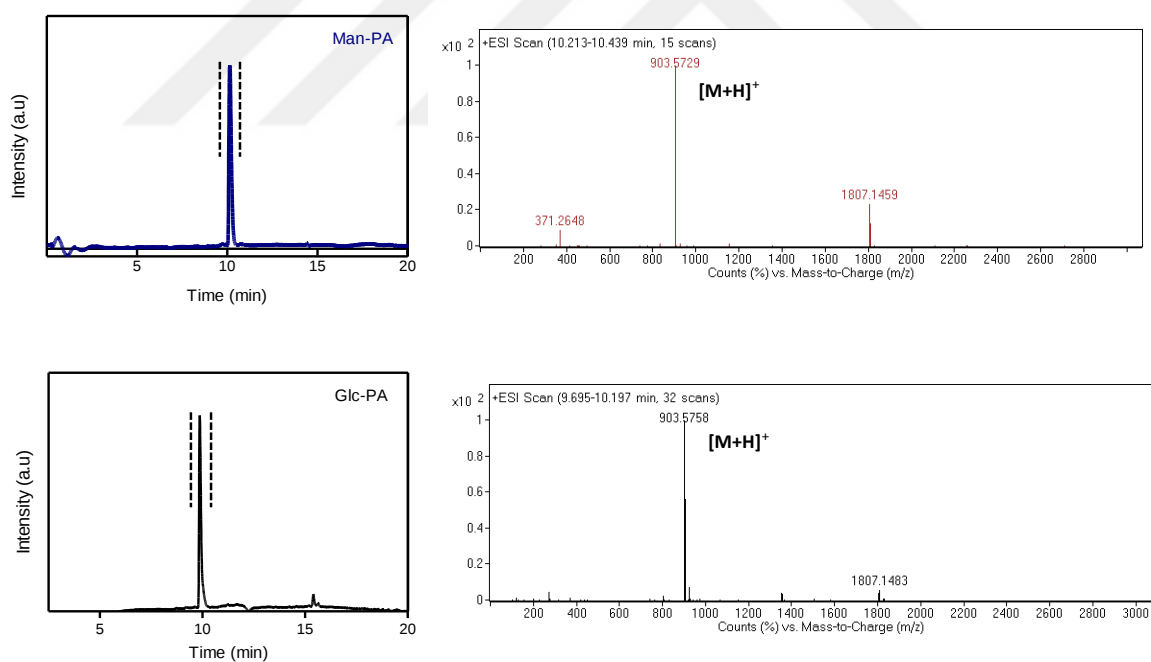


Figure 13 Liquid chromatograms and mass spectra (LC-MS) of amphiphilic glycopeptides. Man-PA, $[M+H]^+$ (calculated) = 903.5688, $[M+H]^+$ (observed) = 903.5729; Glc-PA, $[M+H]^+$ (calculated) = 903.5688, $[M+H]^+$ (observed) = 903.5758.

Before the integration of fluorescent GM3-MIM into the glycopeptides, the secondary structure and morphological properties of the amphiphilic glycopeptides were investigated at physiological pH. Scanning transmission electron microscopy (STEM) was performed in order to analyze the morphological characteristics of glycopeptides and both glycopeptide samples, regardless of the type of sugar they contain, were found to form high-aspect-ratio nanofibers with diameters in the order of 9-11 nm and lengths reaching several micrometers (Figures 14A and 14B),

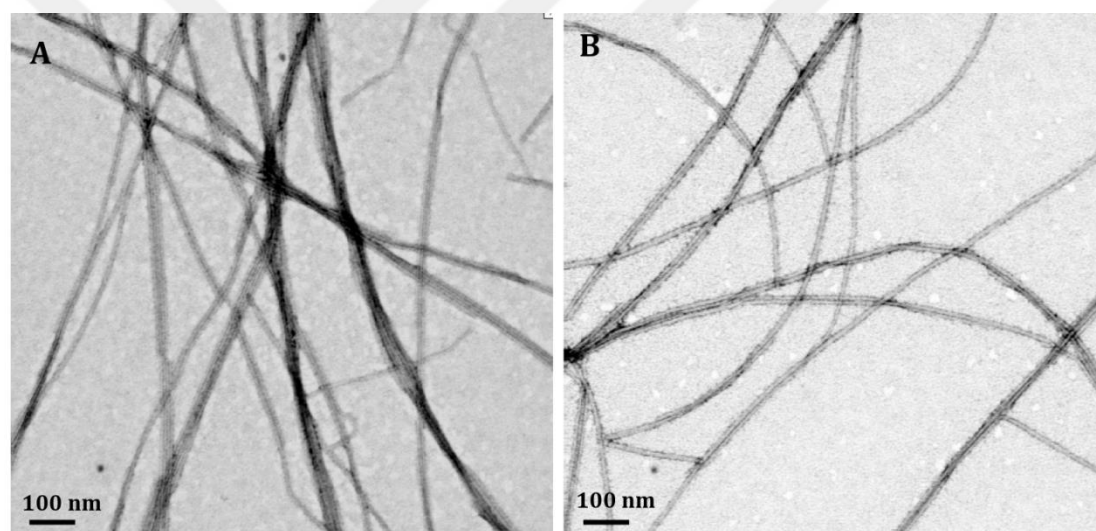


Figure 14 STEM images of (A) Man-PA (B) Glc-PA.

GM3-MIM has poor solubility in water; consequently, molecule was integrated into glycopeptide nanofibers through solvent exchange process to improve its bioavailability (Figure 8). GM3-MIM was solubilized in DCM:MeOH (7:1, v/v %) and glycopeptide solutions were dissolved in slightly acidic water. Glycopeptide solutions were sonicated before the addition of GM3-MIM at 50 °C at pH 6. The rationale behind this step was to disrupt the self-assembled fibers and promote the

generation of glycopeptide molecules as single units, allowing the integration of GM3-MIM into fiber structures during re-assembly. Due to the lysine residues they bear, glycopeptides carry an overall positive charge that increases their solubility in water and promotes their integration during self-assembly. GM3-MIM containing solution was added to glycopeptides separately, and the mixture was heated and sonicated to allow the interaction of the hydrophobic tail region of GM3-MIM with the hydrophobic amino acids and hydrocarbon chain region of glycopeptides. This procedure promoted the transfer of GM3-MIM into water. GM3-MIM displays an amphiphilic structure and is expected to have its hydrophobic tail region face the fiber core following integration into peptide nanofibers, while the hydrophilic ring structure would face the outer region.

GM3-MIM integration was visualized through the use of Bodipy dye-conjugated GM3-MIM molecules (Figure 15A). Quantitative analysis of the GM3-MIM transfer was carried out with fluorescence and UV spectra measurements. GM3-MIM integrated glycopeptides were blue-shifted compared to the non-conjugated GM3-MIM group, as observed both visually and through spectral measurements (Figure 15B and 15C). UV spectra results also provided similar absorbance values for integration into both glycopeptide groups, and suggested that the efficiency of the integration was very close to 100%.

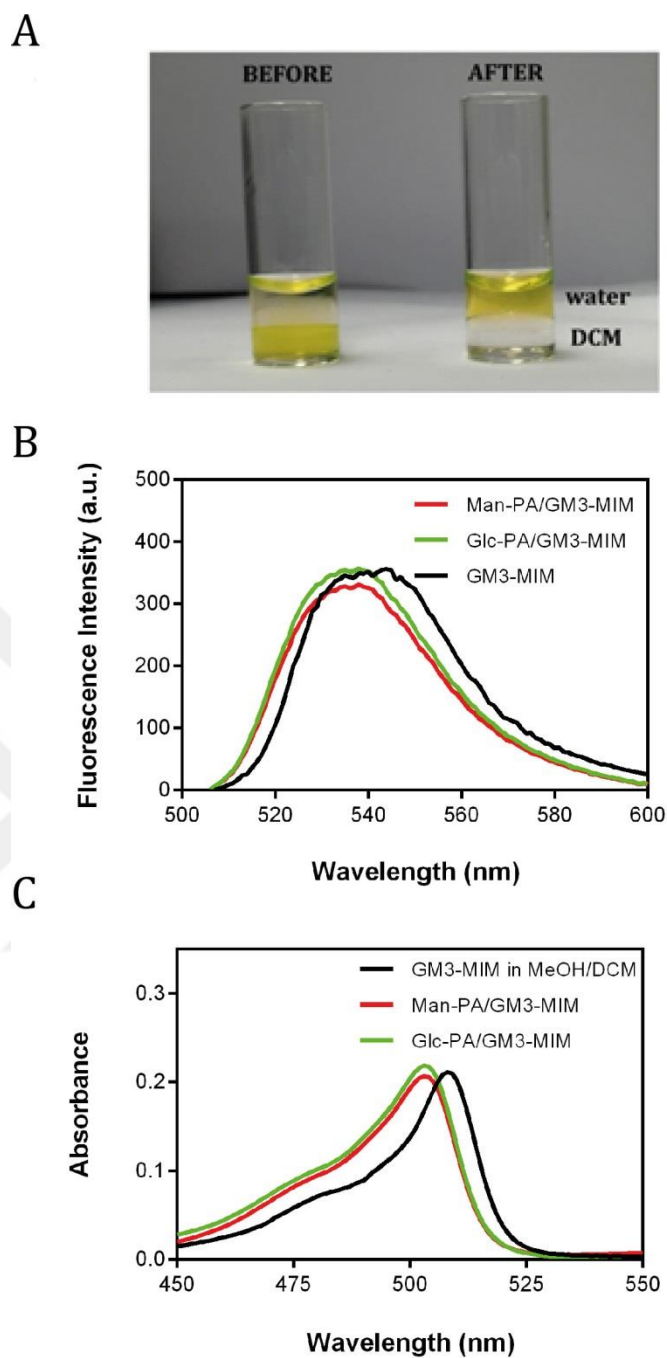


Figure 15 Solvent exchange (DCM:H₂O) process for the integration of the GM3-MIM molecule into peptide structures. (B) Fluorescence and (C) UV spectra of GM3-MIM and GM3-MIM integrated glycopeptide solutions dissolved in DCM:MeOH (7:1) and water, respectively.

After achieving the successful transfer of GM3-MIM, glycopeptides containing the antigen were analyzed for their morphological characteristics through CD and STEM. Diluted solutions of Man-PA and Glc-PA were studied by circular dichroism (CD) spectroscopy and the results revealed that both pure systems were oriented in a β -sheet conformation, exhibiting a positive peak at 202 nm and a negative peak at 218 nm (Figure 16).

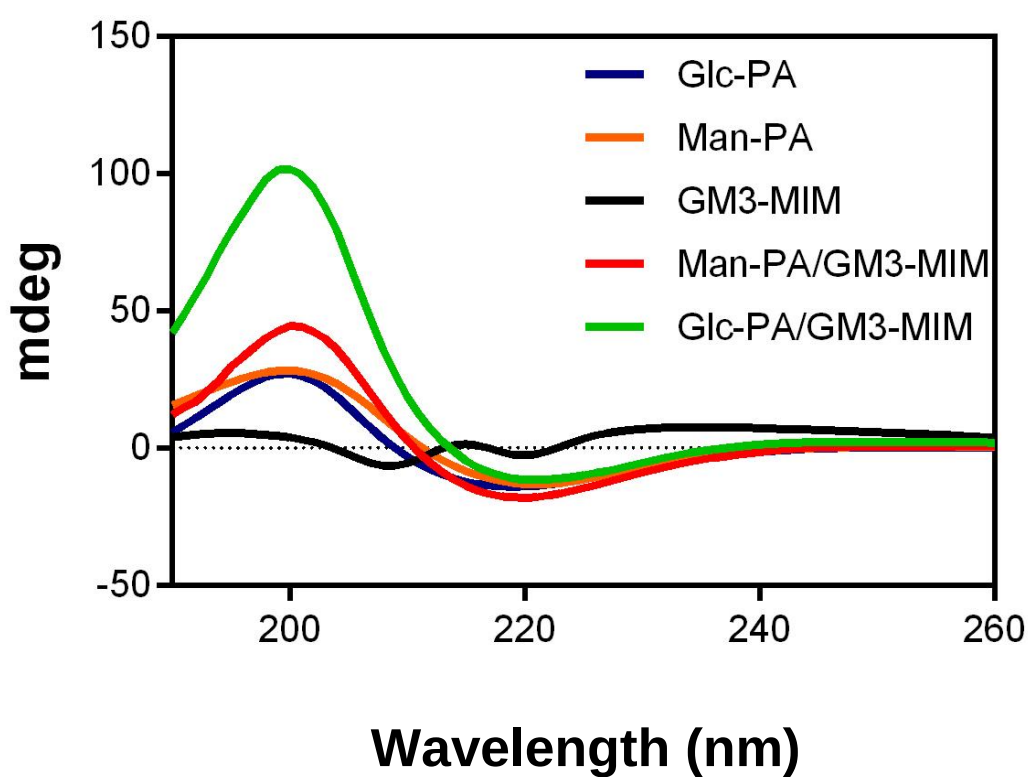


Figure 16 CD spectra of amphiphilic glycopeptide solutions, GM3-MIM and GM3-MIM integrated glycopeptide systems.

Even though bundling to a higher degree was observed after the integration of antigen in STEM images, self-assembly still resulted in the formation of nanofibers with similar diameters, which suggests that there are no significant changes during PA assembly in the presence and absence of the antigen (Figure 17A and 17B). In addition, the antigen contained sulfur and its integration into glycopeptides could also be analyzed through sulfur and oxygen mapping, which was performed by using TEM. This analysis confirmed that GM3-MIM antigen was present in glycopeptides (Figure 18).

