Effects of Different Laminins on Human Neural Progenitor Cells Cultured in 3D Electrospun Fibers

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Effects of Different Laminins on Human Neural Progenitor Cells Cultured in 3D Electrospun Fibers

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Abstract

A low success rate of drug candidates in clinical trials, and especially in brain research, motivates development of better screening models. Hence, in neuroscience and many other fields of Life Science, development of more physiological relevant experimental cell-based models is motivated. It's believed that more *in vivo*-like models will give a better outcome in drug discovery- and development. However, until today, cell models typically include non-human cells, a two-dimensional (2D) culture substrate, which may not include proteins present in the cells' native extracellular matrix (ECM). The aim of this thesis work is, therefore, to explore the potential to generate a more physiological relevant cell-based model for brain research, by using a human brain cell line, three-dimensional (3D) culture substrates and the ECM protein laminin.

Electrospinning was used to fabricate 3D culture fiber scaffolds. A human neural progenitor cell (hNPC) line, with demonstrated capacity to form neurons was used and their innate laminin production at 0 and 20 days *in vitro* (DIV) were characterized. 2D and 3D substrates were coated with one subtype of mouse laminin, six subtypes of human recombinant laminins and non-coated substrates served as control. Bio- and immunochemical assays was used to analyze cell viability and overall and neuronal differentiation potential.

We revealed expression of a variety of laminin chains in hNPC, both in an immature stage and after 20 DIV. Compared to the non-coated groups, cell viability was in general equal or better in all laminin-treated groups, especially for the human biolaminins. Level of differentiation stage, judged by nestin (marker for NPC)- and βIII-tubulin (marker for early neurons) expression in immuno-labeling and western blot, may be affected by different human biolaminins. Specifically, the human biolaminins seem to promote neuronal differentiation compared to the mouse counterpart. Our initial results demonstrate that brain cells have the potential to survive well in functionalized 3D scaffolds, and that different human laminin subtypes most likely affect their viability, differentiation potential differently. However, more studies are needed to confirm the effect of specific human laminins and before a protocol for the culture of *in vivo*-like human *in vitro* brain models can be presented.

Abbreviations and acronyms

2D	two-dimensional
3D	three-dimensional
AB	Alamar Blue
ANOVA	analysis of variance
BM	basement membrane
CCD	charge-coupled device
CNS	central nervous system
DAPI	4,6-diamidino-2-phenylindole
DIV	days *in vitro*
DMEM-F12	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's Phosphate-Saline Buffer
ECM	extracellular matrix
F12	Ham's nutrient mixture F12
FFT	fast Fourier transform
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hbFGF	human basic fibroblast growth factor
hEGF	human epidermal growth factor
hLIF	human leukemia inhibitory factor
HA	Hyaluronic acid
hNPC	human neural progenitor cell
ICC	immunocytochemistry
ND	not determined
NPC	neural progenitor cells
NS	neurospheres
NSC	neural stem cells
PCL	Poly-ϵ-caprolactone
PLL	Poly-L-lysine
PLLA	Poly-L-lactic acid
PNS	peripheral nervous system
PS	Polystyrene
PU	Polyurethane
REDOX	reduction-oxidation
RIPA	radioimmunoprecipitation assay
RT	room temperature
RT-qPCR	real-time quantitative polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	tris-buffered saline
V	volt
v/v	volume/volume
WB	western blot

Popular scientific summary

In brain research, cell culture-based models are widely used as an experimental platform, in both basic research and drug discovery. Such models are useful in initial evaluation on whether a potential drug candidate has promise or not for further development towards use in patients. The average cost from drug-to-market is $2.6 billion, and the time it takes for a new drug from experimental testing to approval from the FDA (sv. Läkemedelsverket) is on average 12-17 years.[1]
If drugs in development with low promise could be detected and discarded earlier, significant amounts of time and money would be saved. This is especially true in brain research, where the success rate of drug candidates is very low in later phases of drug development.

A major issue in drug discovery and development is the lack of physiological relevant models, meaning models that closely mimic the structure and function of the brain. As a start, until today the majority of models are made in two-dimensional (2D) systems - not representing the three-dimensional (3D) environment in the brain. Encouragingly, 3D cell culture systems have shown to more closely mimic the structure and function of the brain, with regard to cell network architecture, affecting cell proliferation, viability, adhesion, and cell development.

Use of 3D hydrogels or physical scaffolds are two different ways for cultured brain cells to receive structural support resembling the cell normal environment. Here we made physical 3D scaffolds by using a technique called electrospinning, by which a melted polymer is spun into thin fibers forming a 3D mesh. Electrospun polymer fiber meshes is a 3D solution to the problems that one can encounter with 2D cultures – it's also a time-efficient and cheap method, has great reproducibility with parameters that are easy to control.

To make physiologically relevant models, not only the topology and 3D architecture need to be mimicked but also the biochemical signature of the cell environment. Most cells of the body grow on specialized, sheet-like extracellular matrix (ECM) structures called basement membranes (BMs). Different cells and tissues require a specific ECM composition for survival and proper function, and many cells themselves produce and deposit ECM proteins. Laminins are abundant components of the BMs and have essential roles in structural organization. In addition, laminins play an essential role in the organization of the BM and the regulation of cell behavior and function. Laminins are multidomain, heterotrimeric glycoproteins, composed of three different subunits: an α-chain, β-chain and γ-chain, combined and expressed in at least 16 different isoforms in the human body.

In this master thesis we investigated the potential to generate a cell-based model for brain research that closely mimics the structure and function of the brain cells and tissue, by using a human brain stem cells, 3D electrospun fiber substrates coated with different human recombinant laminin isoforms, biolaminins. Human brain stem cells were used as a cell tool since they can be expanded into high numbers in culture, which makes them a stable and quite cheap tool to use. We also know from previous studies that they can form the common cell types in the adult brain, neurons and supporting cells (glia), when grown with stimulating factors.

Since cells often lay down the ECM they reside in we wanted to know if hNPCs produce laminin, and if so which subunits, during its different stages by using so-called real-time quantitative PCR (RT-qPCR) which measure the gene expression level, and then we decided which types of laminins to investigate. Human brain cells were cultured for 20 days the effect of 2D versus 3D substrates, and effect of 6 different human laminins (111, 121, 411, 421, 511, 521), mouse laminin 111 and non-coated fibers were studied. Bio- and immunochemical methods were then used to examine cell viability, and the potential for the brain stem cells to mature into neurons.

Our main results show that the brain cells:

- survive equally well up to 20 days at both 2D and 3D scaffold
- on 3D substrates display a better viability with human biolaminin 121 compared to mouse 111
- viability in 2D and 3D cultures were equally and positively affected by the biolaminins investigated
- attach well and integrate into the 3D fiber scaffolds, and a fraction show capacity to form neuron
- in 2D cultures the overall differentiation is favored in all laminin exposed groups
- have a greater potential to form neurons when exposed to all the human biolaminins compared to the mouse laminin at both 2D and 3D surfaces, judged by cell counting of neurons

In conclusion, our initial results demonstrate that brain cells have the potential to survive well in functionalized 3D scaffolds, and that human recombinant laminin subtypes most likely affect their viability, differentiation potential differently. More studies are needed to confirm the effect of specific human biolaminins and before a protocol for the culture of *in vivo*-like human *in vitro* brain models can be presented.

However, our early findings motivate further studies on exploration of novel *in vivo*-like brain models that most likely will contribute to the development of better screening assays that will give a better prediction of the potential of novel drug candidates for diseases affecting the nervous system, and in the longer perspective more treatments available to cure the patients.

Populärvetenskaplig sammanfattning

Inom hjärnforskning används cellkultursbaserade modeller i stor utsträckning som en experimentell plattform, både i grundforskning och läkemedelsupptäckt. Dessa modeller är användbara vid tidig utvärdering av en potentiell läkemedelskandidat. Eftersom nya läkemedel tar extremt mycket tid och pengar att utveckla, vore en så exakt modell som möjligt att föredra.[1]

En viktig fråga i upptäckt och utveckling av läkemedel är bristen på fysiologiskt relevanta modeller, modeller som nära efterliknar strukturen och funktionen i hjärnan. De flesta modeller har historiskt sett varit 2D, något som inte representerar 3D-miljön i hjärnan. 3D-modellerna efterliknar mer strukturen och funktionen i hjärnan, något som påverkar cellförökning, överlevnad, vidhäftning och cellutveckling.

Användning av 3D-hydrogeler eller fysiska ställningar är två olika sätt för odlade hjärnceller att få strukturellt stöd som liknar den normala cellmiljön. Här tillverkade vi fysiska 3D-ställningar med hjälp av en teknik som kallas elektrospinning, där smält polymer spinns till tunna fibrer som bildar ett 3D-nätverk. Elektrospunna polymerfiber är en 3D-lösning på de problem som man kan stöta på med 2D-kulturer – det är också en tids- och kostnadseffektiv metod.

För att göra fysiologiskt relevanta modeller behöver inte bara topologin och 3D-arkitekturen imiteras utan också cellmiljöns biokemiska signatur. De flesta celler i kroppen växer på specialiserade, arkliknande extracellulära matrisstrukturer (ECM). Olika celler och vävnader kräver en specifik ECM-komposition för överlevnad och korrekt funktion, och många celler själva producerar och deponerar ECM-proteiner. Lamininer är en protein i ECM och har väsentliga roller i dess strukturella organisation. Dessutom spelar lamininer en viktig roll i regleringen av cellbeteende och funktion.

I det här examensarbetet undersökte vi potentialen att generera en cellbaserad modell för hjärnforskning som nära efterliknar strukturen och funktionen hos hjärncellerna och vävnaden, med hjälp av humana hjärnstamceller, 3D-elektrospunfibersubstrat belagda med olika humant lamininisoformer, biolamininer. Mänskliga hjärnstamceller användes som ett cellverktyg eftersom de kan öka till ett stort antal inom kulturen, vilket gör dem till ett stabilt och billigt verktyg att använda. Vi vet också från tidigare studier att de kan bilda de vanliga celltyperna i den vuxna hjärnan, nervceller och stödjande celler, när de odlas med stimulerande faktorer.

Sammanfattningsvis visar våra initiala resultat att hjärnceller har potential att överleva väl i funktionaliserade 3D-byggnadsställningar, och att humana lamininsubtyper sannolikt påverkar deras livskraft och differentieringspotential på olika sätt. Fler studier behövs för att bekräfta effekten av specifika humana biolamininer och innan ett protokoll för odlingen av *in vivo*-liknande humana *in vitro*-hjärnmodeller kan presenteras.

Men våra tidiga fynd motiverar ytterligare studier om utforskning av nya *in vivo*-liknande hjärnmodeller som sannolikt kommer att bidra till utvecklingen av bättre screeninganalyser som kommer leda till bättre förutsägelse av potentialen för nya läkemedelskandidater för sjukdomar som påverkar nervsystemet, och i det längre perspektivet fler behandlingar tillgängliga för att bota patienterna.

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Karl Westerlund

Introduction

This thesis work focuses on exploring a three-dimensional (3D) *in vivo*-like brain model by growing human neural progenitor cells (hNPC) on electrospun fibrous scaffolds coated with different forms of the extracellular matrix (ECM) protein laminin. The study is interdisciplinary, involving stem cell biology, neurobiology and nanotechnology. Therefore, the thesis begins with an overall introduction to tissue engineering, both as a concept, and techniques pertinent to this study, followed by a presentation of two-dimensional (2D) versus 3D cell cultures, neural cell tools and the role of the ECM.

Tissue engineering

The term "tissue engineering" was officially coined at a National Science Foundation workshop in 1988, but its history began earlier than that.[2] It can be argued that the first instance of tissue engineering occurred back in 1933 when Vincenzo Bisceglie encased mouse carcinoma in a polymer membrane and inserted them into the abdominal cavity of a pig, to show that the cells could live long enough to not be immediately killed by the immune system.[3] However, the early years of modern tissue engineering can be linked to the beginning of the 1970's, when pediatric surgeon William T. Green at Boston Children's Hospital conducted experiments to grow cartilage tissue by seeding chondrocytes onto bone which was then implanted in mice.[4] While the experiments were ultimately unsuccessful, the conclusion was that the rise of biocompatible materials would then provide the possibility to generate new tissue by seeding the appropriate cells on the appropriate substrate.

As a fairly new and developing interdisciplinary field, it incorporates the principles of engineering with life science by combining biology, materials science and medicine.[5] The idea is to combine cells, culture scaffolds and biologically active molecules into functional tissue with the goal being to rehabilitate, maintain or ameliorate tissue and organs. Regenerative medicine is a field that researches self-healing – the usage of foreign or artificial biomaterials to regenerate cells and also rebuild tissues and organs. Regenerative medicine and tissue engineering is nowadays used interchangeably.[6]

Applications of tissue engineering has since its inception exploded with the advent of biocompatible materials and the continued advancement of biology and medicine. Examples of tissue engineering can be found both in the industry or at research institutions — skin treatment and regeneration from fish skin, 3D-printable tissue via bioink and 3D-printing a human heart.[7, 8, 9] One of the interesting and useful techniques emerging from the discipline is electrospinning, which gives the ability to make fibrous hard scaffolds in a cost-efficient, quick, easily manipulated and reproducible manner.