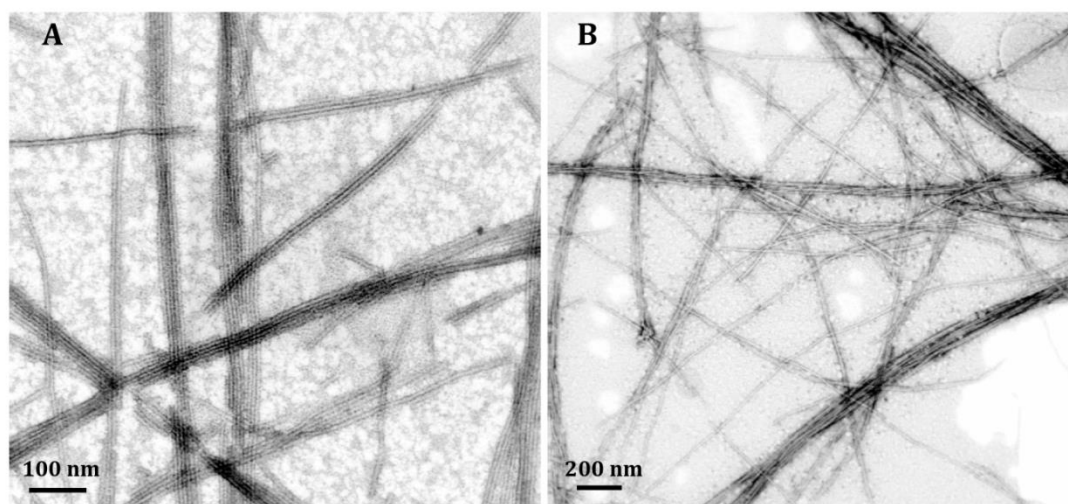


Figure 17 STEM images of (A) Man-PA/GM3-MIM (B) Glc-PA/GM3-MIM.

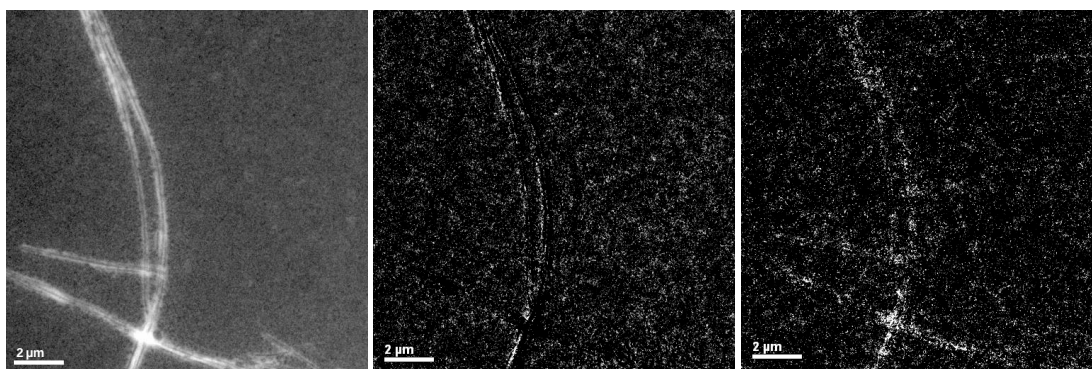


Figure 18 TEM mapping of fluorescent GM3-MIM integrated Man-PA nanofibers, S-pre edge (left), sulfur mapping (middle), oxygen mapping (right).

Charge is a very important factor that may interfere with the internalization efficiency; therefore we analyzed glycopeptides for their charge properties by using Zeta potential measurements. The results indicated a similar positive net charge for both glycopeptides around +27 mV (Figure 19).

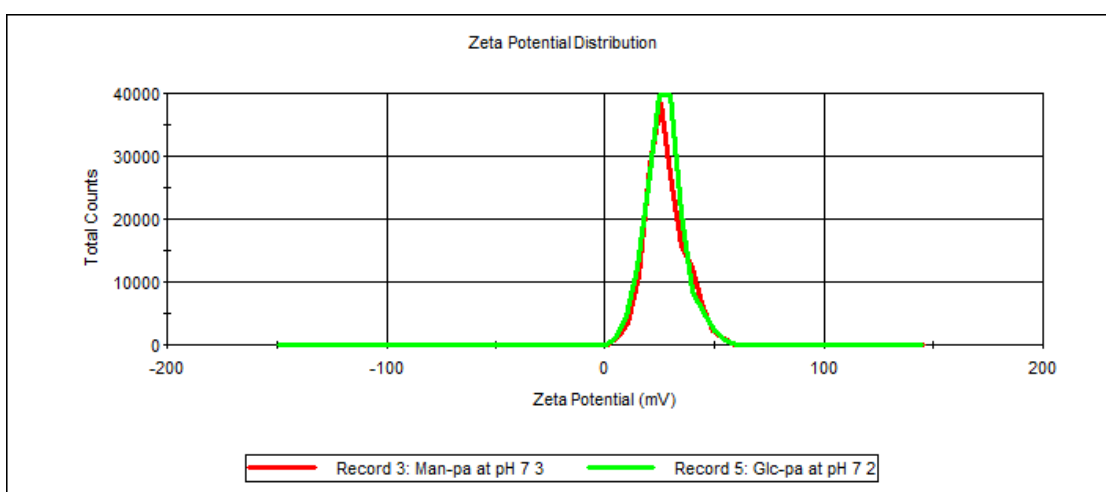


Figure 19 Zeta potentials of Man-PA and Glc-PA.

2.3.2. Cell Viability and iDC Differentiation from THP-1 Monocytes

THP-1 human monocyte cells were used for *in vitro* analyses. Cells were treated with different concentrations of Man-PA and Glc-PA. No significant difference in terms of viability was found between TCP and glycopeptides at 150 μ M (Figure 20), indicating the biocompatibility of both peptide amphiphiles at this concentration. However, toxicity was observed at higher concentrations, possibly due to the stress formed by the accumulation of peptides on the cell membrane.



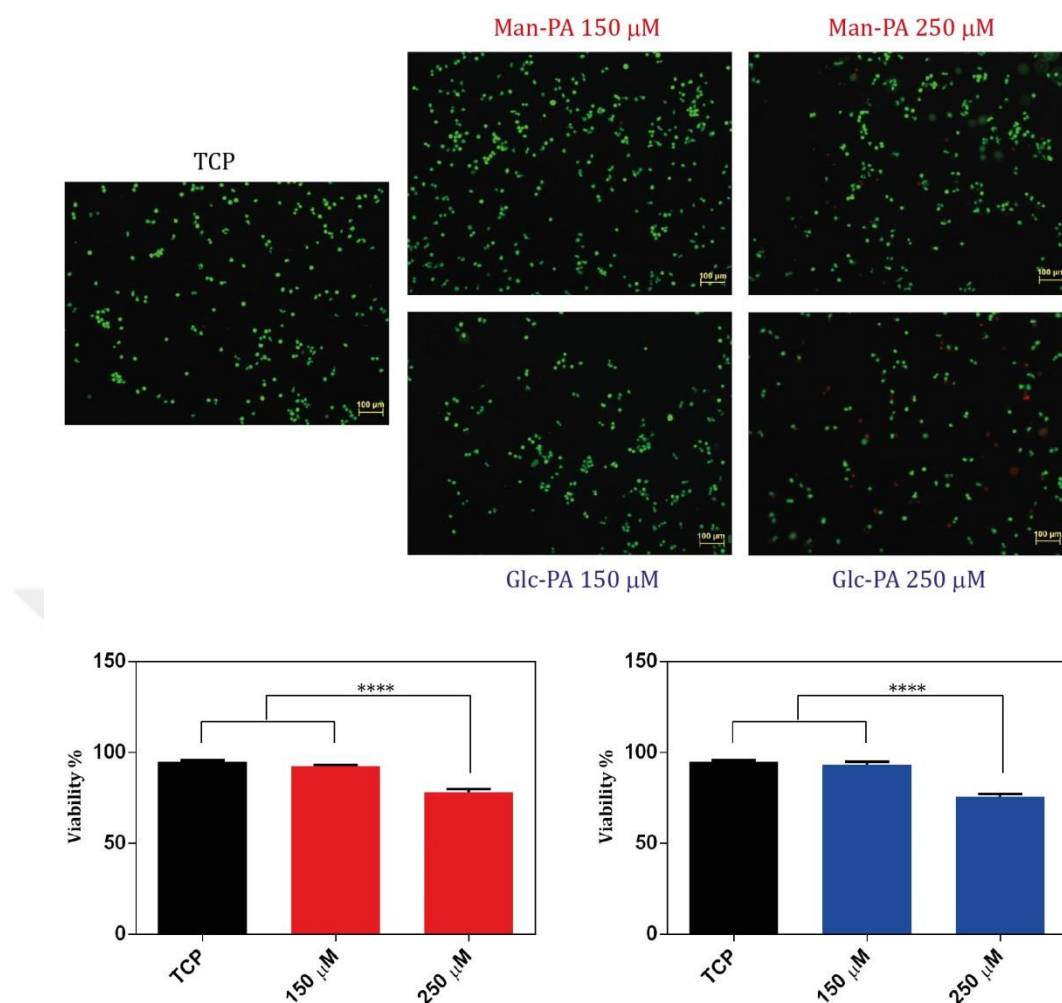


Figure 20 Cellular viability of THP-1 cells treated with different concentrations of Man-PA and Glc-PA, and TCP for 24 h. Values represent mean $\pm$ SEM (****p<0.0001).

After ensuring the biocompatibility of the peptide amphiphiles, human THP-1 monocytes were differentiated into immature dendritic cells (iDC) as previously mentioned (Figure 20 and Figure 21).

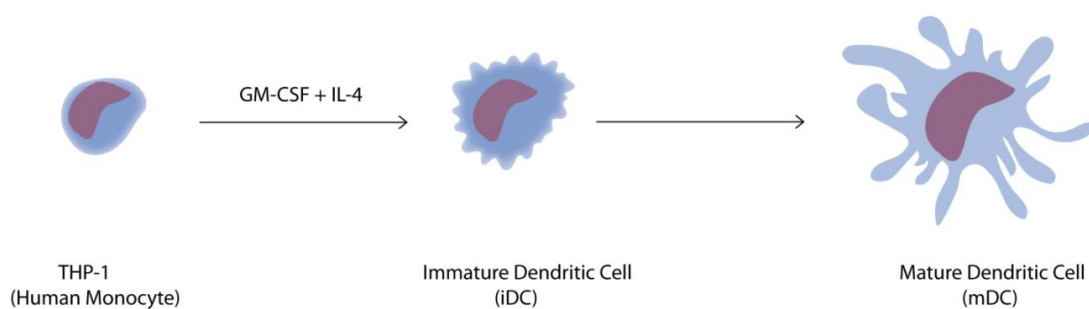


Figure 21 Schematic representation of the transition from human monocytes to mature dendritic cells (mDC).

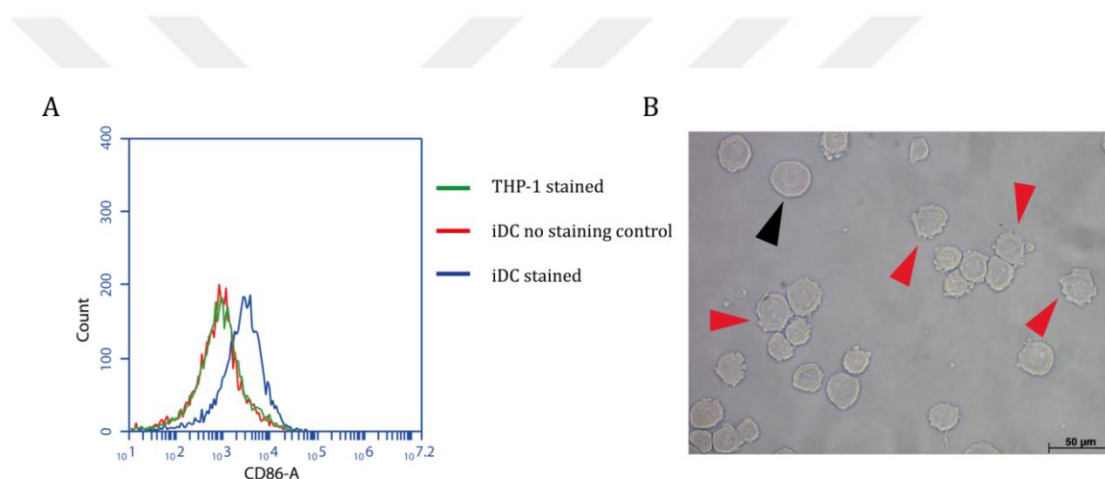


Figure 22 Differentiation of THP-1 monocytes into iDCs in terms of (A) *de novo* surface CD86 expression and (B) morphological changes upon differentiation into iDC.

After differentiation of THP-1 monocytes into iDCs, Man-PA/GM3-MIM, Glc-PA/GM3-MIM, GM3-MIM, Man-PA and Glc-PA were applied to cells for 24 h. At the end of 24 h, light microscope images of the cells were taken in order to examine the morphological changes. Cellular morphology was more indicative of maturation

in Man-PA/GM3-MIM compared to the other groups, indicating the higher delivery of GM3-MIM into iDCs through Man-PA.

2.3.3. Internalization of Fluorescent GM3-MIM Integrated Glycopeptide Nanofibers

After examining the morphological differences, the uptake of PA molecules were assessed through confocal microscopy visualization and flow cytometry quantification. Concentrations were optimized according to the live-dead experiment results. Since GM3-MIM molecule was conjugated with Bodipy fluorescent dye, internalization as well as the morphological changes due to the internalization could be directly visualized.

Confocal microscopy images indicated higher uptake and a more mDC-like morphology in the Man-PA/GM3-MIM group compared to other groups (Figure 22). Results indicate the importance of using Man-PA for the delivery of GM3-MIM into the cells.