Electrospinning

Electrospinning is a tissue engineering technique used to produce 3D scaffolds out of a polymer solution (see fig. 1). The properties of the ECM can be replicated through physical (fiber and pore size), mechanical (elasticity) and chemical (adhesion) modifications during production, creating highly porous meshes of fine fibers that resemble the topography of the ECM, and can be modified towards promoting stem cell proliferation and survival, or advance organization of tissue. The achievability of integrating these scaffolds with topographical and biochemical signals are essential to cell manipulation.[10]

There is a high degree of parameter options, where thickness, porosity and structure can all be controlled with ease by adjusting flow rate, polymer solution, voltage, needle type and collector.[11] Because of this, electrospinning is a cheap, fast and reliable technique that is widely used in 3D scaffolding because of its reproducibility and easy-to-control parameters.

There are two polymers widely used in electrospinning for tissue engineering;

- Poly-ϵ-caprolactone (PCL) is a FDA-approved biocompatible, biodegradable polyester used in 3D scaffolding and other biomedical areas of controlled-release drug delivery, surgical sutures and nerve guides.[12, 13]
- Poly-L-lactic acid (PLLA) is a biocompatible, biodegradable polymer that is FDA-approved as a nanoparticle with good mechanical properties, good biocompatibility and complete degradation in living organisms, without adverse side effects.[14, 15]

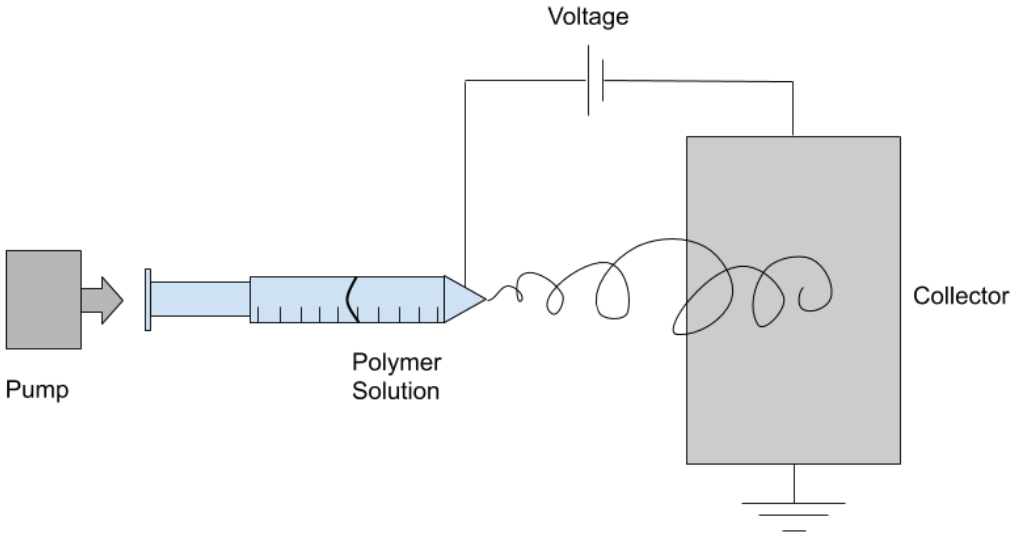

Fig. 1: Schematic representation of an electrospinning setup used for randomized fibers, where the distance between needle and collector, applied voltage and flow rate are some of the parameters affecting the final polymer fiber.

A high voltage is put over a syringe and a grounded collector as the polymer solution is jetted out of the syringe. The solvent will evaporate and left is a polymer fiber that will attach to the collector, creating a low-density fiber matrix (see fig. 1).

Depending on what type of collector used, different structures of polymer fibres can be achieved. For example, a spinning drum collector can create unilaterally linear fibres used in cellular migration studies, a flat collector can create densely packed random fibres used in cell studies.[16] There is also a type of pin needle collector which gives randomized fibres similar to the flat collector, although less dense.

Electrospinning is one way to fabricate 3D culture scaffolds, but there are other ways to go about producing them, and there are other types of such 3D substrates. 3D culture systems have over the last two decades gained lots of attention as they more closely mimic the 3D architecture present in most tissues in the body, including the brain.

Types of 3D culture systems

There are different types of 3D matrices and scaffolds used for cell culturing, all with their respective advantages and drawbacks;

- **Hydrogels** are protein networks swollen with water, with cells either embedded in the gels or seeded on the surface. It provides support for the cells and allows for cell-to-cell interactions.[17]
- **Scaffolds** can be either fibers or sponge-like structures.
- **Cryogels** that have cryogel matrix-like mechanical strength and interconnected porous network as well as the potential for conductive properties.[18]

When it comes to material there are many alternatives, depending on the application and the desired properties;

- **Natural ECM based** (decellularized tissue, collagen, laminin, etc.) - Maximum resemblance to in vivo conditions and good cellular attachment, differentiation and proliferation, but it also has a large variation between matrices, poor mechanical properties and some immunogenic issues. Its applications can be to enhance some native tissue integration, guide cell differentiation by mimicking the ECM environment or induce specific tissue and cell response.
- **Synthetic ECM based** (modified forms of Hyaluronic acid (HA), PCL, Polyurethane (PU), etc.) - Physical and mechanical properties that can be easily reproduced, the polymers used are biodegradable to some degree and can be combined with certain biomolecules such as antibiotics and growth factors. Used as carriers for drug delivery, growth factors, gene transfection and cell therapy.
- **Hybrids of synthetic and biological matter** (PCL-Chitosan, Hydroxyapatite-bioglass-ceramic, PLLA-Hydroxyapatite, etc.) - Possess the predictability and reproducibility of synthetic polymer-based ECM, with added mechanical flexibility and biodegradability. Applications can be to stimulate a healing injury and provide stability to the cells and native tissue until integration, or in vitro 3D models for studying a specific organ.[19]

Other materials and techniques that aren't used in drug research cell cultures are metals (osseointegration and in vivo bone formation and implant material for orthopedic and dental defects), ceramics and bioactive glass (induced bone mineralization) and carbon nanotubes (*in vitro* cardiac tissue constructs, osteoblast growth acceleration, nerve function restoration)

Apart from hydrogels and scaffolds, there is also the scaffold-free technique which lets cells grow into non-adherent aggregates.[20] These are very consistent in size and shape and is a popular use for high-throughput screening.

Despite the different types of cell culture systems, there are still some important properties that one should take heed of when developing them - easiness of fabrication, cost effectiveness, implantability, biocompatibility and biodegradability.

Cell culture in a 2D or 3D environment - does it matter?

Cell culture techniques are ubiquitous utilized in areas of developmental biology and drug discovery. Cell culturing refers to growing cells *in vitro*, (trans. Latin: within the glass). In contrast to *in vitro*, there are *in vivo*-studies that are conducted in animals, humans or plants and two of those forms are animal testing and clinical trials.

In vitro-experiments are conducted in a controlled manner on components of an organism outside its normal environment, such as microorganisms, biological molecules and cells. Cell-free protein expression is a technique useful to quickly express and manufacture small-scale quantities of functional proteins, while cells and tissue can be grown in artificial culture medium; so called cell culture-based assays. Cell culture-based assays are fundamental to life science research, like neuroscience and disease pathology, and drug discovery, since its simple, fast and cost-efficient characteristics allows it to be done without the large scale, expensive studies and, to some, unethical practices of animal testing. The results from these assays are all based on the response from cells, be it from biological compounds or external stimuli. For such an essential method it is therefore necessary to properly explore how cells behave in *in vivo*-like milieus with great accuracy, and it is that desire that ultimately lead to this master's thesis.

Traditionally, cell culture-based assays have used 2D monolayer cells cultured on rigid and flat surfaces such as glass coverslips or tissue culture plasticware such as polystyrene (PS), oftentimes coated with ECM attachment proteins, as an initial model for evaluating the effectiveness of compounds for biological assays. Cells in the physiological environment have constant interaction with the extracellular matrix, regulating complex biological functions like cellular migration, apoptosis, transcriptional regulation, and receptor expression.

It is key, however, that these assays will give a result that translate well when drugs are progressed from drug discovery and preclinical trials into clinical trials. The issue lies when novel drugs show a high efficacy in the discovery phase of drug development in cell culture-based assays, but fail in clinical trials. This is especially prevalent in phase III, the most expensive phase of clinical trials.[21]

One major failure of these assays is that *in vitro*-experimental data cannot be completely translated into clinical trials when cells are grown in 2D conditions since complicated cellular signals between cells and the matrix cannot be reproduced. 3D cell cultures address this challenge and serve as a more accurate representation of *in vivo* physiological conditions, and the difference between some key characteristics is shown in table 1.

More recently, cell cultures based on 3D substrates have become increasingly popular in the drug assays, as these models are much more analogous to *in vivo*, and thus, results gained from them are much more clinically and biologically relevant.

Table 1: Examples of key differences between 2D and 3D cell culture characteristics.[22-28]

Characteristics	2D	3D
Cell shape	Outstretched and flat	Cells natural shape is retained
Cell junctions	Poor resemblance of physiological conditions	Prevalent and enabling cell-to-cell signaling
Differentiation	Poorly differentiated	Well differentiated
Drug metabolism	Low to no metabolism	Enhanced drug metabolism and a higher expression of CYP enzymes
Drug sensitivity	Low resistance and drugs show high potency	Higher resistance and drugs show low potency
Proliferation	High proliferation, higher than in the cells native environment	Depends based on cell type and 3D-cell culture conditions
Viability	Cytotoxin-sensitivity	Less susceptibility to external factors
Stimuli response	Mechanical stimuli induce low response	Mechanical stimuli induce established response

Neural cell tools (fetal and adult)

There are different human cell sources for the generation of neural cell models; including embryonic stem cells, induced pluripotent stem cells and neural stem cells.

Embryonic stem cells (ESC) have the potential for infinite self-renewal and can give rise to all cell types and tissue of its organism, even germ cells and gametes. Induced pluripotent stem cells (IPSC) are somatic stem cells that have been induced to inherit stem cell properties, such as the capability to generate all tissues of an organism. Thus, IPSC hold great potential for therapeutic uses in tissue regeneration and repair for mature individuals.[29] However, the usage of ESCs comes with ethical issues.[30]

Neural stem cells (NSC) is a somatic stem cell class present in the peripheral nervous system (PNS) and central nervous system (CNS) and is found in both fetal and adult neural tissue.[31] NSC, as with all somatic stem cells, possess two fundamental characteristics; infinite self-renewal and, during terminal differentiation, the ability to give rise to the full range of cell types found within neural tissue.[28] A NSC can therefore bring about another NSC or any of other differentiated cell types found in the PNS and CNS — i.e. neurons, astrocytes and oligodendrocytes. It is however important to note that neural stem cells are different from neural progenitor cells (NPC), the latter being incapable of self-renewal and having a limited amount of cell types to be differentiated into.[32]

NPC can be viewed as the further along than NSC, the next differentiation stage, and the stage before fully formed cell type.

hNPC line used in the present thesis work is a fetal cell line, proven highly expandable and multipotent, meaning that it can differentiate into the major cell types of the CNS: neurons, astrocytes and oligodendrocytes.[32]

The ECM is what is produced by and also surrounds the cells in tissue, and it's crucial for proper maintenance. It functions as the structural support for cells, and its structural characteristics is determined by what type of cells produce it which can be seen when examining the ECM of different tissues such as adipose, osseous and cartilage. There are two forms of the ECM, the basement membrane and the interstitial matrix. Cells that lack relevant ECM cues undergo apoptosis which is a natural mechanism that allows for the correct organization of tissues and for the selection of specific cell types. The basement membrane (BM), where we find the glycoprotein family of laminins, is a thin layer that forms between the epithelia and endothelia and almost all stationary cells in the body grow on BMs.[33] Aside from providing the aforementioned structural support, cells bind to laminins via cell-surface receptors, such as integrins, which regulate vital cellular responses, such as proliferation and differentiation, migration, phenotype stability, resistance to apoptosis and homeostasis.[34, 35] Some of these ECM components are present in the embryonic brain; the various forms of laminins, in particular, are thought to play a major role in nervous tissue development as guidance molecules for nerve cell processes.[36] Detailed look at laminin subunits in table 2 and the role of selected laminin trimers in table 3.