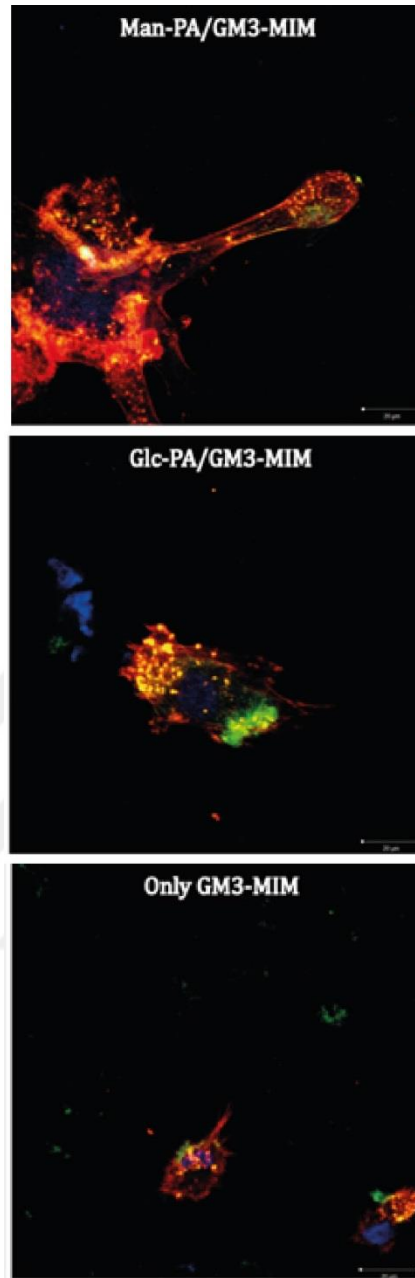


Figure 23 Uptake and morphological changes of iDCs when treated with Man-PA/GM3-MIM, Glc-PA/GM3-MIM and only GM3-MIM for 24 h. Blue indicating To-Pro nuclei staining, red indicating Phalloidin microtubule staining and green indicating bodipy conjugated GM3-MIM.

Quantitative analysis of GM3-MIM internalization was carried out by using flow cytometry. Cells were treated with Man-PA/GM3-MIM, Glc-PA/GM3-MIM, or GM3-MIM for 24 h. Mean fluorescent intensity (MFI) of GM3-MIM for each group was compared between each other and results indicated a significantly greater internalization in the Man-PA/GM3-MIM group compared to other groups (Figure 23). Additionally, there was a significant difference between the Glc-PA/GM3-MIM group and the GM3-MIM group, which suggests that even though there is no specificity of glucose to DC-SIGN, the nanofiber system itself promotes better internalization (Figure 23).

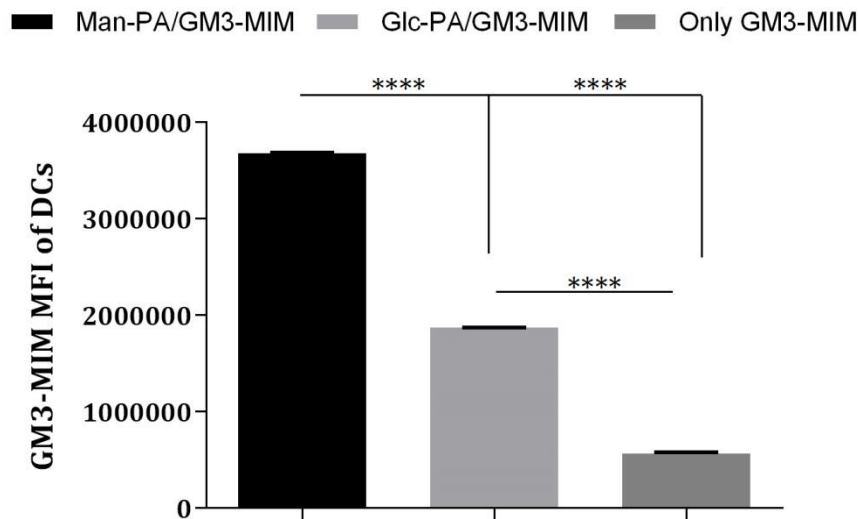


Figure 24 GM3-MIM MFI analyses for Man-PA/GM3-MIM, Glc-PA/GM3-MIM and only GM3-MIM for 24 h. Values represent mean $\pm$ SEM (**** $p < 0.0001$).

2.3.4. Surface Marker Analyses of Dendritic Cell Activation

Activation and maturation state of DCs were measured by analyzing the expression of CD86, CD83 and MHC-II receptors. Increased expression of these molecules was

previously shown to be associated with the maturation and activation status of dendritic cells [67, 71]. CD86 is a co-stimulatory molecule that interacts with CD28 on T cells and is required for proper activation [47]. Man-PA/GM3-MIM group significantly increased the expression of CD86 compared to other groups, as measured by flow cytometry (Figure 24A). CD83 is another activation marker for DCs and its expression is up-regulated during the transition from immature state to mature state [49]. Man-PA/GM3-MIM also significantly increased the expression of CD83 compared to the other compounds (Figure 24B), and the same pattern was observed for the expression of MHC-II (Figure 24C), which is a molecule essential for the proper presentation of antigens [62]. Expression profiles obtained by flow cytometry analysis were in agreement with fluorescent imaging (Figure 22) and flow cytometry analyses (Figure 23) of GM3-MIM internalization. In summary, considering the necessity of the up-regulation of these molecules for DC activation and maturation, Man-PA/GM3-MIM was shown to have a significant effect on inducing the activation and maturation of DCs in terms of surface expression of CD86, CD83 and MHC-II receptors.

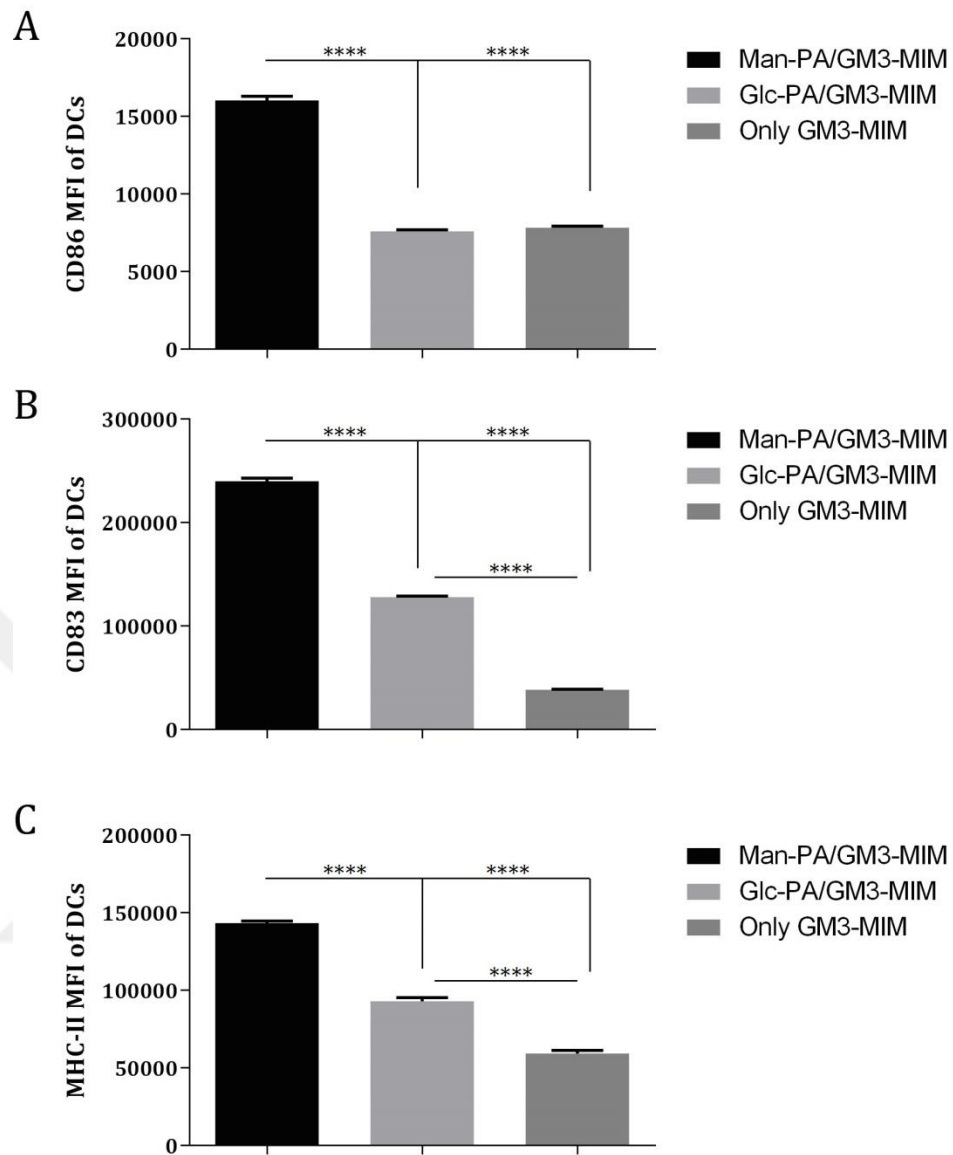


Figure 25 Surface expression MFI values of (A) CD86, (B) CD83 and (C) MHC-II under Man-PA/GM3-MIM, Glc-PA/GM3-MIM and GM3-MIM treatment for 24 h. Values represent mean $\pm$ SEM (****p<0.0001).

2.3.5. Morphological Analyses of Dendritic Cell Activation

Morphological changes occurring during the activation of iDCs into mDCs were also analyzed with SEM imaging. Cells treated with Man-PA/GM3-MIM matured to a greater extent than other groups, although mDC-like morphologies were also observed in Glc-PA/GM3-MIM and only GM3-MIM groups. Additionally, the effects of Man-PA and Glc-PA in the absence of GM3-MIM loading were also analyzed in order to ensure that the maturation of the cells was due to antigen delivery rather than the PA molecules (Figure 26).

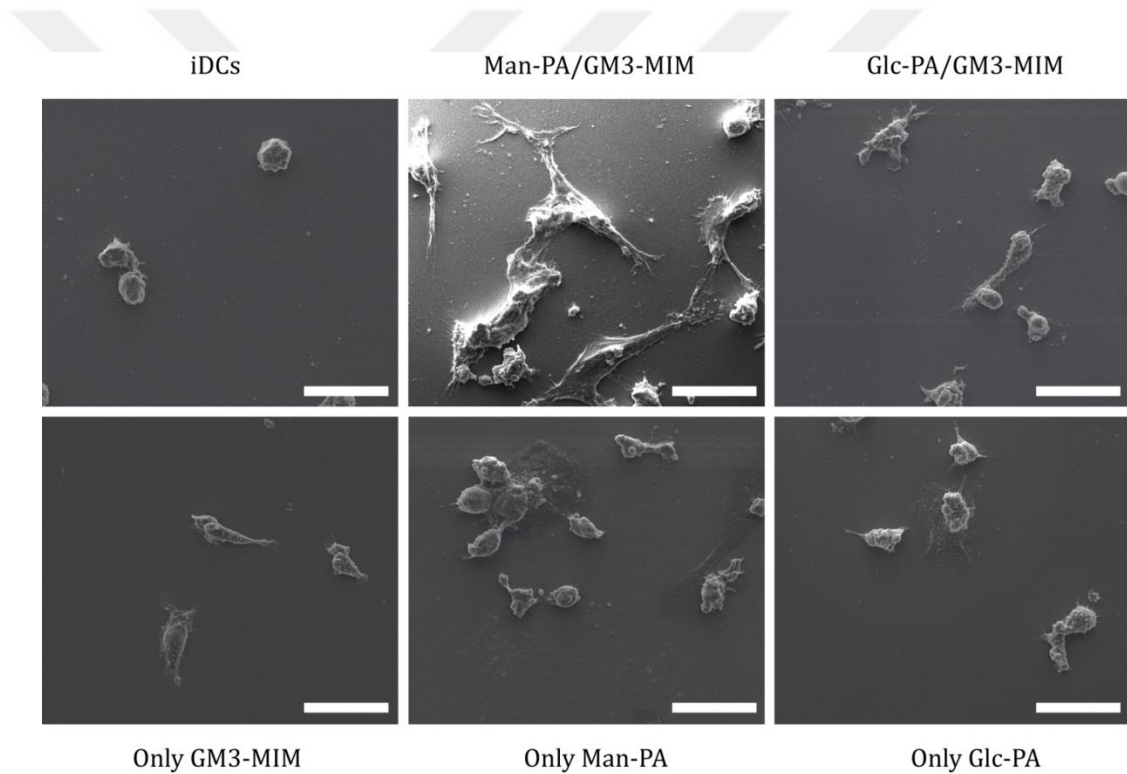


Figure 26 Morphological analyses of the differentiation in iDCs when treated with Man-PA/GM3-MIM, Glc-PA/GM3-MIM, only GM3-MIM, only Man-PA and only Glc-PA for 24 h. Scale bars are 30 μm.

2.4. Conclusions and Future Perspectives

In this study, peptide amphiphile molecules bearing mannose moieties were used for the targeting of the DC-SIGN receptor and the delivery of an immunogenic mimetic of GM3-lactone antigen to induce DC maturation and activation [39]. GM3-MIM presented on Man-PA nanofibers were more efficient than Glc-PA/GM3-MIM and GM3-MIM in terms of antigen internalization, stimulation of CD86, CD83 and MHC-II expression and the induction of a mature DC-like morphology, suggesting that mannose/DC-SIGN interactions are primarily responsible for uptake. Considering the instability of GM3 lactone molecule and its low concentration under physiological conditions, Man-PA is a promising delivery vehicle for the internalization of this immunogenic antigen through DC-SIGN receptors. We showed that the targeted delivery of antigens by using Man-PA to activate iDCs can play important roles in inducing cellular immunity against cancer conditions, and that GM3-MIM can be delivered to the immune system through carrier nanofibers.

Peptide amphiphiles are biocompatible and biodegradable materials with a broad range of potential applications for tissue-specific targeting and delivery. Unlike conventional delivery vesicles, such as liposomes, PA systems do not need to be functionalized with targeting molecules and can naturally localize to tissues of interest due to the bioactive epitopes expressed on their surfaces. These epitope sequences can also be designed to interact with specific cell surface receptors, promoting entry into target cell types. In the present study, the integration of GM3-MIM into the peptide nanofiber structure enabled efficient internalization of the molecule. Considering that highly mannosylated structures are suitable candidates

for DC-SIGN receptor targeting, using sugar moieties for delivery purposes holds high potential for future studies.