Laminins are the proteoglycan part of the basement membrane, and makes up the structure of it together with Collagen IV. It is a cross-shaped heterotrimeric molecule consisting of an α-chain, a β-chain and a γ-chain, and while it can form 60 trimeric combinations, there are only 16 known heterotrimers as of the present.[33] It is named after its α, β, and γ chains, respectively, so a laminin comprised of an α_5, a β_2, and a γ_1 chain is hereafter referred to as laminin 521.[37]

Table 2: Selected tissue expression of laminin subunits.[38]

Laminin subunit	Select tissue expression
α_1	early embryo, fetal and newborn kidney, neuroretina and brain
α_2	skeletal and cardiac muscle, placenta, peripheral nerve, brain, capillaries
α_3	skin and other epithelia
α_4	mesenchymal cells
α_5	epithelia, kidney, developing muscle and nerve
β_1	most tissue
β_2	neuromuscular junction, glomerulus
β_3	skin and other epithelia
γ_1	most tissue
γ_2	skin and other epithelia
γ_3	distribution of non-basement membrane in brain, epithelia and nerve

Table 3: Laminin heterotrimers and their receptors/cell surface binding proteins and selected cell effects.[38]

Selected laminins	Receptors/cell surface binding proteins	Selected cell effects
111	integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_7\beta_1$, dystroglycan, LAR receptor phosphatase, heparan sulfates, sulfatides	neurite outgrowth, cell migration, receptor/cytoskeletal clusters in myotubes
121	ND*	ND
411	integrin $\alpha_6\beta_1$	ND
421	ND	ND
511	integrin $\alpha_3\beta_1$, $\alpha_6\beta_1$	ND
521	integrin $\alpha_3\beta_1$, $\alpha_6\beta_1$	stop signal for Schwann cells

Aim

The aim of this master thesis was to examine if physiologically relevant *in vivo*-like conditions could be achieved by constructing a 3D brain model with the use of human brain cells and engineered 3D substrates with laminin functionalized surfaces.

Research questions

The study was designed to answer the following questions:

1. Do a 3D culture substrate affect cell viability, and overall- and neuronal differentiation?
2. Do human laminins affect cell viability, and overall- and neuronal differentiation?
3. If yes, are there any differences between different subtypes of laminin and their effect on hNPC viability and differentiation?

*ND = Not determined.

Materials and methods

Fabrication of functionalized electrospun fibrous scaffolds

PCL pellets (M_n: 80,000, Sigma Aldrich, USA), 100 mg/mL were dissolved in a mixture of chloroform and methanol (1:1 v/v). The polymer solution was mixed for 24 h at room temperature (RT) (23°C). A 1 mL syringe with a 22 gauge blunt tip needle (Nordson EFD, USA) was filled with polymer solution and attached to a syringe pump (Aladdin-1000, World Precision Instruments, USA). The positive terminal of a high voltage power supply (HCP 35-35,000, FuG Elektronik GmbH, Germany) was attached 1 mm from the tip of the needle. A rectangular metal plate (135 × 90 mm) was used as a collector. To produce randomly oriented fibers, the flow rate was 1.5 mL/h, the strength of the electrical field was 17.5 kV and the syringe-target distance was 20 cm. PLLA plastic film was used as the collector film for all the fibers. The polymer fiber matrix was then laser cut as discs to the dimension of each respective well type, 48-well plate and 12-well plate. The discs were then sterilized in 70% ethanol, mounted in the plates and left to completely dry before coating and incubation with the various laminin groups and/or PLL.

For the flat substrates, trials were initially performed to produce flat 2D PCL substrates by melting PCL pellets (M_n: 80,000, Sigma Aldrich) between two glass slides at 150°C in the oven, then removed to chill in an ice bath and put in vacuum overnight. It was ultimately decided to not use these 2D surface since they couldn't be manufactured in a reproducible and functionable manner, and attempts to find similar flat PCL substrates on the commercial market were unsuccessful.

Expansion of hNPCs, cell culture and seeding

The hNPC line used for this study was originally established by Drs L. Wahlberg, Å. Seiger, and colleagues at the Karolinska University Hospital, Stockholm, Sweden.* The cell line was established from forebrain tissue, isolated and obtained from one 7-week (post conception) human embryo. Cells were cultured as free-floating cell aggregates (neurospheres) in defined DMEM-F12 medium (Invitrogen, Paisley, UK) supplemented with 2.0 mM L-glutamine (Sigma, St. Louis, MI, USA), 0.6% glucose (Sigma), N2-supplement (Invitrogen), 2.0 µg/ml heparin (Sigma) at 37°C in a humidified atmosphere of 5% CO_2 and 90% RH, see fig. 2. At the initiation of the culture, and thereafter every third day, human basic fibroblast growth factor (hbFGF, 20 ng/ml; Invitrogen), human epidermal growth factor (hEGF, 20 ng/ml; PROSPEC, Rehovot, Israel), and human leukemia inhibitory factor (hLIF, 10 ng/ml; PROSPEC) were added to the culture. By using mechanical dissociation, the neurospheres were passaged every 10-14 days and reseeded as single cells at a density of 2×10^5 cells/ml. Viable cells (opalescent cells excluding Trypan Blue; Sigma) were counted in a haemocytometer.

The hNPCs were seeded (see appendix B) onto different pre-soaked (laminin and poly-L-lysine (PLL)) coating overnight, 1 h in culture medium to make them hydrophilic before seeding) substrates, and was further cultured with differentiation media, i.e., without hEGF, bFGF and hLIF, and supplemented with 1% fetal bovine serum (Life Technologies, Europe BV), and cells passaged 16-19 times were used for seeding. The cells were seeded at a density of 90 x 10^3 cells/cm^2 for the 48-well plates and 86 x 10^3 cells/cm^2 for the 12-well plates, with the same cell density for both 2D and 3D. Seedings were performed at three independent sessions and days. Starting at 3 days *in vitro* (DIV), half of the medium volume was changed to fresh medium every 3rd day until the end of the experiment at day 20, see fig. 4.

**(DNr 344-96. Etikkommittén, Huddinge sjukhus)*

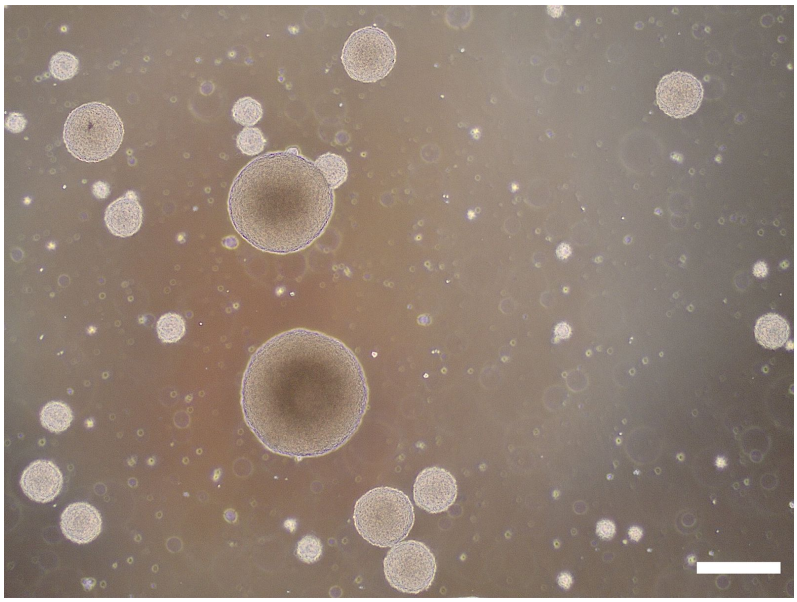

Fig. 2: Free-floating hNPCs in expansion media as both single cells and aggregates, called neurospheres (NS), 8 days since previous passage. Scale bar is 200 µm.

Experimental plan

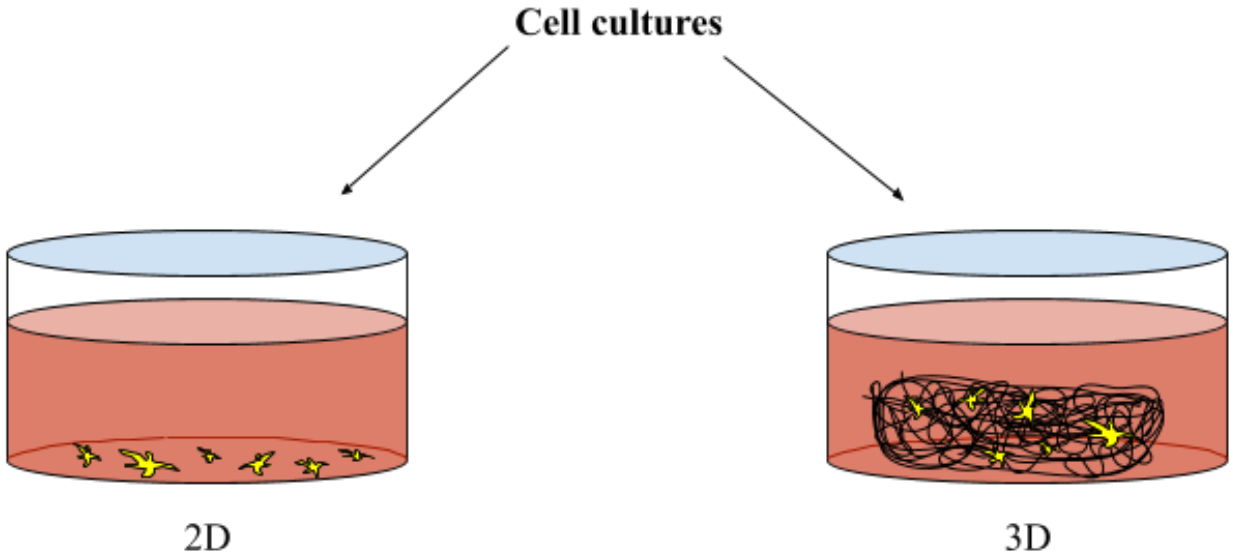

Fig. 3: Wells for 2D and 3D, where the electrospun random PCL fibres can be seen in the right well.

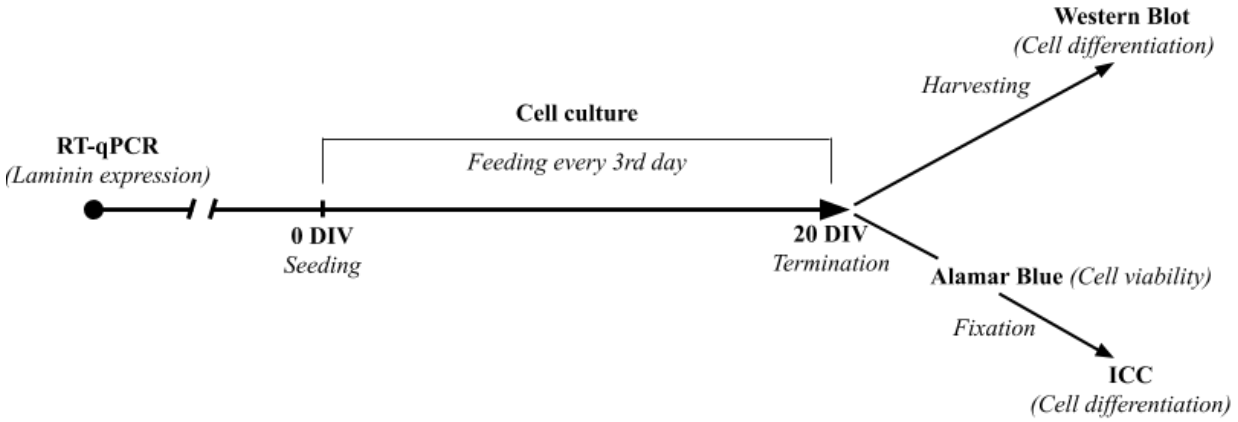

Fig. 4: Timeline of the thesis project.

Work plan

8 coating groups were used for the electrospun fibers; BioLamina AB provided the 6 human biolaminins in this study — 521, 511, 421, 411, 121 and 111. The other two groups were mouse laminin m-111 (laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, L2020, Sigma) and an uncoated naked group as control. The substrates for m-111 and naked coating was first treated with PLL, which was done to ensure a positive charged surface for the negatively charged neural cells to attach to.[39] Plasma treatment was also tried, but ultimately PLL seemed like the much more efficient choice. The eight groups coating were used in two parallel groups, a 2D PS surface and a 3D electrospun PCL scaffold, resulting in a total of 16 experimental groups, see fig. 3.

For the cell viability assay and immunostaining, one 48-well plate was used per 8 coating groups, giving two 48-well plates per seeding. For Western Blot, one 12-well plate was used per experimental group, giving 16 12-well plates in total per seeding. This upgrade in cell quantity was to ensure that there were enough protein when performing Western Blot.

Cell seedings were terminated after 20 DIV, meaning 20 days after cell seeding upon substrates.