Chapter 3

3. Three-dimensional laminin mimetic peptide nanofiber gels for *in vitro* neural differentiation

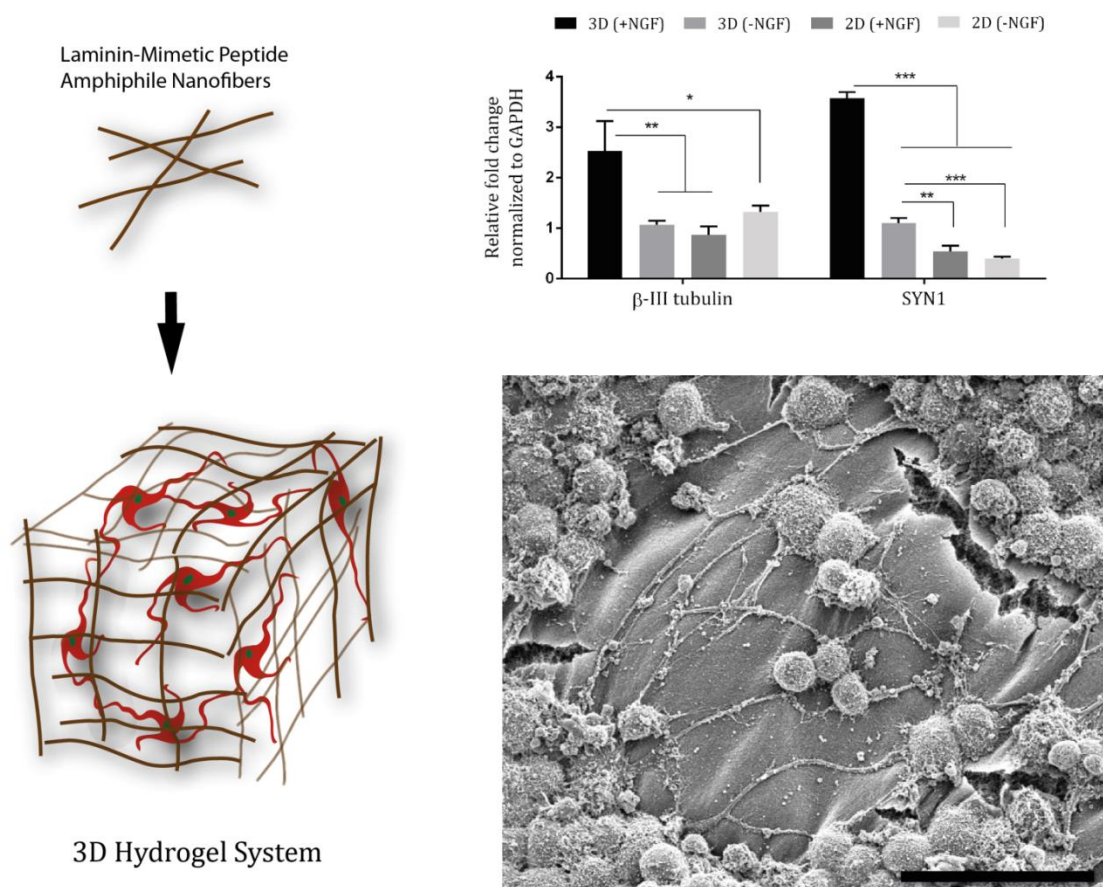
This work is partially described in the following publication:

Effect of 2D and 3D Culturing on *in vitro* Neural Differentiation Under Bioactive Signaling

Melike Sever, Gokhan Gunay, Mustafa O. Guler*, Ayse B. Tekinay*

(Under revision in Biotechnology Journal)

3.1. Abstract



The extracellular matrix (ECM) provides biochemical signals and structural support for cells and its functional imitation is a fundamental aspect of biomaterial design for regenerative medicine applications. In this study, we investigated the stimulation of neural differentiation by a laminin-derived epitope in two-dimensional (2D) and three-dimensional (3D) environments. The 3D gel system was found to be superior to its 2D counterpart for the induction of neural differentiation, even in the absence of a crucial biological inducer, the nerve growth factor (NGF). In addition, cells cultured in 3D gels exhibited a spherical morphology that is consistent with their natural form under *in vivo* conditions. Overall, the present study underlines the impact of bioactivity, dimension and NGF addition, as well as the cooperative effects thereof, on the neural differentiation of PC-12 cells. These results underline the significance of 3D culture systems in the development of scaffolds that closely replicate *in vivo* environments for the formation of cellular organoid models *in vitro*.

3.2. Introduction

The extracellular matrix (ECM) provides biochemical cues and structural support for cells and mimicking the ECM for the investigation of cellular responses has been a great challenge for scientists. Design of biomaterials aims to mimic the native environment of cells (Figure 26) and the interactions of these biomaterials with cells could vary accordingly with the culturing technique. Recently, 3D culturing studies indicated that the cellular responses might significantly change compared to 2D culturing in terms of cellular morphology, viability [72, 73], proliferation [74], signaling [75, 76], differentiation [77, 78] and release molecules [79, 80].

3D hydrogels such as Matrigel[®], collagen and alginate are extensively used in cell culture studies, and their similarity to the natural extracellular matrix contributes strongly to their bioactivity in regenerative medicine applications as delivery systems of bioactive agents and their usage in wound healing and cellular transplantation purposes [81]. 3D Collagen gels on the other hand are very suitable systems for mimicking connective tissue [82]. Matrigel is another 3D gel used for regenerative medicine applications; it contains laminin, collagen IV and entactin in high amounts [83], providing a wide range of applications for cellular studies. 3D collagen gels, for example, are highly suitable for mimicking the connective tissue, while Matrigel[®] is applicable for the culture of a broad range of cells and tissues due to its incorporation of basement membrane elements such as laminin, collagen IV and entactin. However, these materials present significant batch-to-batch variation and do not necessarily provide the full range of ECM-mimetic signals found in natural tissue environments [84, 85]. Consequently, there is considerable potential in developing easy-to-use and tailorable 3D culture systems through the use of synthetic biomaterials.

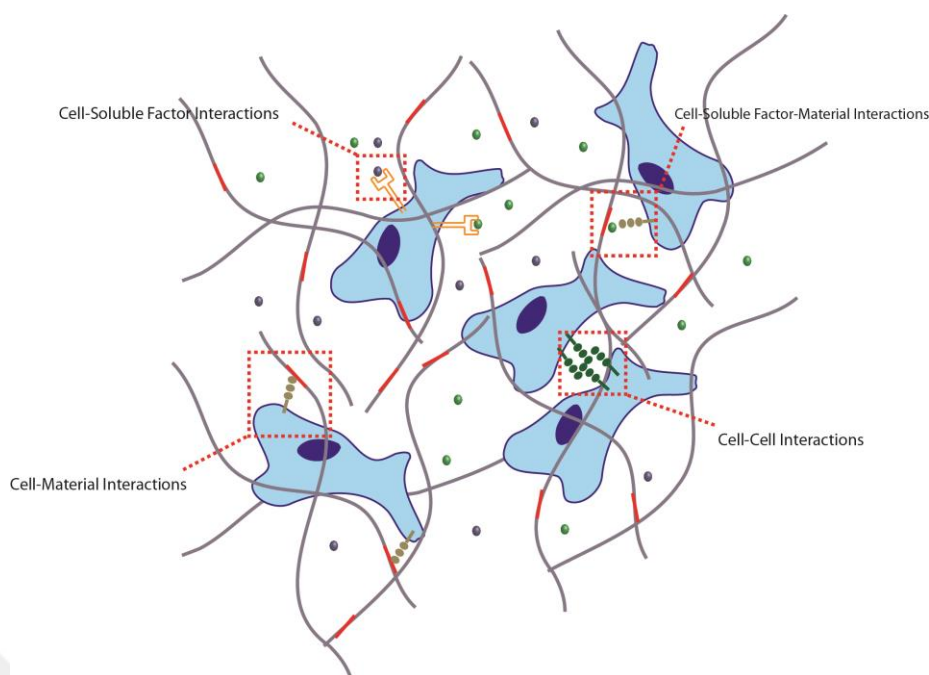


Figure 27 Schematic illustration of cellular interaction with ECM.

Peptide amphiphile systems have been extensively used in neural regeneration applications, and bioactive epitopes derived from ECM proteins, such as the laminin-derived IKVAV and YIGSR, fibronectin-derived RGD, and tenascin-C-derived VFDNFVLK sequences, have been shown to induce neural differentiation, attachment and migration [86-89]. The imitation of ECM proteins is especially important in this context, as integrin receptors and similar mechanotransduction elements are essential for cellular adhesion, homing and differentiation [90]. Many types of integrins, in turn, are directly bound to ECM components [91] and initiate signaling cascades through focal adhesion kinase (FAK) and phosphoinositide 3-kinase pathways. $\beta 1$ integrins, for example, were shown to be effective for stem cell differentiation and self-renewal [92]. Previous studies also indicated that $\beta 1$ integrins regulate the self-renewal and differentiation of neural stem cells (NSCs) through

Notch and EGF receptors [93]. Laminin is another major ECM component of both central and peripheral nervous systems (CNS and PNS) and serves as an important mediator of axonal outgrowth [94]. IKVAV is one of the most extensively studied bioactive epitopes and is located on the α -laminin chain [95], which is essential for the ability of laminin to regulate the overall cellular fate [96]. IKVAV sequence, derived from laminin induces neural differentiation [97], axonal growth after spinal cord injury *in vivo* [98] and neural differentiation of PC-12 cells *in vitro* [31, 87] as a part of peptide nanofiber system. However, no 3D study has so far demonstrated the effect of a laminin-mimetic environment on neuronal differentiation, which is a critical step for the translation of biomimetic scaffolds to clinical applications.

Cell behavior is influenced strongly by the interactions that occur at the cell-biomaterial interfaces, and the development of novel biomaterials that can exercise precise control over cell function are of fundamental importance for tissue regeneration. Consequently, physical properties should also be considered in addition to biological and chemical properties when biomaterials designing for tissue-engineering applications. Parameters such as stiffness and dimensionality are important for scaffold functionalization to induce neural differentiation. When designing a scaffold for neural differentiation, the mechanical properties of the scaffold should be similar to that of brain tissue, the elastic modulus of which is below 1 kPa [99]. Stiffness is also important for differentiation into different cell types. For instance, NSCs prefer to differentiate into neurons under an intermediate substrate stiffness (500 Pa), while increased stiffness favors differentiation into the astrocyte lineage [100]. Dimensionality is another important parameter, and although two-dimensional (2D) cell cultures are commonly used in differentiation studies,

three-dimensional cell cultures are important *in vitro* models to fill the gap between 2D cell culture experiments and *in vivo* studies. 3D models are especially important for studying the regeneration of neural tissue, which has a very low regeneration capacity that may be improved by closely mimicking the native extracellular matrix of neural cells to provide support and enhance the diffusion of oxygen and nutrients [101].

In this chapter, the neural differentiation potential of PC-12 cells was investigated on 2D and 3D laminin-mimetic environments produced through the self-assembly of peptide nanofibers. Effects of bioactivity, dimensionality and NGF addition on neural differentiation were analyzed by monitoring the expression of neurogenic markers and proteins, and scanning electron microscopy imaging was used to observe the overall morphology of cells in 2D and 3D scaffolds. Overall, the present study compares the neural differentiation potential of PC-12 cells under different culturing conditions and highlights the importance of presenting bioactive IKVAV sequence on 3D hydrogels.

3.3. Experimental Section

3.3.1. Materials

All protected amino acids, lauric acid, 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin (Rink amide MBHA resin), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), and diisopropylethylamine (DIEA) were purchased from Nova-Biochem, ABCR, or Sigma-Aldrich. Cell culture materials were purchased from Invitrogen. All other chemicals and materials used in this study were purchased from Thermo Scientific or Sigma-Aldrich.

3.3.2. Peptide Amphiphile Synthesis and Purification

PA molecules were synthesized by using an Fmoc-protected solid phase peptide synthesis method on Rink amide MBHA resin. Positively charged LN-PA (lauryl-VVAGKKIKVAV-Am) was used as a bioactive laminin-mimetic PA molecule. Positively charged KK-PA (lauryl-VVAGKK-Am) and negatively charged EE-PA (lauryl-VVAGEE-Am) were used as nonbioactive molecules for charge neutralization and as epitope-free controls. Couplings of amino acids were done by mixing 2 equivalents of amino acids with 1.95 equivalents of HBTU and 3 equivalents of DIEA for 2 h. Fmoc removal was carried out with 20% piperidine–dimethylformamide (DMF) solution for 20 min. After each coupling step, 10% acetic anhydride–DMF solution was used to permanently acetylate the unreacted amine groups. After each step, DMF and dichloromethane (DCM) were used as washing solvents. Cleavage of PA molecules and protection groups from the resin was carried out by using a mixture of trifluoroacetic acid (TFA): triisopropylsilane (TIS): H₂O at

a 95:2.5:2.5 ratio for 3 h. Rotary evaporation was used for the removal of excess TFA. Ice-cold diethyl ether was used for the precipitation of PAs in the remaining solution. 1 day later, centrifugation was used to collect the precipitate, which was dissolved in ultrapure water. The resulting solution was frozen at -80 °C for 4 h and then lyophilized for 3-4 days. Characterization of PAs was carried out by using liquid chromatography-mass spectrometry (LC-MS). Mass spectra were obtained with Agilent LC-MS equipped with Agilent 6530 Q-TOF with an ESI source and Zorbax Extend-C18 2.1x50 mm column for basic conditions and a Zorbax SB-C8 4.6x100 mm column for acidic conditions. A gradient of (a) water (0.1% formic acid or 0.1% NH₄OH) and (b) acetonitrile (0.1% formic acid or 0.1% NH₄OH) was used for LC-MS. In order to remove the residual TFA, positively-charged PAs were treated with 0.1 M HCl solution and then lyophilized. Peptide purification was carried out with an Agilent preparative reverse-phase HPLC system equipped with a Zorbax Extend-C18 21.2x150 mm column for basic conditions and a Zorbax SB-C8 21.2x150 mm column for acidic conditions. A gradient of (a) water (0.1% TFA or 0.1% NH₄OH) and (b) acetonitrile (0.1% TFA or 0.1% NH₄OH) was used for HPLC. All peptide batches were freeze-dried and dissolved in ultrapure water before use.