RT-qPCR

Before deciding on which human recombinant laminin subtypes to explore, we characterized the expression of the different laminin chains in hNPC in expansion phase and at 20 DIV after culture at 2D and 3D surfaces, respectively. Real time quantitative polymerase chain reaction (RT-qPCR) is used to measure the amount of a specific RNA, and in the scope of this thesis the method was used to examine whether hNPCs have an innate ability to produce laminin on their own, and if so, how much. This is achieved by monitoring the amplification reaction using fluorescence, and it's routinely used for analysis of gene expression analysis. RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from messenger RNA (mRNA). The cDNA was then used as the template for the RT-qPCR reaction.

Livak's method ($2^{-\Delta\Delta Ct}$) was used for expression fold change for relative quantification between the different cultures, the method is a convenient way to analyze the relative changes in gene expression. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene the process was done for 40 cycles, primers that had not amplified at 33 cycles were deemed non-expressed.

Alamar Blue

Alamar Blue (AB) is a colorimetric cell viability assay reagent which contains the cell permeable, non-toxic and weakly fluorescent blue indicator dye called resazurin. AB quantitatively measures viability in human, animal, bacterial, fungal and mycobacterial cells. It is useful for cell viability assays and *in vitro* cytotoxicity determinations as well as cell growth monitoring.

The active compound in AB, resazurin, is used as an oxidation-reduction (REDOX) indicator that undergoes colorimetric change in response to cellular metabolic reduction. The reduced form resorufin is pink and highly fluorescent, and the intensity of fluorescence produced is proportional to the number of living cells respiring. Through detecting the level of oxidation during respiration AB acts as a direct indicator to quantitatively measure cell viability and cytotoxicity.[40] Therefore, resorufin fluorescence/absorbance is proportional to the amount of viable cells.

Fluorescence signals were measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

Three experimental replicates (n=3) were used for the AB assay.

Immunocytochemistry

Immunocytochemistry (ICC) is a method used to identify proteins and other macromolecules in cells and tissues. It uses antibodies to localize structures in cell cultures and tissue sections and is an extremely powerful method and responsible for many important discoveries.

Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in tris-buffered saline (TBS) for 10 min and thereafter rinsed 3 × 10 mins in TBS. The cells were then incubated in 1% bovine serum albumin (BSA) in TBS and 0.25% Triton X-100 for 30 min with the primary antibodies; rabbit α-nestin protein (1:500; ABD69, Merck), as an NPC marker, and mouse α-βIII-tubulin (1:2000; T8660, Sigma-Aldrich), as an early neuronal marker, overnight at 4˚C. After rinsing in TBS, cells were incubated with secondary antibodies; an Alexa Fluor 488 donkey α-rabbit IgG antibody (1:200; Abcam, Cambridge, UK) and an Alexa Fluor 594 goat α-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA, USA). For counterstaining of nuclei and cover-slipping 4,6-diamidino-2-phenylindole (DAPI)-containing Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) was used and the specimens thereafter stored at −20˚C.

One experimental replicate (n=1) was used for ICC and per group.

Western Blot

Western blot (WB) analysis is one of the most common methods of detecting proteins and determining specific protein concentrations in biological samples. It involves a Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) step, a membrane transfer step and an immunoblotting step.

When terminating the cell cultures, the cells were harvested from the plates by dislodging them from the substrate with Trypsin and then lysed with Radioimmunoprecipitation assay (RIPA) buffer to extract the protein, involving several Dulbecco's Phosphate-Saline Buffer (DPBS) washing steps along the way. Cell debris was discarded and the protein concentration was quantified using Pierce Rapid Gold BCA (Thermo Fisher Scientific, Waltham, MA, USA). From the protein concentration assay, we could determine the sample volume needed for 5 µg of protein from each experimental group, which were mixed with NuPage LDS Sample Buffer (4x), NuPage Reducing Agent (10x) and ddH_2O to mix a protein sample, which was then boiled at 70˚C. They were then loaded in each well of the gel (NuPAGE™ 4-12% Bis-Tris Protein Gels).

After the proteins were transferred from the gels to the membranes, Coomassie Brilliant Blue staining protocol was performed on the gels to confirm that there were no protein degradation in the protein samples used.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an extensively used protein for loading control in WB, due to the general assumption that its expression levels are insensitive to the influence of various physiological conditions and treatments.[41] GAPDH was used as a loading control and results were normalized against the expression of GAPDH in each individual well.

Primary antibodies (1:10000, rabbit α-nestin; 1:2000, mouse α-βIII-tubulin; 1:8000, rabbit α-GAPDH) mixed with blocking solution (10% non-fat dry milk in 1x TBS 0.1% Tween 20) and the membranes were incubated overnight.

Secondary antibodies were marked by a chemiluminescent reaction with an equal mix of SuperSignal West Pico PLUS Luminol/Enhancer Solution and Stable Peroxide Solution (Thermo Scientific), whereas the chemiluminescent signals were then measured with a charge-coupled device (CCD) camera and displayed in the software Image Lab (Bio-Rad) where further analysis was performed.

One experimental replicate (n=1) was used for WB and per group.

Data and Analysis

RT-qPCR

Livak's method was used for calculating the two-fold expression.

Cell counting

Labelled cells were counted manually by taking 4 pictures of different areas in the same ICC-stained sample, then counting the total number of DAPI-stained nuclei. Nestin-positive cells and βIII-tubulin-positive cells were identified and then averaged against the total number of cells, giving a fraction of positive immuno-labelled cells.

Normalization

For cell viability, fluorescence data for each coating group was averaged and normalized against the control group (naked coating) in 2D. For protein quantification in WB, protein expression was normalized against the loading control GAPDH.

Microscopy and imaging

Zeiss Pro 2 (Zeiss, Germany) was used for fluorescence microscopy for ICC. Images were processed using ImageJ (National Institutes of Health, USA). The scanning electron microscope images were acquired using the field emission SEM JSM-5600 LV (JEOL Ltd., Japan), and the diameter of the fibers were calculated using a fast Fourier transform (FFT) in ImageJ.

Statistics

Two-way ANOVA in GraphPad (Prism, USA) was used for calculating significance, where a p-value below 0.05 was assumed to show significance.

Results

Laminin chain expression in hNPC in expansion phase and at 20 DIV

We used RT-qPCR for examining the relative expression of the laminins subchains, i.e. α_1-X, β_1-X and γ_1-X.. The samples used were taken from a previous study where the cells had been grown on substrates for culturing, i.e. group B. For comparison between passages of the cell line also a second group was added, i.e. A, consisting of cells in expansion phase. No later time-point could be added to group A, due to time restrictions. In fig. 5, only the laminin chains demonstrating gene expression is shown, the laminin chains of α_2, α_3 and γ_2 were unexpressed. With regard to group B, some laminin chains are more extensively expressed and even more at 20 DIV. In general, more laminin is expressed in 2D cultures compared to 3D cultures. Among the laminin chains, specifically α_1, α_5, β_2 and γ_1 are strongly expressed (see fig. 5).

With the hNPC laminin chain expression in mind six human biolaminin subtypes were further studied, by coating of the culture substrates. The human laminins 521, 511, 421, 411, 121 and 111 were studied.

The hNPC line has only been cultured on m-111 in previous studies. Therefore, we were interested in the effect of its human recombinant counterpart. Since α_4 and α_5 chains are known to play a role in progenitor cell maintenance, endothelial cell niche maintenance and for different neural phenotypes in the brain they were added. Lastly there is an important distinction in the effect of the β_2 chain compared to the β_1 chain, although the mechanism is not yet fully understood, but developmentally there is usually a shift in the β-chains and was therefore added for initial examination.

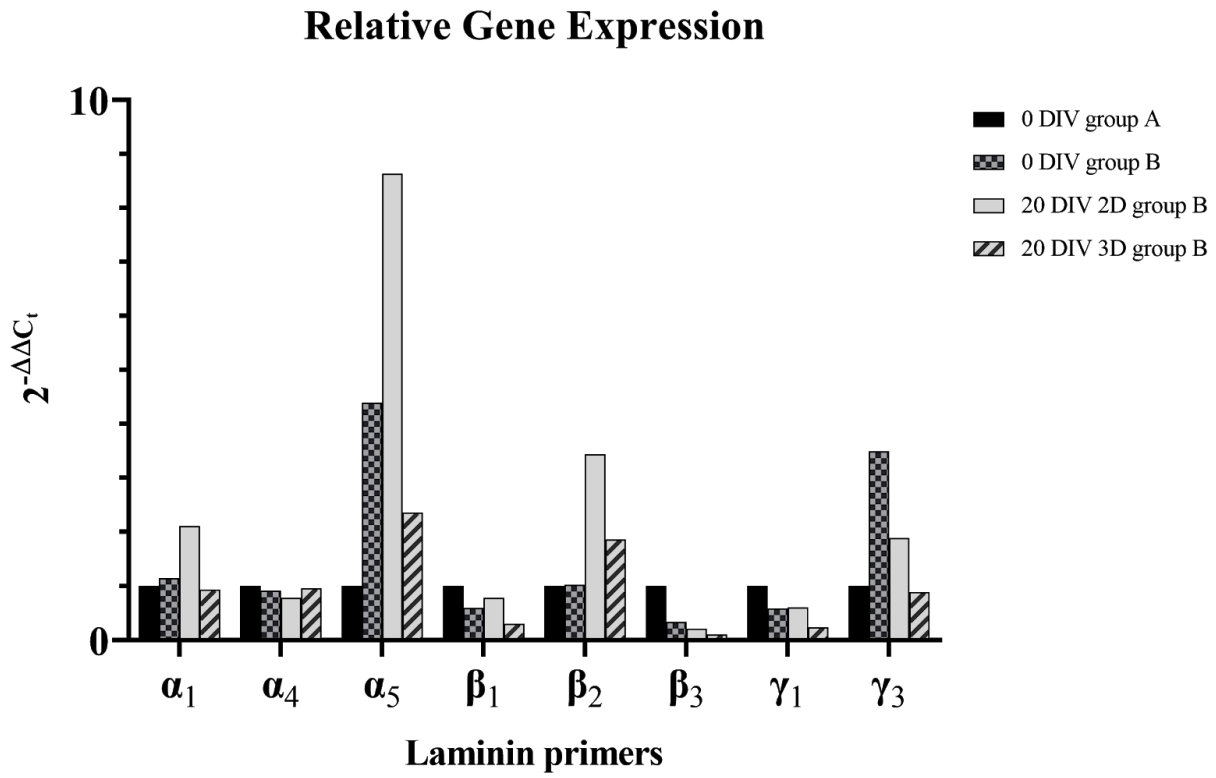

Fig. 5: Relative gene expression for hNPCs using laminin primers from four different conditions. Laminin primers that were deemed non-expressed are not shown in the figure, n=1.

Fabrication of electrospun PCL fibers

From the SEM images, the fiber diameter of the random PCL fibers (see fig. 6a) had a diameter of 0.6-0.8 µm (see fig. 6b).

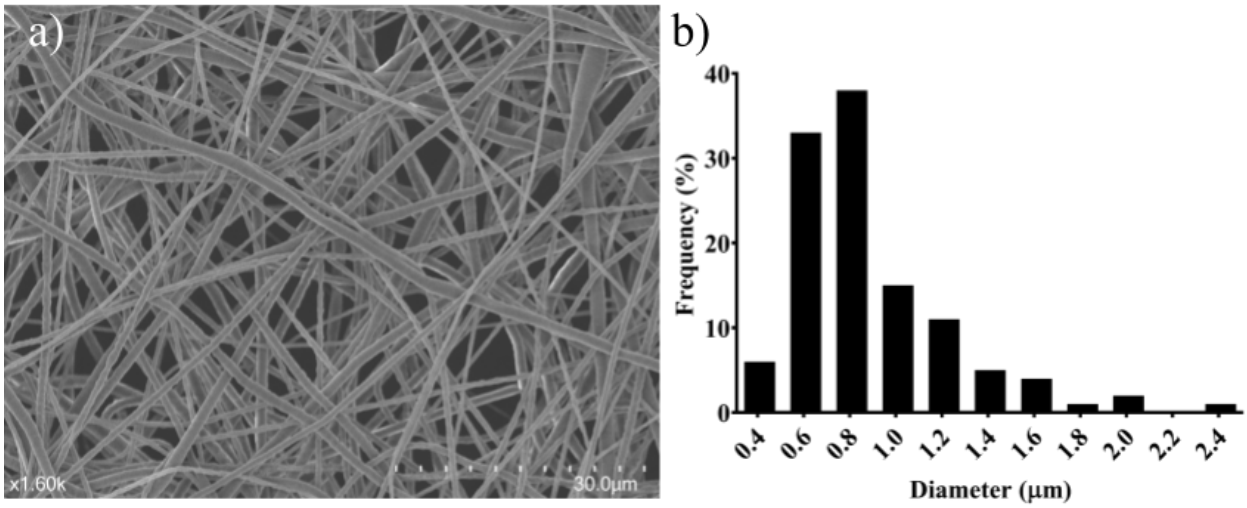

Fig. 6 : a) Scanning electron microscope (SEM) picture of the electrospun random PCL fibers and b) thickness of the fibers between 0.6-0.8 µm. (Image courtesy: Cellevate)

Cell viability

The effect of substrate surface (2D versus 3D) and laminin type on the viability of hNPCs was evaluated using the AB assay. No significant differences in cell viability was found at 20 DIV with regard to only substrate topography (2D vs. 3D), except for 421 were there was a significant higher cell viability. However, for all 8 coating types, including naked, slightly lower values were found for the 3D groups.

Notably, for both 2D and 3D cultures viability was higher with all human laminin coatings, including m-111, compared to naked controls (see fig. 7). Overall trends in effect on viability for the laminin subtypes can be seen and with the same pattern in the 2D and corresponding 3D groups. Cultures exposed to the α-chain 1 display the highest values (i.e. 111 and 121). This is followed by the groups exposed to laminins with the α-chain 5 and especially β-chain 1, and subsequently α-chain 4 exposure show the lowest values. Statistical analysis within each substrate group, revealed a significant higher cell viability for the laminin 121 compared to naked control in the 3D group. In addition statistical analysis based on the effect of laminin, revealed a significantly higher viability in the 2D group compared to the 3D group.

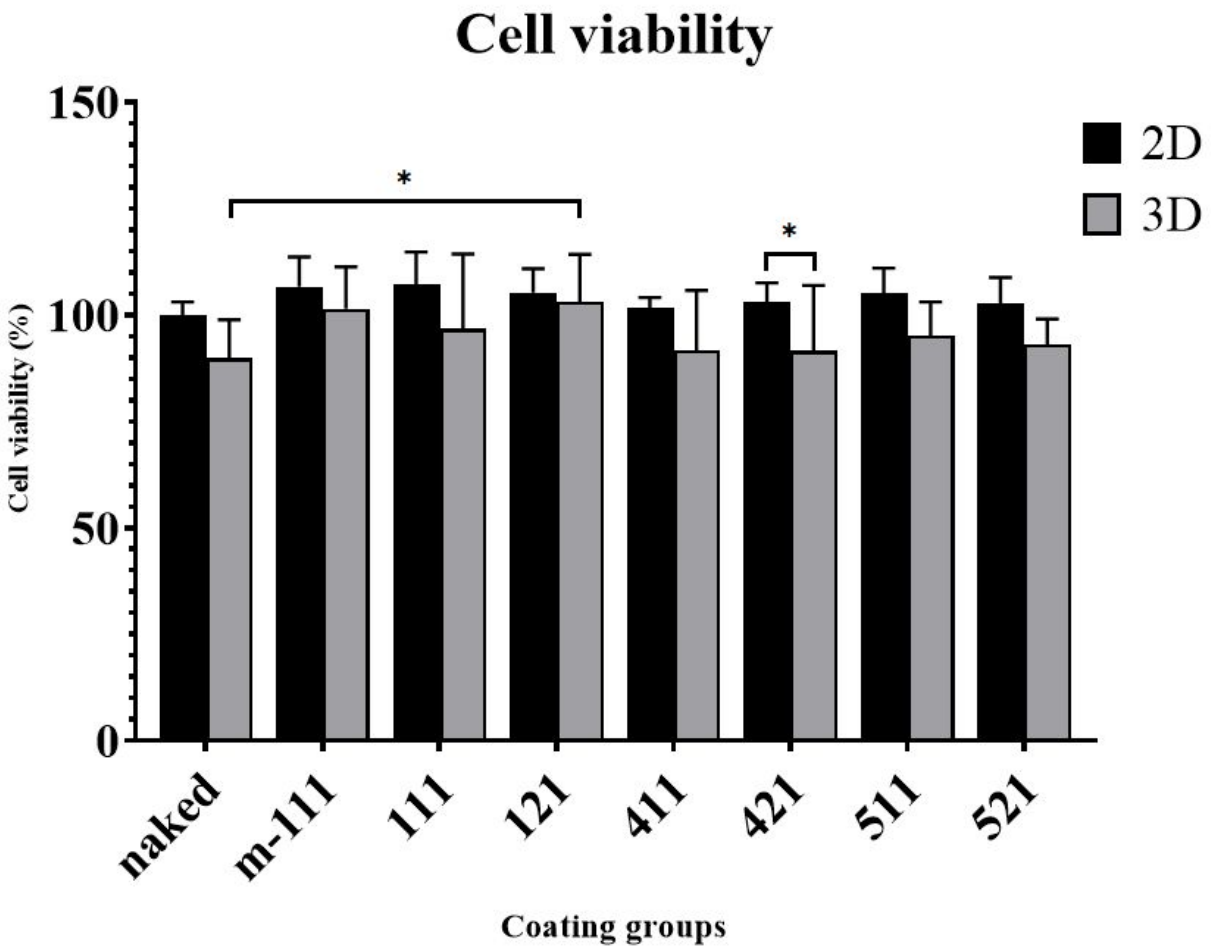

*Fig. 7: AB performed on the cells after 20 DIV to examine cell viability between the two different substrates as well as the 8 different coating groups, n=3. Data are expressed as mean ± SD (n=3). Statistical analysis was made using two-way analysis of variance (ANOVA) followed by post-hoc test Sidak, *p < 0.05.*

Immunocytochemical analysis

Next we wanted to assess the effect of the culture substrate and laminin type on overall differentiation capacity of the hNPC. This was at first studied at 20 DIV, by quantification of numbers of DAPI-positive nuclei expressing a marker for hNPC in a neural progenitor stage, i.e. nestin. Overall, samples stained from all 16 groups displayed nestin-positive cells (see fig. 8 and 9). In both 2D and 3D cultures, a large fraction of nestin-positive cells were found (see table 4).

In 2D, naked and 411 showed a high level of expression for both nestin and βIII-tubulin. m-111 showed a very low number of βIII-tubulin-positive (early neuronal marker) cells compared to the other coatings in both 2D and 3D, but average nestin-positive expressing cells. Both laminins with α_4 chains showed a higher number of βIII-tubulin-positive cells than the average for the laminin types.

The numbers of marker-expressing cells for the 3D substrate has less differences between coatings used than the 2D (see table 4). For βIII-tubulin and nestin, the fraction of cells expressing the markers was between ~50-68%, except for m-111. m-111 showed almost half of the numbers expressing nestin compared to the naked type and human laminin types. However, the amount of cells expressing βIII-tubulin was similar for all human laminin coated groups and naked fibers, but with a very low fraction expressing the marker in the m-111 exposed culture (i.e. 14%).

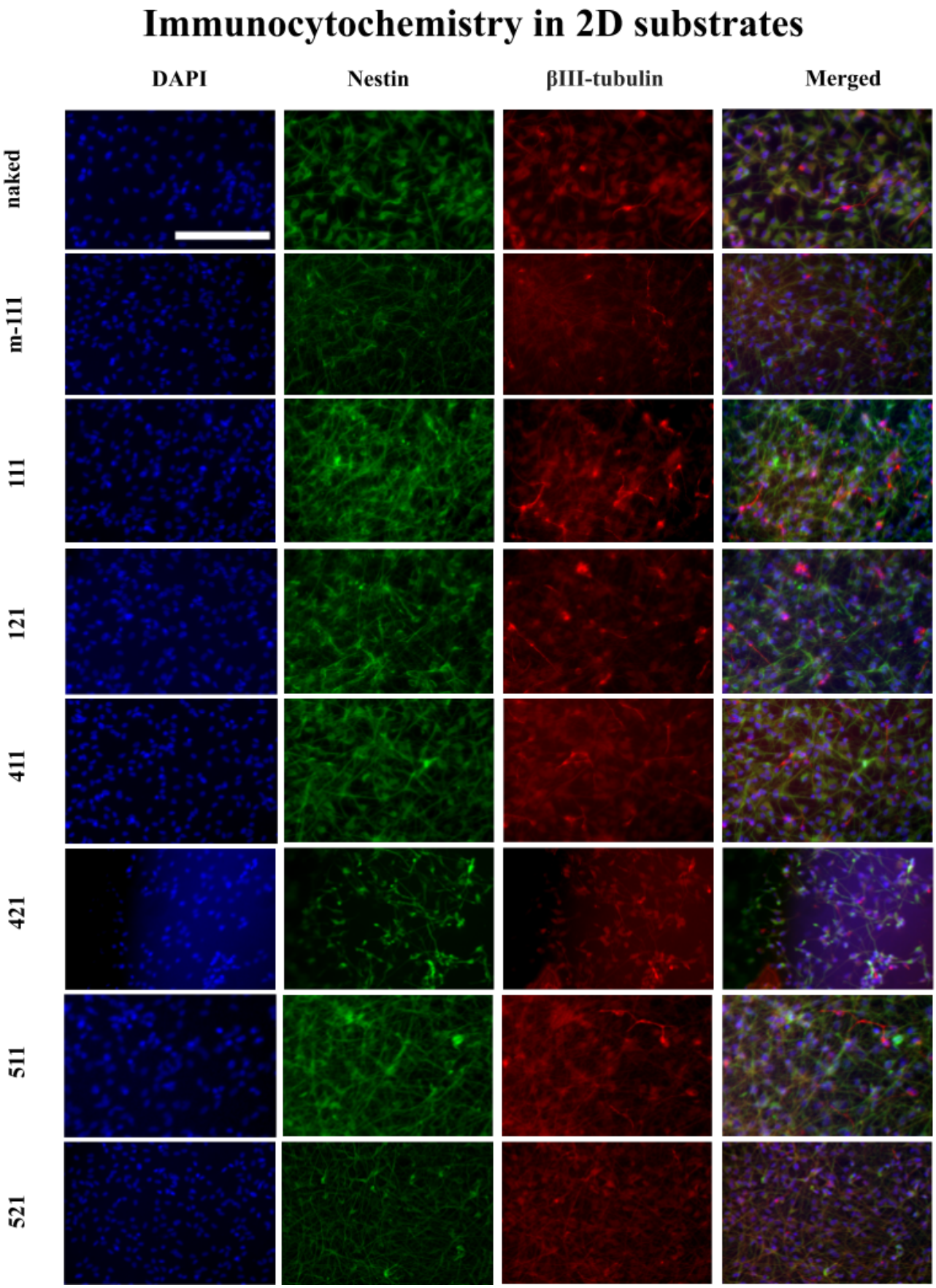

Fig. 8: ICC for nuclei (DAPI, blue), NPC marker (nestin, green) and early neuronal marker (βIII-tubulin, red) on 2D substrates. Scale bar is 0.1 mm.

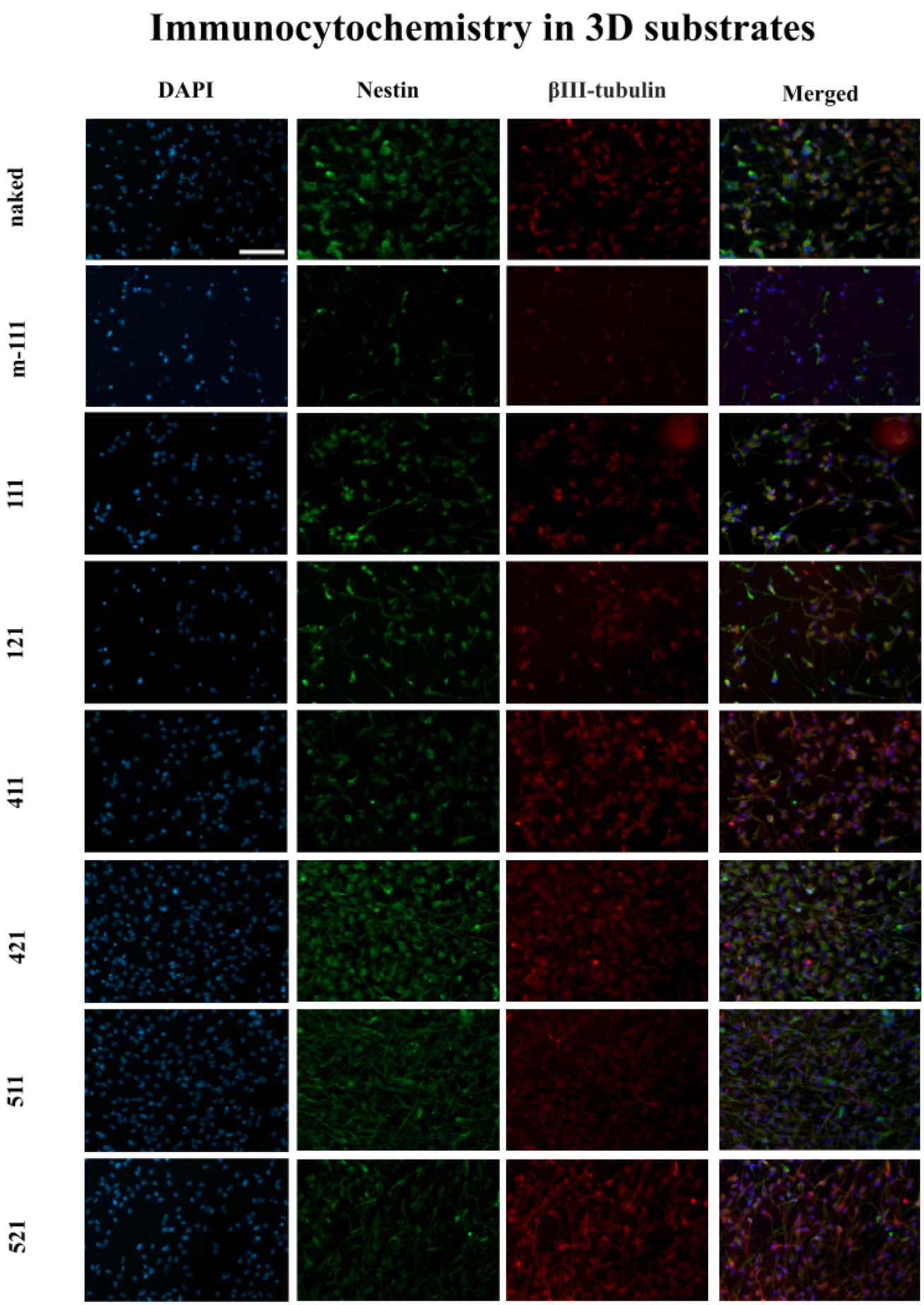

Fig. 9: ICC for nuclei (DAPI, blue), NPC marker (nestin, green) and early neuronal markers (βIII-tubulin, red) on 3D substrates. Scale bar is 0.1 mm.