3.3.3. Scanning Electron Microscopy

SEM was used to visualize the PA nanofiber networks. 30 µL of oppositely charged PA solutions were mixed to produce gel structures through charge neutralization. Gels were prepared on silicon wafers and then dehydrated by using increasing ethanol concentrations of 20%, 40%, 60%, 80% and 100% v/v. A Tousimis Autosamdri 815B critical point dryer was used to remove the residual ethanol and dried gels were coated with 4 nm Au/Pd prior to imaging. SEM (FEI Quanta 200

FEG), images were taken using an Everhart–Thornley Detector (ETD) system at high vacuum mode at 5 keV beam energy.

3.3.4. Characterizations of Self-Assembled PA Nanofibers

Oppositely charged PA solutions were mixed at a concentration of 2.5×10^{-4} M in appropriate volumes in order to obtain nanofibers with neutral charges. 120 μ L of LN-PA and 180 μ L of EE-PA were mixed to obtain charge neutralization for laminin mimetic peptide nanofibers. 150 μ L of KK-PA was mixed with 150 μ L EE-PA for epitope free control peptide nanofibers with neutral charge (1:1 ratio for KK-PA/EE-PA and 2:3 ratio for LN-PA/EE-PA nanofibers). Measurements were carried out using a Jasco J815 CD spectrometer from 300 nm to 190 nm by adjusting data interval and data pitch to 0.1 nm and scanning speed to 100 nm min⁻¹. Digital Integration Time (DIT) was adjusted to 1 s and band width to 1 nm. All measurements were repeated 3 times.

3.3.5. Oscillatory Rheology Measurements

An Anton Paar Physica RM301 Rheometer, operating with a 25 mm parallel plate configuration at 25 °C, was used for the measurements. Different concentrations of PA molecules were prepared in a total of 250 μ L and loaded onto the lower plate center, and gels were then incubated for 10 min before each measurement to ensure complete charge neutralization and gelation. After reaching equilibrium, the upper plate was lowered to have a gap distance of 0.5 mm. Storage modulus (G') and loss modulus (G'') values were scanned from 100 rad s⁻¹ to 0.1 rad s⁻¹ of angular frequency, with a 0.5% shear strain. All measurements were repeated 3 times.

3.3.6. Zeta Potential Measurements

Zeta potential of the nanofibers was measured by a Malvern Nanosizer/ZetaSizer Nano-ZS ZEN 3600 (Malvern Instruments, USA) instrument. Measurements were performed in quartz cuvettes and repeated three times. Samples were prepared by dissolving each component in water at a concentration of 250 μ M.

3.3.7. *In vitro* Studies

PC-12 cells were used in all cell culture experiments. 25 cm² flasks were used for culturing cells in a humidified incubator at 37 °C, supplied with 5% CO₂. Cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% horse serum (HS), 5% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (P/S). Medium was changed every 3 days.

2D cell culture was carried out by coating positively and negatively charged PA solutions to 6-well plates, which were incubated at 37 °C for 30 min and placed in a laminar flow hood at room temperature overnight for the evaporation of solvent. The PA matrix formed on the plates was UV-sterilized for 45 min prior to cell seeding. PC-12 cells were seeded onto the plates at a density of 1.5×10^5 cells/well. For 3D gel formation, positively charged PA molecules were dissolved in 30 μ L of 0.25 M sucrose and negatively charged PA molecules were dissolved in 30 μ L of culture medium. Initially the first layer of the 3D gel was prepared, and gels were placed into the incubator for stabilization for 1 h. On this gel, positively charged PA solution was added, and negatively charged PA solution containing PC-12 cells at a density of 5×10^5 cells/well was added very slowly in order not to impair gel structure during self-assembly. Gels containing the cells were placed into the incubator for stabilization, and medium was added to each well after 1 h. Cellular differentiation

was induced after 1 day with differentiation medium supplemented with 20 ng/mL NGF and the experiment was ended at the end of 7 days.

3.3.8. Neurite Extension Assay

2 mM LN-PA and 3 mM EE-PA were used to form gels on 6-well plates and 3 mM KK-PA and 3 mM EE-PA were used as a control group. After coating, plates were incubated at 37 °C for 30 min, and excess solvent was evaporated in a laminar flow hood at room temperature. Plates were UV-sterilized prior to cell seeding. PC-12 cells were seeded onto the plates at a density of 1.5×10^5 cells/well. Cells were initially seeded in RPMI medium supplemented with 10% HS, 5% FBS, 2 mM L-glutamine and 1% P/S, and the medium was changed after 1 day with differentiation medium containing MEM with 2% HS, 1% FBS, 2 mM L-glutamine and 1% P/S along with 20 ng mL⁻¹ NGF. At the end of 7 days, light microscope images were taken at 6 random points for each well at 200x magnification. Image J program was used to quantify neurite length and these values were normalized by cell number/image. Neurite-bearing cells were also analyzed by using Image J. Overall results were quantified by counting cells bearing neurite/total cell number (%). Statistical analysis of the results was performed by one way ANOVA.

3.3.9. SEM Imaging of PC-12 Cells Cultured on 2D and in 3D Scaffolds

SEM (FEI Quanta 200 FEG) imaging was carried out in order to evaluate the morphology and neurite extension pattern of PC-12 cells by using an ETD detector at high vacuum mode at 5 keV beam energy. After 7 days of culturing, cells were washed once with 1X PBS and fixed with 2% glutaraldehyde/PBS and 1 wt % OsO₄ for 1 h each. After fixation, cells were washed with ddH₂O and dehydrated by using increasing ethanol concentrations of 20%, 40%, 60%, 80% and 100% v/v. A tousimis

Autosamdri 815B critical point dryer was used afterwards and dried samples were coated with 4 nm Au/Pd prior to imaging.

3.3.10. Gene Expression Analyses

Quantitative real time-PCR (qRT-PCR) was used to quantify the gene expression profiles of PC-12 cells cultured on 2D and in 3D scaffolds. RNA was isolated from cells at the end of 7 days using TRIzol (Invitrogen) according to the manufacturer's instructions. Nanodrop 2000 (Thermo Scientific) was used to examine the yield and purity of the extracted RNA. Primer sequences were designed by using NCBI database. cDNA synthesis from RNA and qRT-PCR were carried out using SuperScript III Platinum SYBR Green one-step qRT-PCR kit according to the manufacturer's instructions. Reaction conditions for gene expression analyses were: 55 °C for 5 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, X °C for 30 s (varies with regards to primer sets), and 40 °C for 1 min, followed by a melting curve analysis in order to confirm product specificity. A standard curve was plotted to evaluate the reaction efficiencies for each primer set by using 5-fold serial dilutions of total RNA. Target genes used for qRT-PCR are; β III tubulin, synaptophysin as neural differentiation markers and GAPDH as a reference gene. Primary gene expression data was normalized to GAPDH and Ct method (Pfaffl) was used for analyses.

3.3.11. Western Blot Analysis

Total protein samples of PC-12 cells cultured in different conditions (2D or 3D culture, in the presence or absence of NGF) were isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific). Equal amounts of proteins (50

μg, 15 μL) were separated on 12% SDS-PAGE gels under denaturing and nonreducing conditions and then transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBST at room temperature for 1 h and then incubated with β-III tubulin antibody (Abcam, ab78078, 1:1000) at 4 °C overnight. After washing in TBST, the blots were incubated with an HRP-conjugated secondary antibody (Millipore, 12–349 goat anti mouse IgG, 1:1000). Signals were visualized using a chemiluminescent signal enhancement system (Invitrogen, Novex ECL). GAPDH was used as the internal control (Millipore, MAB374, 1:500). Gels were visualized by enhanced chemiluminescence (Bio-Rad) according to the manufacturer's protocol on a ChemiDoc™ Imaging System with Image Lab™ Software (Bio-Rad), and protein concentrations in gel slabs were quantified by using Image J. Intensities of β-III tubulin bands were normalized by GAPDH.

3.3.12. Statistical Analysis

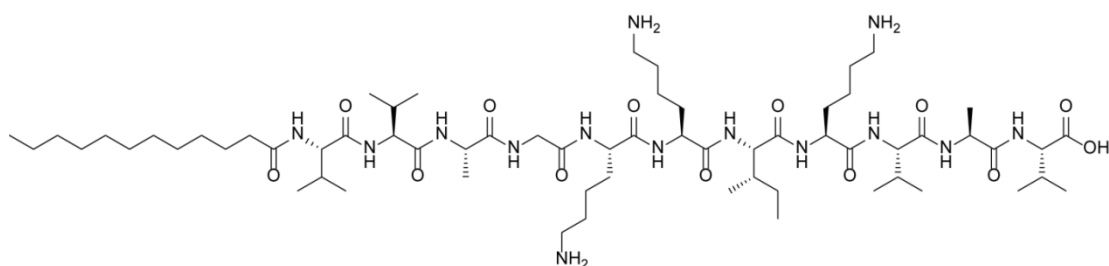
All quantitative values are presented as mean ± SEM (standard error of means), and experiments were carried out with at least three replicates. One-way ANOVA was used for statistical analyses, and Bonferroni was used as a post-hoc test with ANOVA. All groups were compared with each other, and p-values of less than 0.05 were considered statistically significant.

3.4. Results and Discussion

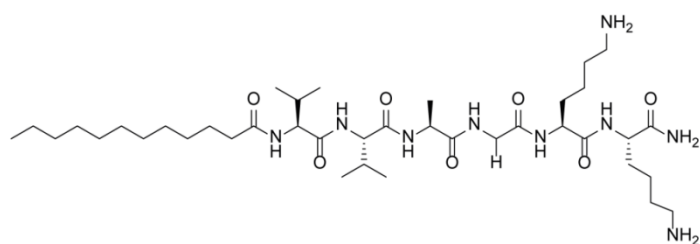
3.4.1. Design and Characterization of Peptide Amphiphile Nanofibers

In this study, laminin-derived epitope-presenting 3D peptide nanofiber gels were constructed by using LN-PA (lauryl-VVAGKKIKVAV-Am) to examine the neuronal differentiation potential of PC-12 cells in a 3D environment. Positively

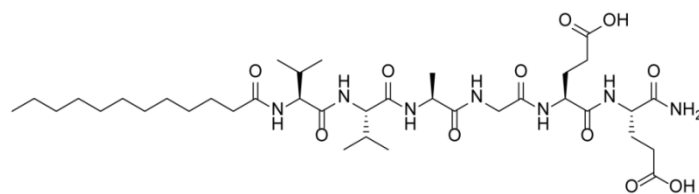
charged KK-PA (lauryl-VVAGKK-Am) and negatively charged EE-PA (lauryl-VVAGEE-Am) (Figure 27) were used as nonbioactive molecules for charge neutralization and as epitope-free controls. Peptides were synthesized by solid phase peptide synthesis, characterized by LC-MS and purified by HPLC (Figure 28). Self-assembling PA molecules were designed with a hydrophilic peptide that is conjugated to a hydrophobic fatty acid, triggering the self-assembly of PA molecules into nanostructures in aqueous solution [102]. It was shown that when two oppositely charged peptide amphiphiles carrying different bioactive epitopes are mixed, they can self-assemble into nanofibers at physiological conditions through electrostatic interactions between ionic amino acids of peptide amphiphiles [22]. Noncovalent forces such as hydrogen bonding, hydrophobic and electrostatic interactions between PAs trigger and stabilize the fiber formation [103]. In this study, the KK-PA and LN-PA molecules formed nanofibrous systems upon mixing with negatively charged EE-PA molecule through electrostatic interactions, assembling into a H-bonded β -sheet secondary structure [22]. Secondary structure analyses of coassembled PAs were performed using circular dichroism (CD) spectroscopy and confirmed that LN-PA/EE-PA and KK-PA/EE-PA systems exhibited β -sheet conformation, displaying a negative minimum peak around 220 nm and positive ellipticity around 202 nm (Figure 29).



LN-PA: Lauryl-VVAGKKIKVAV-Am



KK-PA: Lauryl-VVAGKK-Am



EE-PA: Lauryl-VVAGEE-Am

Figure 28 Chemical structures of peptide amphiphile molecules (LN-PA, KK-PA and EE-PA).