Table 4: Percentage of cells that express nestin and βIII-tubulin in the 2D cell cultures, (379-2026 nuclei counted) and in the 3D cell cultures (121-332 nuclei counted), n=1.

	2D expression		3D expression	
	Nestin (%)	**βIII-tubulin (%)**	**Nestin (%)**	**βIII-tubulin (%)**
naked	73.5	61.2	67.9	57.6
m-111	28.2	9.7	31.4	14.0
111	38.1	17.6	63.0	66.1
121	34.1	15.4	63.0	63.7
411	60.6	57.6	66.4	57.9
421	35.1	38.8	63.3	63.3
511	26.9	26.7	63.1	63.1
521	35.0	22.6	62.3	50.3

Protein expression analysis

In order to more in detail study the differentiation potential, westen blot analysis was made for revealing quantitative measurement of protein expression of nestin and βIII-tubulin in the respective groups. Immunoblotting for nestin revealed several bands (corresponding to isoform), see for example lanes 411, 121, 111, m-111 and naked in fig. 10 or lanes 421, 111 and naked in fig. 11. The number of bands can vary depending on the amount of protein loaded into each well during the electrophoresis process as well as the concentration of antibodies used during the immunoblotting stage.

In some of the lanes there is no detection of nestin nor βIII-tubulin (see lane for 521, 511 and 421 in fig. 10), even though ICC results confirm the presence of these proteins in our samples. This relation is further shown in quantification of nestin, see fig. 12 which show next to no nestin expression in 2D for coating groups 521, 511 and 421. In fig. 11 there's no detection of nestin for lanes 521, 411 and m-111, but there's a clear expression of that protein in the ICC pictures in fig. 9. A similar discrepancy can be seen while comparing the quantified βIII-tubulin expression in fig. 13 and the immunoblots in fig. 10 and 11.

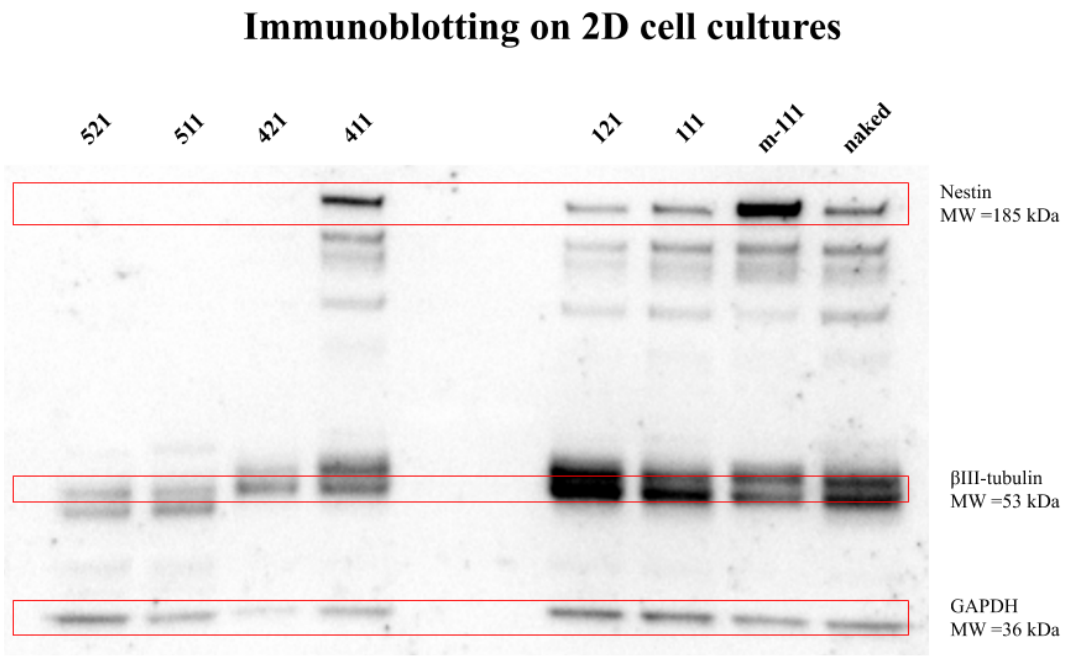

Fig. 10: Immunoblot of nestin, βIII-tubulin and loading control GAPDH from the 2D cell culture.

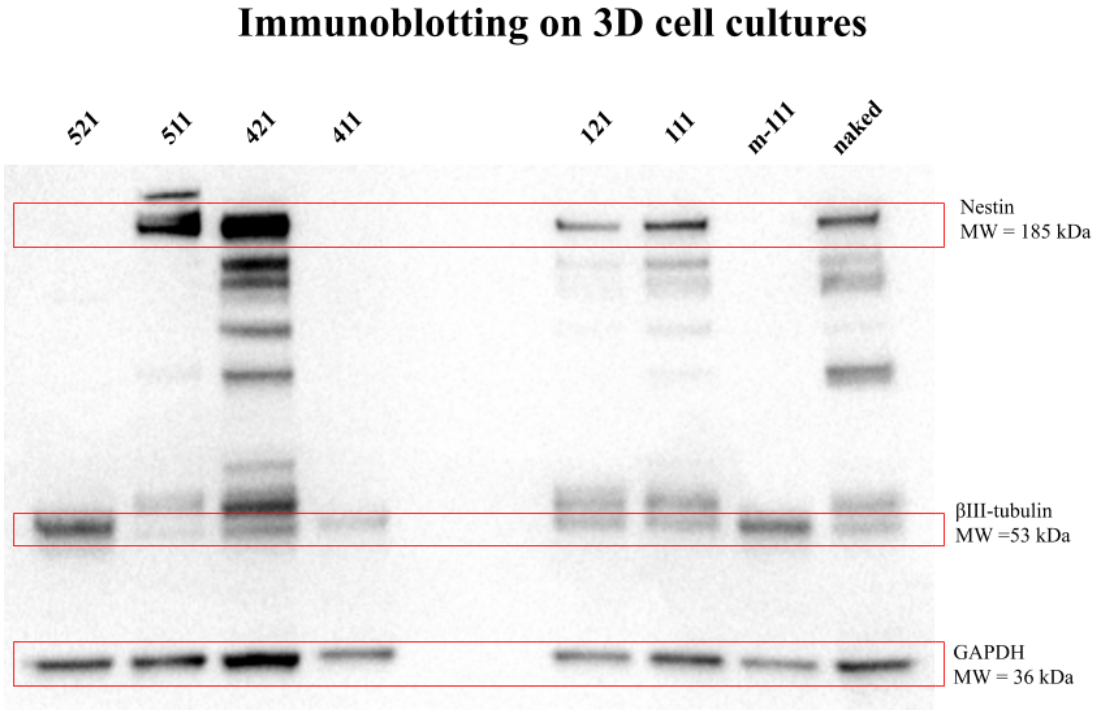

Fig. 11: Immunoblot of nestin, βIII-tubulin and loading control GAPDH from the 3D cell culture.

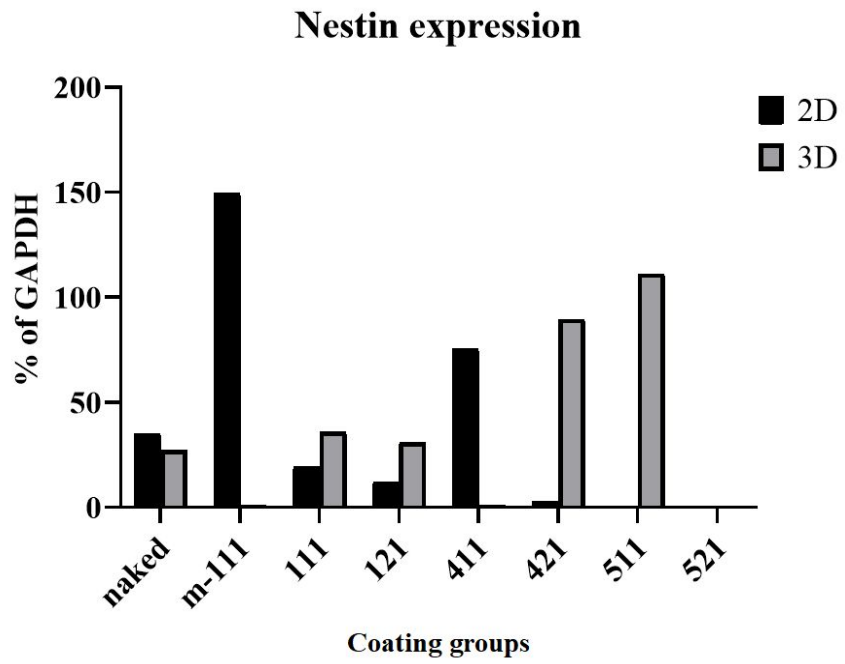

Fig. 12: Comparison of nestin expression in 2D and 3D culture, n=1.

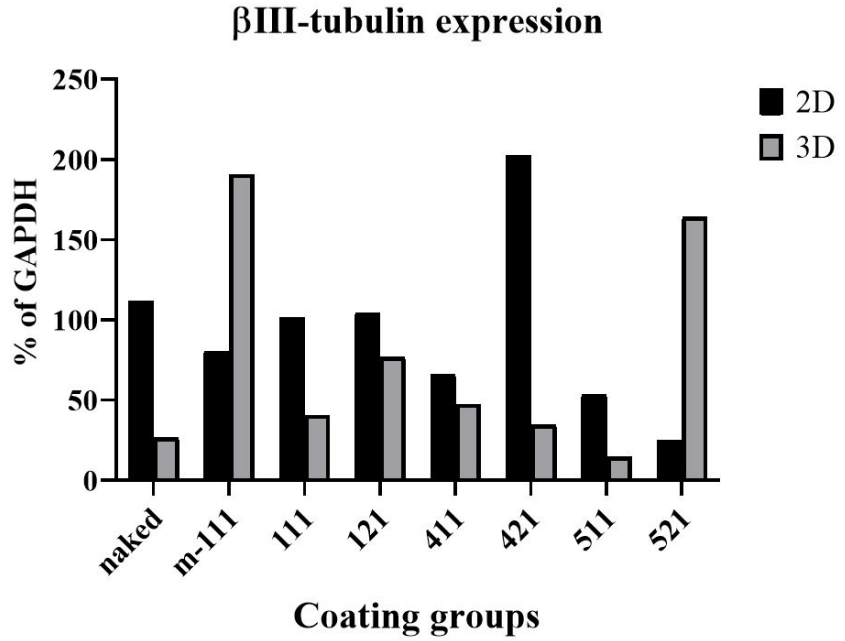

Fig. 13: Comparison of βIII-tubulin expression in 2D and 3D culture, n=1.

Discussion

The aim of this thesis work was to explore the potential to generate a more physiological relevant cell-based model for brain research, by using a human brain cell line, three-dimensional (3D) culture substrates and the ECM protein laminin. Here, we could, indeed, demonstrate the ability to generate 3D human neural cultures, most likely containing a substantial fraction of neuronal cells. This result is in line with previous studies using the same cell line and in a similar culture setting.[11, 42] Together, successful reproducibility in culture of hNPC in non-functionalized 3D electrospun scaffolds for long-term holds great promise in the further development of functionalized scaffolds.

A sub-aim was to characterize the hNPC production of laminin subunits during proliferation and differentiation stage. We found gene expression of especially subchains α_1, α_5, β_2 and γ_1 were strongly expressed.

We were able to answer the research questions raised in this study. Our main results show that the hNPC:

- survive equally well up to 20 days at both 2D and 3D scaffold
- viability in 2D and 3D cultures were equally and positively affected by the biolaminins investigated
- attach well and integrate into the 3D fiber scaffolds, and a fraction show capacity to form neuron
- in 2D cultures the overall differentiation is favored in all laminin exposed groups
- have a greater potential to form neurons when exposed to all the human biolaminins compared to the mouse laminin at both 2D and 3D surfaces, judged by cell counting of neurons

Effect on cell viability — 3D and laminin

The cell viability data is the strongest since we could use three biological replicates, compared to ICC and WB where we could only use one biological replicate. AB showed statistical significant difference between human laminin 121 and naked on 3D substrate, and between 2D and 3D in the human laminin 421 type.

As we can see in the cell viability chart (fig. 7), while there is a difference in viability between the different cultures, in both 2D and 3D, the only two significant differences were between human laminin 421 in the 2D and 3D culture and between human laminin 121 and naked in the 3D culture. It's generally hard to give a complete answer about viability when it comes to AB. Results can be skewed by several factors. For example, take two different, hypothetical wells:

- **Well 1**: Few cells in unfavorable conditions, they can be stressed and respirate more than a healthy cell. This could with AB seem as though there's higher cell viability than it is.[43]
- **Well 2**: More cells than in well 1 that are in favorable conditions will have a normal respiration, This could with AB be registered as less cellular respiration than well 1.

In this example well 1 could potentially show higher cell viability than well 2, which might not be true. It's important to think critically about the environment the cells are in before reaching a conclusion.

In table 1, where some key differences between 2D and 3D systems are outlined, we can see that cell proliferation is higher in 2D than in 3D and even higher in 2D than in the native tissue. This might be one

explanation to why the cells grown in 2D show consistently higher viability than those in 3D. It might not be that cells actually have a higher viability in 2D, in fact, most of the data we have available to us says that 3D is more suited for cells when it comes to mimicking the native environment and providing a suitable milieu.[22-28] A simple explanation could be that cells are generally more stressed in a foreign environment and thus respirate more, combined with a higher rate of proliferation, leading to the data suggesting that a 2D substrate is a more viable environment than a 3D substrate.

Our cell viability results are in line with previous studies in the lab, especially for the naked and m-111 groups.[42]

Effect on cell differentiation using ICC – 3D and laminin

Here stainings with the marker nestin was used to initially analyse the fraction of cells in an NPC stage. For all 16 groups nestin-positive cells were found, however, in different numbers. Especially in the 2D cultures a trend toward lower numbers of nestin-expressing cells in the groups exposed to mouse and human laminins (except 411) compared to non-treated fibers was noted. Hence, this result may suggest that some laminins promote differentiation. However, countings of βIII-tubulin did not show a higher number in laminin treated groups compared to naked fibers. But, the human laminin seemed to facilitate neuronal differentiation more than the mouse laminin.

Cell countings in the 3D samples revealed very similar numbers in all groups, which makes it difficult to draw any conclusions.

Cell counting done here is semi-quantitative, rather than fully quantitative, because only one technical/experimental replicate was added. It's not an infallible technique, as what constitutes as protein expression is subjective to a certain degree and thus the result can vary from person to person. There's also the layer effect - 2D cell cultures are a monolayer with next to no cell overlap making it easy to distinguish between them, whereas 3D has several layers of cells that have integrated into the fibers because of the scaffold structure resulting in cell overlap as observed in the fluorescent microscope. This means that one can mark a cell as false positive for expressing a certain protein, when in reality it could be a cell underneath that's expressing it. The problem outlined above could be one explanation why the protein expression for both nestin and βIII-tubulin seems so much higher in 3D than in 2D. Using a confocal microscope could be a viable way of counting layer per layer in a 3D system.[44] All in all, the practice of counting cells certainly highlight the need for actual protein amount quantification, and that's why Western Blot is a natural next step when determining the protein expression in cells.

Another reason could be that the physical effect of the 3D environment has such a large effect on cell differentiation, so that it drowns out the chemical effect from the different laminin types. That might be why the protein expression in 3D is much more homogenous.

There's a larger fraction of both nestin and βIII-tubulin expression in 3D compared to 2D, which could be due to the counting, and the layer effect described above, but it could also be an effect of the 3D environment having an effect on cell differentiation and morphology. Previous studies on growing mouse neural stem cells on electrospun fibers has shown increased rates of differentiation and improved neurite outgrowth.[45]

Effect on cell differentiation using western blot – 3D and laminin protein expression

Three technical replicates were performed in the western blots, for training purposes and for trying to achieve a level of consistency required for further studies on this subject (see appendix B for all 6 immunoblots). What became apparent was that there was a huge variation between the technical replicates. Coating groups where nestin and βIII-tubulin expression was detected could in the next immunoblot show no detection at all, even though all the other conditions of the previous experiment were consistent. One explanation could be that my technique isn't robust enough, resulting in mistakes along the way. There are many stages in Western Blot that are crucial and where it's essential to not make mistakes, such as the sample preparation, protein loading and membrane transfer, where one small lapse of judgment can lead to a complete redo of the experiment being a necessity.

The membranes in figures 10 and 11 reveals bands that may be isoforms of nestin and/or βIII-tubulin or unspecific bands. High primary or secondary antibody concentration can be a reason, although the primary concentration for nestin was lower than in other successful immunoblots. As mentioned earlier, protein degradation was ruled out by performing a Coomassie Brilliant Blue staining protocol on the gels after protein transfer.

There were also some instances during the immunoblotting when the secondary antibodies weren't detected during exposure. This could maybe suggest the usage of less than ideal aliquots of antibodies or false dilutions, due to antibodies not always being extracted from the stock vial.[46] There could be old aliquots with inactive antibodies, or someone diluting an antibody and forgetting to label it as the correct dilution. In some cases, proteins could be detected during immunoblotting after either letting some time lapse so the enzymatic reaction with the antibodies and the chemiluscent solution was allowed to take place, or pouring more solution in case it didn't cover all of the membrane.

Generally it's hard to give an answer regarding the protein expression in the cell cultures as of right now, due to the contradictory nature between ICC and WB, and also because of the lack of performed experimental replicates. That's something that should be focused on in later studies. This suggests that the protocol must be further optimised, since previous studies have shown a consistent and clean result for both βIII-tubulin showing one band, and nestin showing 1-2 bands.[11]

However, what is clear from the immunoblotting, although inconsistent between session, is that the laminins seem to have different impact on the hNPC which we will investigate further.

Overall

Fetal bovine serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors. When used at appropriate concentrations it supplies many defined and undefined components that have been shown to satisfy specific metabolic requirements for the culture of cells. However, its composition is quite undefined which can result in large variations between batches.[47] This might have an effect on the cell cultures and thus the end result, leading to too high variance. However, the only test I performed on more than one experimental replicate was AB, and the respective substrate:laminin combinations gave similar results from seeding to seeding.

Conclusion

This thesis's results demonstrate that brain cells have the potential to survive well in 3D scaffolds, and that human laminin subtypes most likely affect their differentiation potential differently. However, more studies with more biological replicates are needed to confirm the effects of the specific laminins and before a protocol for the culture of *in vivo*-like human *in vitro*-brain models can be presented.

Another goal is to further optimize the western blot protocol to get better and more consistent results, further strengthening the conclusions regarding protein expression, and confirm them via immunocytochemistry.

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APPENDIX A - recipes

L-glutamine P/S HNPC Culture Medium

Ingredients	50 ml	100 ml	500 ml
BASIC MEDIUM			
DMEM/F12 1x *ThermoFisher no 21331-020*	47 ml	94 ml	470 ml
N2-supplement, 100x *ThermoFisher no 17502-001*	0.5 ml	1 ml	5 ml
L-Glutamine P/S (Stock 200mM, 10000 units/ml Penicillin, 10000 µg/ml streptomycin) *ThermoFisher no 10378016*	0.5 ml	1 ml	5 ml
Glucose 0,6% (Stock 30%) *Sigma no G70213*	1 ml	2 ml	10 ml
Heparin 2 µg/ml (Stock 10mg/100ml) *Sigma no H3149*	1 ml	2 ml	10 ml
Can keep media for approx 1 month	***STERILE FILTER***		
COMPLETE MEDIUM			
EGF, rec. human EGF (Final conc 20 ng/ml) (Stock conc 100 µg/ml) *Prospec, CYT-217*	10 µl	20 µl	100 µl
bFGF, rec. basic human FGF (Final conc 20 ng/ml) (Stock conc 100 µg/ml) *Prospec, CYT-218*	10 µl	20 µl	100 µl
LIF, recombinant human LIF (Final conc 10 ng/ml) (Stock 10 µg/ml) *Sigma, no L5283* *Prospec CYT-644*	50 µl	100 µl	500 µl
Can keep media for approx 1 week			

APPENDIX B - protocols

cDNA synthesis

QuantaBio qScript cDNA synthesis kit manual.[1]

RT-qPCR

Quantabio qScript One-Step SYBR Green gRT-PCR Kit manual.[2]

Thawing and expansion of hNPCs

1. Get frozen cells in the cryovials in dry ice
2. Prepare 50 ml tubes for every 2 cryovial of the same batch and add 7 to 10 ml of basic medium.
3. Quick thaw the cryovials in a 37°C water bath.
4. Add 1 ml of basic medium to the cells to dilute the freezing medium, as DMSO is toxic to the cells and immediately transfer the content to the rest of the basic medium in the 50 ml tube.
5. Centrifuge at 300 x g for 5 min.
6. Aspirate supernatant and resuspend the pellet in 2 ml expansion medium.
7. Transfer the cells into a T75 flask containing 18 ml of expansion medium.
8. Check cells under the microscope.
9. Incubate in a cell culture incubator at 37°C, 5% CO2, 95% humidity.
10. Two (2) days after, add 20 ml of fresh expansion medium to each T75 flask and then remove 20 ml of the suspension into a new T75 flask.
11. Incubate in a cell culture incubator at 37°C, 5% CO2, 95% humidity.
12. Feed cells (each flask) with 3 to 4 ml every 3rd to 4th day until the neurospheres are big enough and ready for dissociation.

[1] "Manuals - Quanta BioSciences." http://www.quantapcr.com/page/manuals. Accessed 6 Sep. 2019.
[2] "Manuals - Quanta BioSciences." http://www.quantapcr.com/page/manuals. Accessed 6 Sep. 2019.

Seeding of hNPCs

Preparations

- Warm all the culture media to 37°C
- Clean the hood and the pipettes before use

Note: Rinse tips to be used for the cells before using them for the cells in DMEM-F12 (they stick to surfaces).

Seeding

1. Look at the cells in the microscope. Cells should look round, healthy and not too big. If they can clearly be seen without a microscope they are too big.
2. Knock the flasks to detach spheres from the bottom and sides of the flask (if there are any). Shake the spheres to the bottom at an angle.
3. Transfer medium and spheres into a 50 ml tube.
4. Draw up 5 ml of DMEM and rinse out the flask.
5. Centrifuge at 1500 rpm for 5 min.
6. Aspirate supernatant (leaving few µl so the cells don't dry).
7. Add 100 µl of DMEM/F12 (rinse the tip before use!) to the pellet and resuspend carefully.
8. Remove the liquid (probably >100 µl) into a 1.5 ml tube.
9. Take a second 100 µl and use it to rinse the angled sides and bottom of the 50 ml tube. Add this to the previous tube.
10. Repeat step 7 three times trying to get off as many cells as you can.
11. Set the 100 µl-pipette to 80 µl and rinse the tip well. Dissociate the pellet by vigorously triturating till satisfied. Advise to keep cells resting after trituration and before counting and measuring viability.
12. Count cells using the TC20 cell counter. Use trypan blue for viability
13. Seed the cells according to experiment. For e.g. cell expansion, add 2.4 million cells into 1 T75 flask containing 19-20 ml complete medium.

hNPC Freezing

FREEZE MEDIUM			
	5 ml	**10 ml**	**50 ml**
Basic medium	4.1 ml	8.25 ml	41.25 ml
DMSO 100 % (end conc 7.5%)	0.4 ml	0.75 ml	3.75 ml
BSA (end conc 10%)	0.5 g	1 g	5 g

Freeze medium

1. Add BSA to basic medium. To dissolve BSA warm the mixture at 37°C water bath. Note: BSA and basic medium can stay for 1 week at 4°C.
2. Add DMSO (just before the freezing).
3. Sterile 0.2 µm filter. (Store at 4°C if not used immediately)

Freezing cells

1. Passage as usual and seed them at 4 million per flask
2. Two days after the passage (small spheres) spin down the cells (no passaging!) and add 1ml of freezing medium (approximately 4 million per cryovial)

Thus, take one T75 with 4 approx million cells per flask to one cryovial. The numbers will not be exact, but it is not so important here

3. Put the cryovials with cells in a freezing container into -80 degrees for 1-2 days and then finally store at -150°C (liquid nitrogen tank)

Coating protocols with adhesion factors

1. Coating volumes table

Culture plate	**Surface area/well**	**Coating volume**
96 well	0.33 cm^2	70 - 100 µl/ well
48 well	0.83 cm^2	250 µl/ well
4 or 24 well	1.9 cm^2	300 - 500 µl/ well
MEA (3brain)	0.36 cm^2	90 µl/ well

2. Poly-L-Lysine solution 0.01%, sterile-filtered (P4707, MW: 70 – 150 kDa Sigma)

- Add X µl (according to table) of the 0.01% PLL solution to each culture well.
- Rock gently to ensure uniform coating of culture surface.
- Incubate at room temperature (RT) for at least 5 min to 1h.
- Dry at RT.
  Note: Coated slides will be stable for one year if protected from dust.