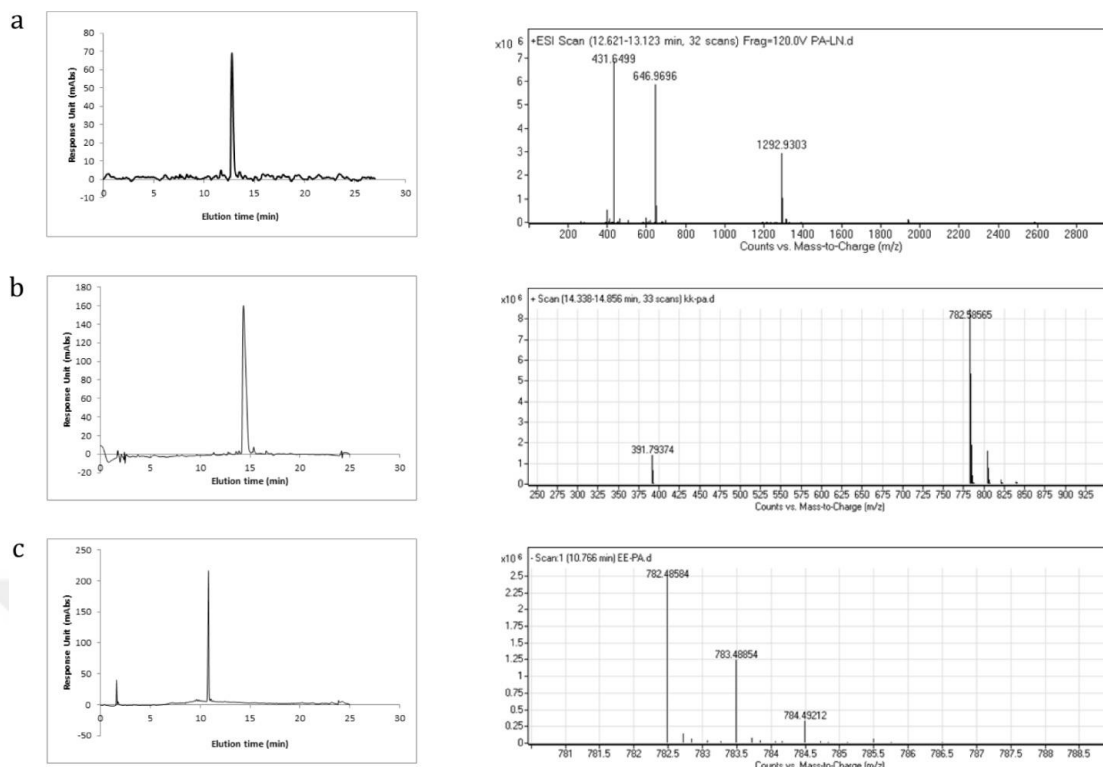


Figure 29 Liquid chromatography and mass spectroscopy of LN-PA (A), KK-PA (B) and EE-PA (C). Mass spectrometry of LN-PA; $[M+H]^+$ (calculated): 1292.93, $[M+H]^+$ (observed): 1293.93, $[M+2H]^{+2/2}$ (calculated): 646.96, $[M+2H]^{+2/2}$ (observed): 646.97, $[M+3H]^{+3/3}$ (calculated): 431.64, $[M+2H]^{+2/2}$ (observed): 431.65. Mass spectrometry of KK-PA; $[M+H]^+$ (calculated): 782.58, $[M+H]^+$ (observed): 782.59, $[M+2H]^{+2/2}$ (calculated): 391.79, $[M+2H]^{+2/2}$ (observed): 391.79. Mass spectrometry of EE-PA; $[M-H]^-$ (calculated): 782.47, $[M-H]^-$ (observed): 782.48.

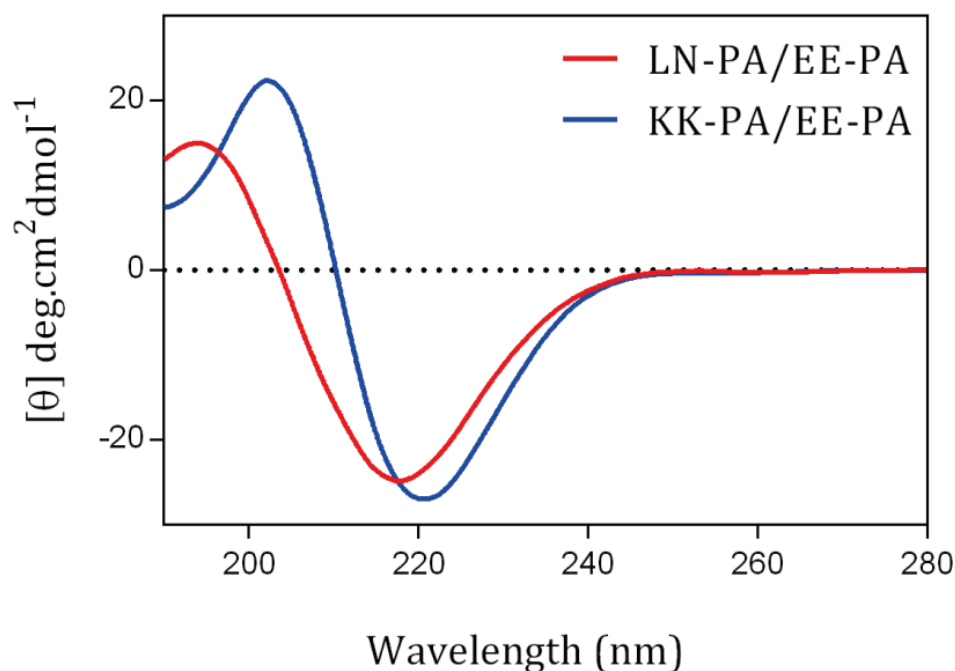


Figure 30 CD spectra of LN-PA/EE-PA and KK-PA/EE-PA nanofiber systems.

Mechanical properties of LN-PA/EE-PA and KK-PA/EE-PA were analyzed by oscillatory rheology. Since the elastic modulus of the ECM has major influence over cellular differentiation patterns [104], LN-PA/EE-PA and KK-PA/EE-PA gels were prepared to imitate the mechanical properties of the brain ECM, which has an elastic modulus of ~ 1 kPa. Gel components were optimized to the ratios of (4 mM) LN-PA/(6 mM) EE-PA and (10 mM) KK-PA/(10 mM) EE-PA, as these mixtures exhibited a comparable elastic modulus to brain ECM while retaining an overall neutral charge. According to equilibrium modulus values, storage modulus was greater than the loss modulus for both mixtures, indicating that the resulting gels show elastic solid behavior (Figure 30). Both gels were prepared at physiological pH.

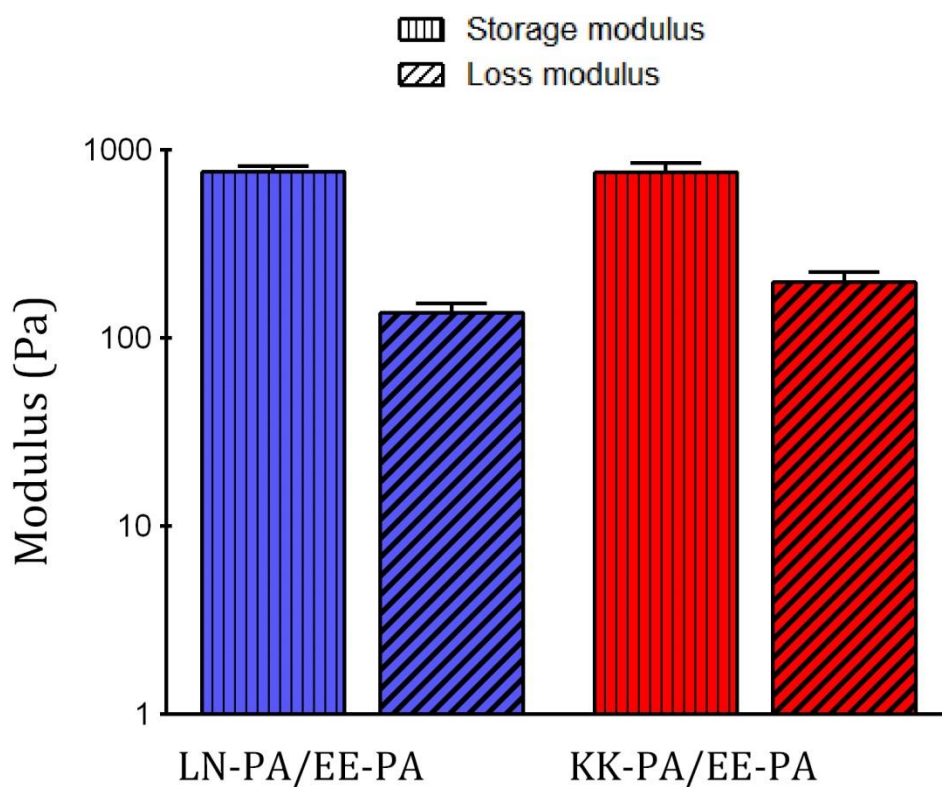


Figure 31 Equilibrium storage and loss moduli of LN-PA/EE-PA and KK-PA/EE-PA hydrogels prepared in water at pH 7.

Scanning electron microscopy (SEM) was utilized in order to analyze the morphological characteristics of 3D nanofiber network of LN-PA/EE-PA and KK-PA/EE-PA gels. Structural organization of LN-PA/EE-PA and KK-PA/EE-PA gels was similar to native ECM and could be expected to provide a supportive physical, chemical and biological environment to cells (Figure 31A and 31B). In addition, zeta potential analysis was carried out in order to analyze the charge of the both epitope-free control and laminin-mimetic nanofibers. Charges were found to be around -10 mV, which is in acceptable range of neutral charge (Figure 32).

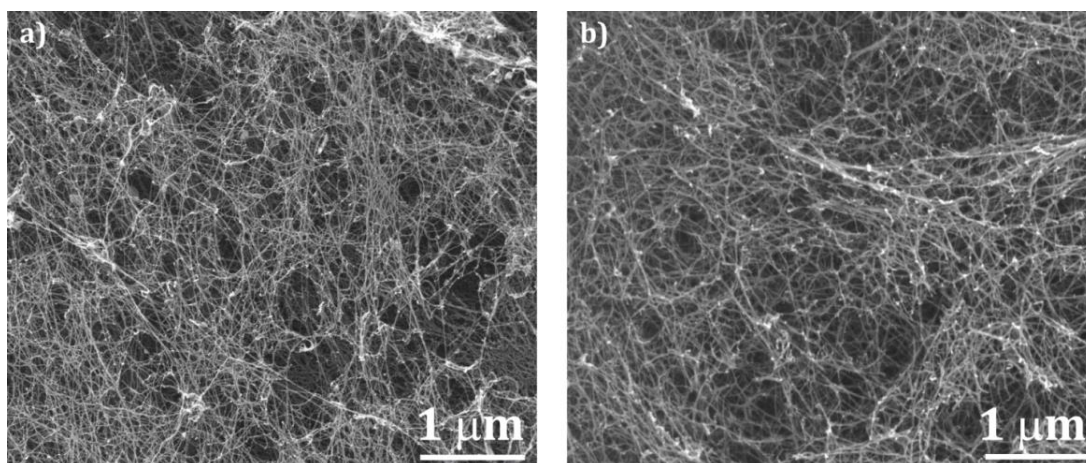


Figure 32 Microscopic characterization of peptide nanofibers. SEM images of a) LN-PA/EE-PA and b) KK-PA/EE-PA.

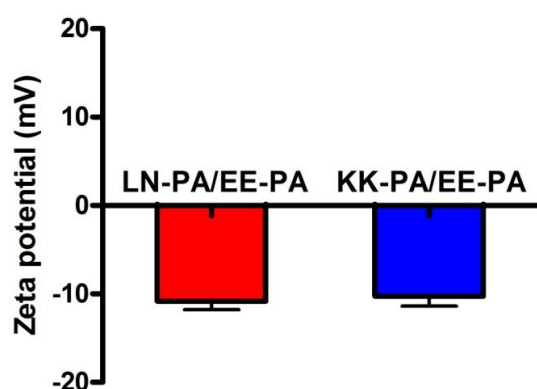


Figure 33 Zeta potential measurements of the peptide nanofibers.

3.4.2. Neural Differentiation Potential in Laminin Mimetic 2D and 3D Environment

PC-12 cells were cultured on LN-PA/EE-PA and KK-PA/EE-PA nanofibers (2D) in the presence of NGF to analyze the effect of 2D culturing on neural differentiation. Neurite length (μm) and neurite-bearing cell (%) analyses were performed using

Image J from images taken at the end of the 7-day culture period. The LN-PA/EE-PA scaffold significantly enhanced neurite length (Figure 33C) and the number of cells bearing neurites (Figure 33C) compared to the non-bioactive control scaffold. Representative images of cells cultured on LN-PA/EE-PA (Figure 33A) and KK-PA/EE-PA (Figure 33B) indicated that cells exhibited a flat morphology and only bidirectional neuronal outgrowth on 2D culture. Additionally, no neurite outgrowth was observed in either group without NGF addition (Figure 34).

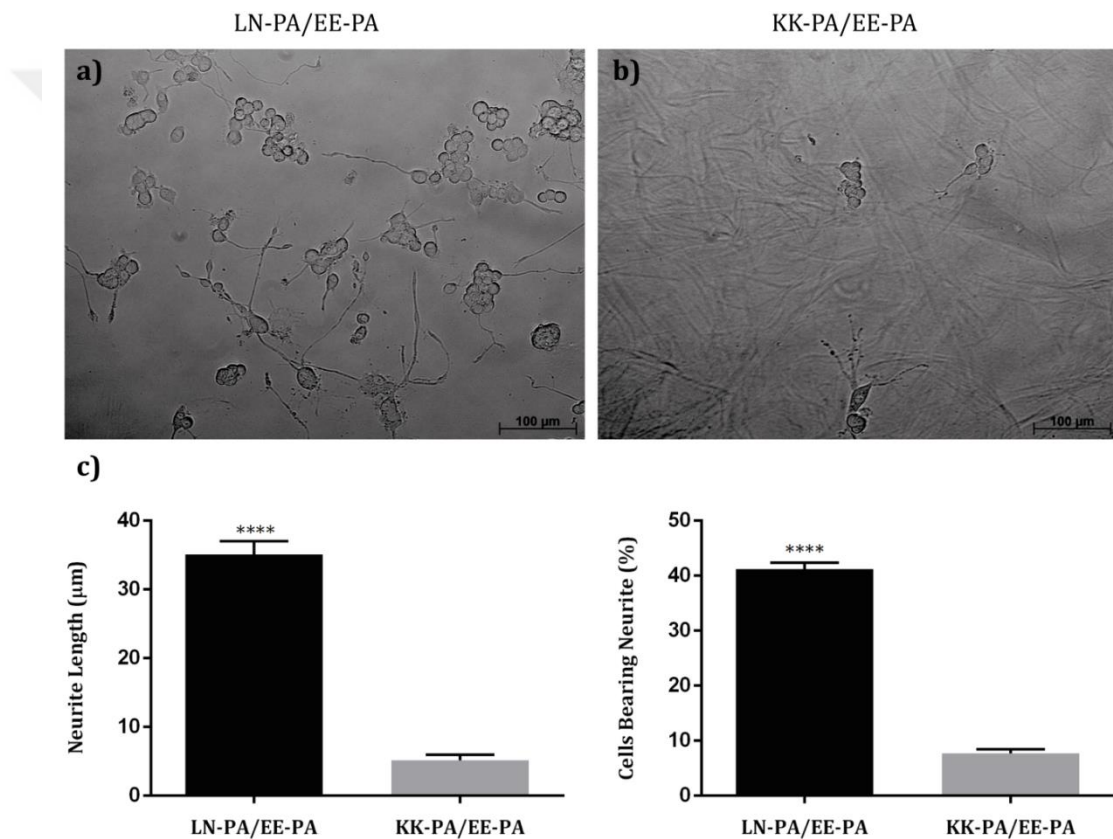


Figure 34 PC-12 cells cultured on a) LN-PA/EE-PA and b) KK-PA/EE-PA nanofibers (2D) for 7 days, scale bars: 100 μm . c) Quantification of neurite length and percentage of neurite bearing cells for 7 days. Values represent mean $\pm$ SEM (**** $p < 0.0001$).

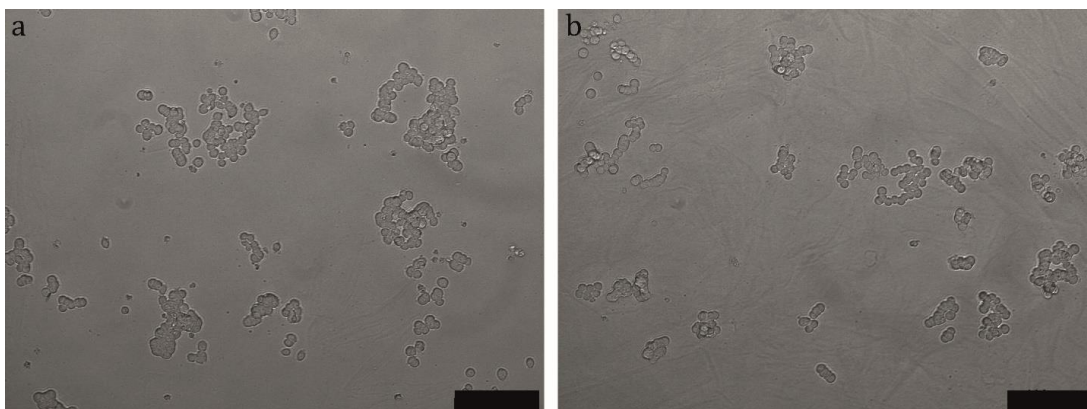


Figure 35 PC-12 cells cultured on LN-PA/EE-PA (a) and KK-PA/EE-PA (b) nanofibers (2D) for 7 days without NGF addition, scale bars: 100 μm .

3.4.3. Neural Gene Expression Analysis

To further investigate the neural differentiation of PC-12 cells, β -III tubulin and SYN1 gene expression analyses were carried out by culturing cells on both LN-PA/EE-PA and KK-PA/EE-PA nanofibers (2D) and gels (3D) for 7 days in the presence and absence of NGF. β -III tubulin is a microtubule element found in the nervous system [105] and synaptophysin (SYN1) is a major synaptic vesicle membrane protein that is expressed in neurons [106]. qRT-PCR analysis showed that the 3D LN-PA/EE-PA gel environment significantly enhanced the expression of β -III tubulin and SYN1 genes, as the cooperative effect of NGF and 3D culturing increased the expression of β -III tubulin more than twoFolds and SYN1 more than threeFolds compared to other groups. The twoFolds increase in SYN1 expression in 3D (-NGF) scaffolds compared to the 2D (+NGF) group is especially striking and suggests that the regulation of SYN1 is affected by both bioactivity and dimension (Figure 35A). As SYN1 expression was significantly increased in the 3D (-NGF) group compared to both 2D (+NGF) and 2D (-NGF) groups, it is also evident that the

bioactive 3D environment is more effective in eliciting neural differentiation than its 2D counterpart, even when the latter is supplemented by NGF.

In the control groups, expressions of both neural genes were strongly influenced by the presence or absence of NGF, and differences between the groups were not as sharp as under bioactive conditions (Figure 35B). These results demonstrated that, when cells were cultured on nonbioactive scaffolds, NGF presence is more important than scaffold morphology for altering gene expression levels. In contrast, cells cultured on bioactive environments responded both to NGF presence and scaffold morphology, suggesting that a level of interplay occurs between the physical microenvironment and bioactive signaling pathways for the regulation of gene expressions. Additionally, PC-12 cells showed the highest neural differentiation potential in terms of gene expression when cultured in 3D (+NGF) LN-PA/EE-PA (Figure 36), further confirming the synergistic effect of bioactivity, dimensionality and NGF addition.

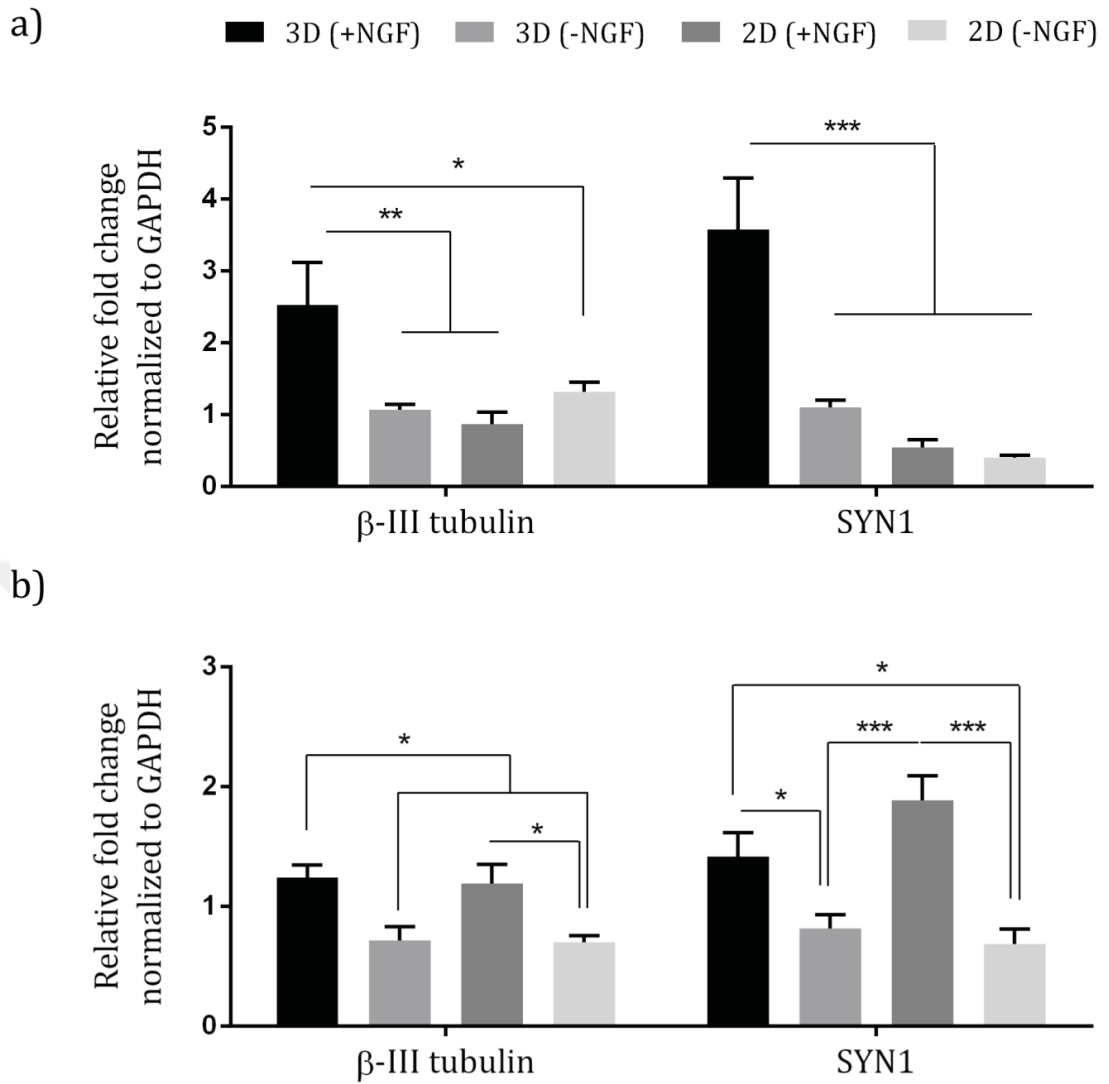


Figure 36 Gene expression analyses of β -III Tubulin and SYN1 on day 7 in 2D nanofibers and 3D hydrogels with and without the addition of NGF. Expression level of each gene was normalized to GAPDH. a) Gene expression levels of PC-12 cells cultured on LN-PA/EE-PA nanofibers and hydrogels. b) Gene expression levels of PC-12 cells on KK-PA/EE-PA nanofibers and hydrogels. Values represent mean $\pm$ SEM (*** p <0.001, ** p <0.01, * p <0.05).

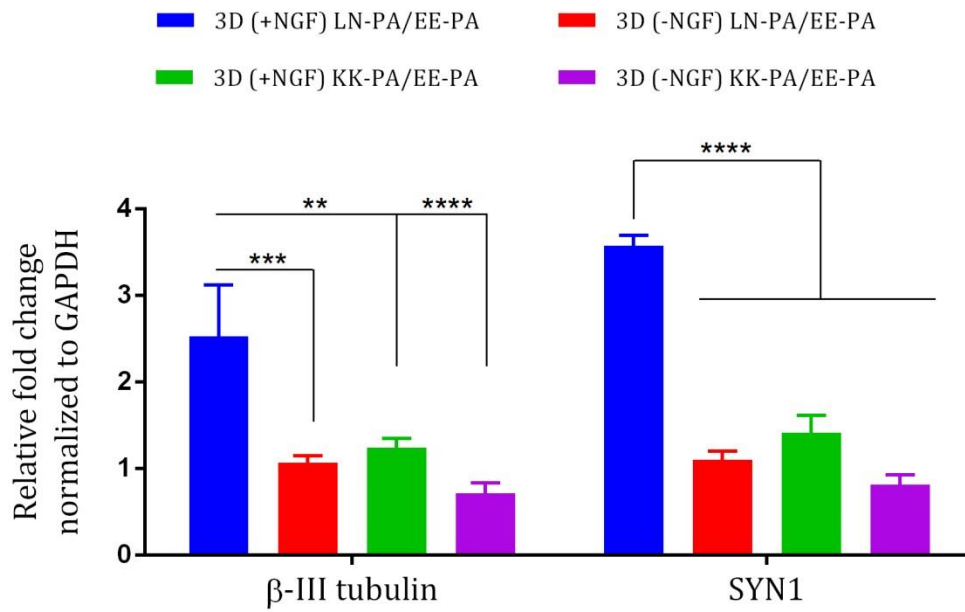


Figure 37 Gene expression analyses of β -III tubulin and SYN1 on day 7 in 3D hydrogels with and without the NGF addition. Expression level of each gene was normalized to GAPDH. Values represent mean $\pm$ SEM (**** p <0.0001, *** p <0.001, ** p <0.01).

3.4.4. Western Blot Analysis

Neural differentiation of PC-12 cells in 3D scaffolds was further characterized by Western blot analysis to investigate the changes in β -III tubulin levels at later stages of culture. β -III tubulin was significantly upregulated at the protein level in 3D LN-PA/EE-PA gels in the presence of NGF (Figure 37). Moreover, cells cultured in 3D KK-PA/EE-PA gels with NGF addition exhibited greater β -III tubulin expression compared to samples lacking NGF, demonstrating the influence of this growth factor in regulating β -III tubulin expression when culturing cells in a 3D environment.

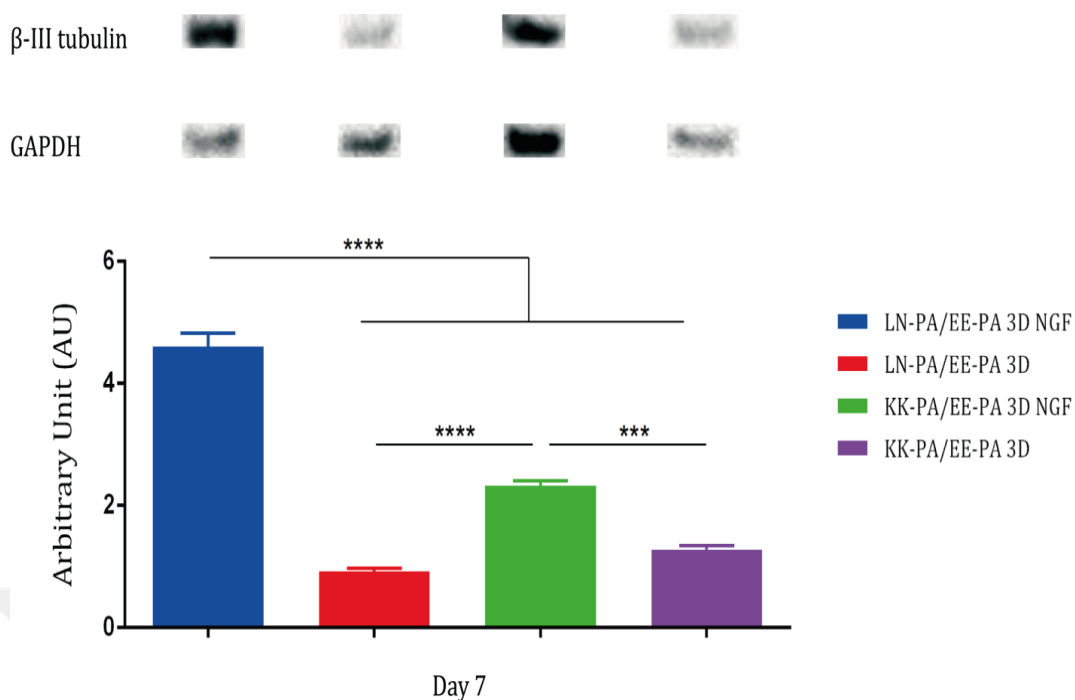


Figure 38 β -III tubulin expression of cells cultured in 3D hydrogels with and without NGF addition on day 7. The density of the bands were analyzed by Image J and normalized to GAPDH. Values represent mean $\pm$ SEM (**** p <0.0001, *** p <0.001).

3.4.5. SEM Imaging

Neurite extension and the morphology of PC-12 cells were visualized through SEM imaging after 7 days of culturing on LN-PA/EE-PA and KK-PA/EE-PA nanofiber surfaces and gels with and without the addition of NGF. Cells cultured on LN-PA/EE-PA nanofiber surfaces and gels showed more neurite extension in the presence of NGF. However, it is notable that neurite extension was also observed without NGF addition in cells cultured in 3D (Figure 38), which underlines the importance of 3D culturing for neural differentiation. Cells cultured in 3D environments also had round morphologies, whereas they had a more flat morphology when cultured in a 2D environment. Different cell morphology and the

outgrowth patterns of neurites in different dimensions are the result of cell-to-3D material and cell-to-cell interactions (Figure 38 and Figure 39).

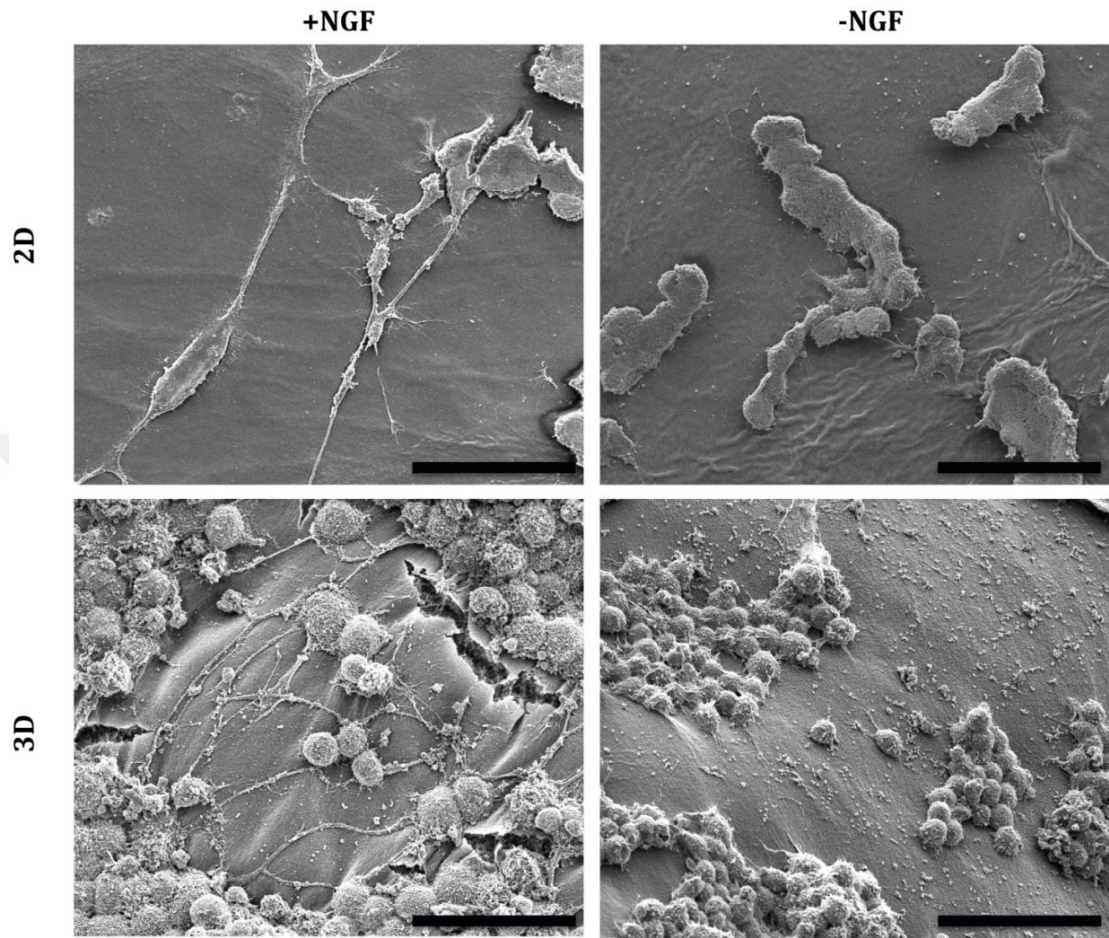


Figure 39 Neurite extension and morphological characteristics of PC-12 cells cultured on 2D and 3D LN-PA/EE-PA with and without the addition of NGF, scale bars: 40 μm.

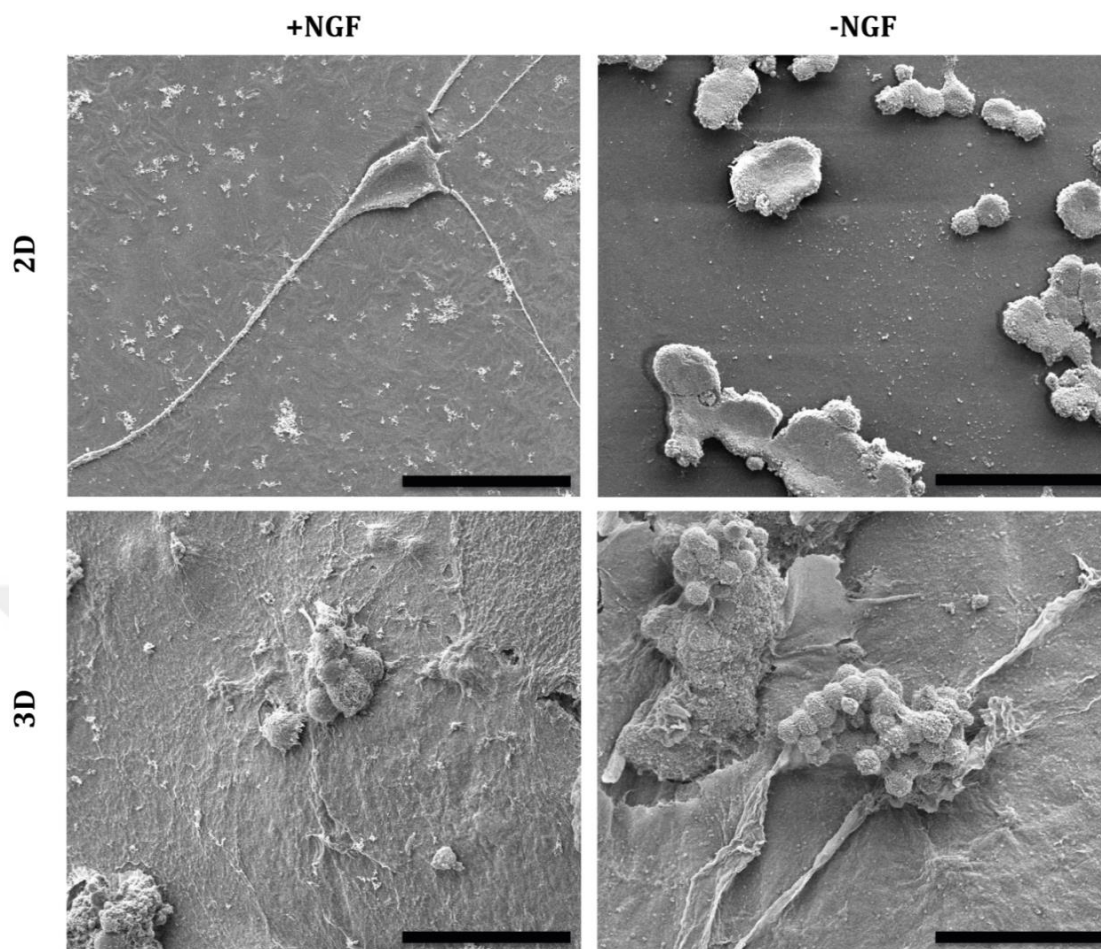


Figure 40 Neurite extension and morphological characteristics of PC-12 cells cultured on 2D and 3D KK-PA/EE-PA with and without the addition of NGF, scale bars: 40 μm .

3.5. Conclusion and Future Perspectives

Depending on the application of the materials, different types of matrix materials have been used to support 3D cell culture, which includes synthetic polymers, natural polymers, natural-synthetic hybrids, ceramics, glass and carbon nanotubes [107]. Within these materials, hydrogel scaffolds, both natural and synthetic hydrogels, are a great option for engineering the neural tissue due to easy modification to mimic the

elastic properties of nervous system. Although synthetic hydrogels are preferred because of their ease to control their physical and chemical properties, they lack the biological activity of natural polymers. Natural hydrogels of macromolecules such as laminin, collagen and hyaluronic acid have biological activity; however, their use in clinical application is limited due to risk of immune reaction [108]. Synthetic hydrogels with controlled chemical and physical properties are also biodegradable. They can be degraded by cells while the cells synthesize their own ECM [109]. More recent examples of synthetic hydrogels use self-assembling peptide hydrogels which have porous structure similar to natural ECM, and bioactivity can be introduced by incorporating ECM-derived signals. RADA peptides functionalized with IKVAV epitope were used to enhance NSC survival and reduce glial cell formation in a 3D system [110]. In another example, neurons encapsulated within aligned nanofibers with Tenascin-C mimetic signals displayed enhanced neurite outgrowth, and also these nanofibers were found to promote neurosphere-derived cell migration [87].

The present study underlines the differences between 2D and 3D cell culturing for neural differentiation. Neural differentiation potential of PC12 cells was investigated on 2D and in 3D peptide nanofiber scaffolds presenting laminin mimetic bioactive signal or control sequence, with or without the addition of external NGF. Compared to controls, the laminin epitope IKVAV could better stimulate neurite outgrowth and neurogenic gene expression when cells were cultured on 2D surfaces, suggesting that bioactive signals have a greater impact on neural differentiation under 2D culture environments. Despite offering more modest improvements over controls, the IKVAV epitope also had synergistic effects with 3D culture conditions, as the combination of the bioactive gel and 3D environment stimulated neurogenesis to a

greater extent than all other groups. Strikingly, synaptophysin gene expression was significantly higher in the (NGF-) 3D bioactive group compared to the (NGF+) 2D bioactive group, indicating the effect of dimensionality on gene expression. Additionally, Western blot analysis indicated that cells cultured in bioactive 3D scaffolds showed significant upregulation in β -III tubulin protein expression, and neurite extension was also enhanced when cells were cultured in a bioactive laminin-mimetic 3D microenvironment. This distinction is vital for mimicking the native extracellular environment of neural systems, as mammalian brains permit cell-cell and cell-ECM interactions in all three dimensions – indeed cells were only observed to extend their neurites in two dimensions on 2D surfaces, while they could extend their neurites in three dimensions when cultured in 3D scaffolds.

Overall, the neural differentiation potential of PC-12 cells was investigated on 2D and in 3D peptide nanofiber systems presenting laminin-mimetic bioactive signals with or without exogenous NGF addition, and scaffold morphology was found to be crucial in determining the extent of differentiation and responsiveness to NGF. It is essential to understand and replicate cellular behavior in *in vitro* conditions for developing better *in vivo* applications; and the 3D scaffold described in the present study accomplishes this by presenting a three-dimensional environment similar to that found in native ECM, mimicking the mechanical characteristics of brain tissue, and incorporating a laminin mimetic IKVAV bioactive epitope for a close representation of *in vivo* conditions. Overall, it is possible to combine biological signals and mechanical properties into peptide nanofiber scaffolds and further improve their usage for regeneration under neurodegenerative disease conditions.

Culturing cells in 3D engineered scaffolds holds high potential for future clinical studies; many studies indicate that, cellular behaviours significantly changes upon 3D culturing in terms of gene expression, protein expression, response to external growth factors and drugs. Therefore constructing 3D scaffolds bearing different bioactive cues for the investigation of cellular responses holds high potential for clinical studies. Moreove 2D *in vitro* neurodegenerative disorder models are limited and do not reflect the natural conditions, on the other hand 3D systems are promising candidates for the construction of more *in vivo* like conditions.

The studies highlighted in this thesis demonstrated the construction of bioactive peptide amphiphile nanofibers for biomedical applications such as targeted drug delivery and tissue engineering. Biological systems are highly complex and constructing peptide amphiphiles with bioactive moieties, which can mimic the microenvironment of cells, have high potential for treating injury and disease conditions. Targeting cells with tailored bioactive moieties are effective in terms of delivery purposes, and construction of scaffolds, which can mimic the extracellular matrix enables more *in vivo* like conditions for cell culture studies.

Pulsing dendritic cells with desired antigens enables the induction of adaptive immunity, however, dendritic cells can also be used to inactivate the adaptive immunity through the induction of immune tolerance by these cells. Within the central nervous system, neuronal cells and immune cells are in constant interaction and changes in the immune cell activation may lead to the progress of neurodegenerative diseases such as multiple sclerosis. Multiple sclerosis is a common autoimmune disease and is characterized by the inflammation of the central nervous system where immune cells attack the myelin sheath and cause

degeneration. Even though there are many studies carried out in 2D cultures for model construction and treatment of MS, overall understanding of its progression is not fully discovered. There are very limited studies about the culturing of immune cells in 3D scaffolds. Considering their activation, migration and ability to induce other cell types within the body, 3D constructed scaffolds are promising candidates for the investigation of immune cell activation as well as the interaction between immune cells and the cells of central nervous system.

Although many studies focus on targeting cells in 2D cell cultures, there is a need for 3D culture in order to properly investigate cellular behaviors of cells. There are many studies indicating that cellular responses significantly changes upon 3D culturing. In order to fully understand the natural responses of the cells, 3D cell culture studies hold high potential for drug delivery and tissue engineering purposes. Additionally, the combination of 3D systems with other systems such as microfluidics may provide more controlled culturing conditions. Media could be provided continuously, and the concentration of growth factors, small molecules, drugs etc. can be better defined and desired mechanical stimulus can be applied. Additionally, directing cellular differentiation through bioactive signals and tissue specific mechanical properties may enable the construction of cellular organoid models *in vitro*.

Even though supramolecular peptide amphiphile nanostructures were designed and widely examined for biomedical applications, their usage in clinical studies are still limited which is caused from safety concerns and technical limitations. Collaboration between industrial companies and scientific community is absolutely required in order to commercialize the nano biomaterials for biomedical and clinical applications.

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