3. Laminin 1mg/ml (L2020, Sigma or other types of laminin)

Recommendation: coating at 1 – 2 µg/cm²

- Check exact laminin concentration and volume provided in each vial, by checking lot#
- Thaw laminin at 4°C to avoid gel formation
- Prepare X mg/ml of laminin solution (according to substrate in use)
    - 10 µg/ml in DPBS for coating on glass cover slips, TC culture plastic, nanofiber scaffolds
    - 80 µg/ml laminin solution in complete BrainPhys medium pre-dot solution
- Add x µl of laminin solution in each culture well
- Incubate:
    - for at least 2h at 37°C
    - overnight at 4°C
    - If not used immediately, the culture ware must be sealed to prevent evaporation of the laminin solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 2 weeks after coating. Allow stored coated cultureware to come to room temperature (15 - 25°C) for 30 minutes before plating cells.
- Aspirate solution from the wells immediately before plating the cells. Do not let laminin to dry.

Immunostaining protocol for fixed cultured cells

(e.g: dissociated neural cultures on glass cover slips or nanofibrous scaffolds)

Before starting stainings:

- Prepare blocking solution (PBS + 1%BSA + 0.25% Triton X) and have it at RT
- Prepare calculations and solutions with Ab. Note: primary and secondary antibodies diluted in "blocking" solution (1x PBS or TBS + 1%BSA + 0.25% Triton X)
- Have 1xPBS or 1 x TBS at RT
- Bring slides/ culture plates with cells from the cold room and keep them at RT (at least 30 min). When at RT ready to use

Note: Be consistent when using the salt buffer (PBS or TBS)

Protocol:

Day 1

1. **Optional:** Pre-incubate samples in blocking solution with 5% serum for 30 min
2. Aspirate and add primary antibody solution and incubate for at least 16 hrs at 4°C. Remember do not let the cells be without solution for more than 10-15 seconds

Day 2

3. Collect primary Ab solution and immediately add 1xPBS/ TBS for washing (15 min "incubation")
4. Wash once more (15 min "incubation")
5. Aspirate the washing buffer and add secondary ab solution. Incubate for 1 hr (minimum 45 min) at RT in the DARK!
6. Collect secondary Ab solution and immediately add 1xPBS/ TBS for washing so that cells don't dry
7. Wash once more

8. Mount using anti-fading media containing DAPI:
    a. Cultures in **round glass coverslip**:
        i. Add a drop of mounting medium on the glass slide
        ii. With a forcep, carefully pick up the cover slip from the well and place it onto the droplet of mounting medium (avoid bubbles formation). Note to turn the coverslip upside down so that the cells are the ones in contact with the mounting medium.
        iii. Optional to put a big coverslip on top
    b. Cultures on **fibrous scaffolds**:
        i. Remove the plastic ring
        ii. With a forcep, pick up the fiber scaffold and place it onto the glass slide face up (cells up). Perhaps try to remove the excess buffer from the fibers, by touching sideways a paper mat
        iii. Immediately (fast) add a drop of the mounting medium on top of the fiber scaffold, and make sure that it cover all fiber mesh.
        iv. Place a cover slip on top of the sample avoiding bubbles.
9. Place the glass slides into the carton folder and store it at -20°C freezer

Alamar Blue assay

Materials and Reagents:

- AlamarBlue kit
- Culture media
- 96 well plate with opaque black walls for fluorescence measurements
- 3% SDS solution
- Aluminium foil

1. On the day of viability assay, prepare AlamarBlue (AB) solution by mixing 1:9 (1/10th volume) of AlamarBlue:Culture medium. Note: prepare some extra for blank/negative controls
2. Aspirate all culture medium from each culture well and immediately add 400 µl (if using a 24w plate) of freshly prepared AB solution
3. Wrap the plates with aluminium foil (**protection from direct light)**
4. Incubate at 37°C. Determine the time of incubation by checking the plate every hour
5. Add 50 µl of 3% SDS solution into each well to wells in a 96 w plate (black walls). Note: only the number of wells necessary)
6. Transfer 100 µl from each well of the medium after incubation with AB solution to individual wells in a 96 w plate containing 3% SDS. SDS will stop the reaction.

NOTE: Plate can be stored at RT for up to 24hrs (or 1-3 days at 4°C) before recording data, provided that the contents are protected from light and covered to prevent evaporation.

7. Record results using fluorescence or absorbance as follows:
    - **Fluorescence** measured at 530-570 nm excitation wavelength and 590nm (580-620nm) emission wavelength
    - **Absorbance** is monitored at 570 nm and 600 nm

Protein concentration measurements using Pierce Rapid GoldBCA protein assay kit

- Prepare BSA standards according to the kit protocol.
- Prepare 96-well plate design. 2 replicates per sample
- Keep standards and experimental samples on ice
- Prepare appropriate volume of Working Reagent (WR) required for assay procedures. Follow the formula to determine the total volume of WR required:

(# of wells for all samples including standards and 3 extra wells) x 0.2 ml = X ml

Prepare WR by mixing 50 parts of Rapid Gold BCA Reagent A with 1 part Rapid Gold BCA Reagent B (50:1, Reagent A:B):

Reagent A	Reagent B
10 ml	**0.2 ml**
5 ml	**0.1 ml**
2.5 ml	**0.05 ml**

Note: When Rapid Gold BCA Reagent B is first added to Rapid Gold BCA Reagent A, a pale blue/green precipitate may be observed, but, upon vortexing or mixing for < 5 seconds, the precipitate should dissolve to yield a clear, green solution. After making the WR, it is recommended to use the fresh solution. Upon standing at room temperature, it is normal to see a slight color shift to darker green for the WR. The standard curve is NOT affected when the signal is subtracted from the blank signal as long as the WR is used within 1.5 hours.

- Add 20 µL of standard solution to each well and 10 µl of experimental samples (3 wells/sample). This will allow a *2 factor dilution* of the samples.
- Add 200 µl of WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
- Incubate at RT for 5 min.
- Measure the absorbance at or near 480 nm on a plate reader.

Note: Because the assay acts quickly, the plate should be read within 20-30 minutes to ensure color development of the top standard (2 mg/mL BSA) is not out of instrument linearity. In the event plate reading must be done after 30 minutes, the reaction can be stopped by using 50 µL of 1 N HCl. Using a stop solution allows the plate to be read up to 1 hour post-incubation with < 7% difference in results.

Prepare a standard curve by plotting the average blank-corrected 480 nm measurement for each BSA standard versus its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Note: Using the spectrophotometer at Magnus lab, the standard curve and sample concentrations can be automatically done. Just need to select the parameters and then export to an excel file.

Western blot

Preparations in advance:

- Fill in experiment protocol. Calculate the volume of protein you want to load (ex. 2.5 µg - 20 µg of total protein).
- Turn on Heating block to 70°C, at least 1h before use.
- Prepare 1 x Running Buffer (NuPAGE) by mixing 50 mL stock with 950 mL ddH2O.
- Prepare 200 ml of running buffer with 500 µl antioxidant
- Prepare Blocking buffer (5% nonfat dry milk in TBST)
- Prepare TBS-T 0.1%
- Prepare blocking buffer (5% nonfat dry milk in TBST) e.g. by mixing 5g of dry milk with TBST
- Prepare all loading samples, according to the following table.

Note: the maximum loading volume for the 12w, 1.0mm Bis-Tris mini gels is 20 µl.

Components	Reduced samples 10 µl	Reduced samples 20 µl
Sample	x µl	x µl
NuPage LDS Sample Buffer (4x)	2.5 µl	5 µl
NuPage Reducing Agent (10x)	1 µl	2 µl
Deionized water	to 6.5 µl	to 13 µl
Total volume	**10 µl**	**20 µl**

- Open gel pouch (Novex NuPAGE, 4°C) and remove the cassette. Rinse with ddH2O
- Peel off tape covering slot on cassette
- Prepare running chamber (XCell SureLock, Invitrogen) by inserting gel cassettes (if only one, use Buffer Dam in place of real gel). Well side (shorter wall) should face in towards electrode wire. Insert tension wedge behind buffer core- unlocked. Pull tension wedge lever towards front
- Fill inner core chamber (small chamber) with a small amount **(200 ml)** of the running buffer **with antioxidant** and check if it is not leaking. If not, continue to fill core so that surface is well above top of wells.
- Remove comb and fill wells with running buffer and antioxidant. Be careful and avoid trapping bubbles
- Fill outer chamber with 600 mL Running Buffer (no antioxidant).
- Boil (water bath or hot plate) each cell lysate (experimental sample) in 1x Sample Buffer at 70°C for 10 min.
- Remove from bath (turn off) and put samples on ice
- Vortex and perhaps spin down (short)
- Load gel (mix and vortex before loading gel) with 20 µl/ well of sample (equal amounts of protein).
- For ladder, load 5 µl of Page Ruler (stored at -20°C) in the first well.
- Secure lid.
- Plug electrodes into power supply (Biorad Power Pack 200), pushing firmly to ensure contact. Turn on power supply and run gel, 50 V for 10 minutes, 150 V for 45 minutes and 200 V for 10 minutes.

- Make sure it is running by checking for small bubbles from electrode wire in inner chamber.
- When done (colored from should be near edge or have just run out), shut off the power supply and remove lid

Immunoblot

Removing the gel from the cassette for transfer after completion of electrophoresis:

- Using gloves, open the mini gel cassette using the Gel Knife by inserting the knife into the narrow gap between the 2 plates of the cassette. Push up and down gently on the handle of the knife to separate the plates.
- Slowly remove the gel and place it in 1x running buffer

Note: There is generally no need for any pretreatment of the gel after electrophoresis, but equilibration of the gel in 20% ethanol (prepared in deionized water) for 5–10 minutes prior to performing blotting improves the transfer of proteins >150 kDa

Assemble the transfer sandwich by:

- Wet the filter paper in deionized water
- Open Invitrogen iBlot device. Using gloves, take one anode stack, one cathode stack, one sponge and one filter paper from boxes (Invitrogen/novex)
- Unseal anode stack, keep in plastic tray. Place stack (with tray) on blot device. Align with Gel Barriers on right. Copper strip will point to the left.
- Cut off well and bulge at the bottom and throw away in acrylamide waste.
- Remove gel from electrophoresis plate by placing it in a tray with running buffer. Ease off of plate and place gel on top of anode stack, starting with one side and easing down while remaining centered and not introducing bubbles.
- Immediately, place the wet filter paper on top of gel, run roller over to remove bubbles
- Unseal cathode stack, throw plastic tray away. Place stack on top of filter paper so that electrode side (copper mesh) is facing up
- Make sure all sides are aligned, remove bubbles with roller
- Place sponge in lid of device with the metal contact on the right corner
- Close lid, secure latch and check for red light which confirms that the circuit is closed.
- Set to program 3 and press Start button
- When done, press the Stop button. Using gloves, open lid and carefully lift stack to be sure protein has transferred to membrane under gel (no color left in gel).
- Discard sponge. Remove top half of stack and discard gel in acrylamide waste
- Lift membrane and place it in TBST so it doesn't dry out while handling
- Clip off upper right hand corner, so you know where the beginning is, and cut into several pieces if necessary.
- If you have 2 gels, start blotting the second one

Antibody incubation and development

Optional:

- Briefly rinse the blot in water and stain it with Ponceau S solution to check the transfer quality
- Rinse off the Ponceau S stain with 3 washes with TBST

Antibody incubation:

- Put membrane in a 50 mL tube containing 10 mL blocking buffer (5% dry milk in TBS-T) careful not to roll on itself. Seal tube and run on a roller for 2-2.5 hrs at RT or overnight
- Add primary antibody directly to 4 mL blocking buffer
- Seal tube and run on roller at RT for 4 hrs or in the cold room (4°C) overnight
- Book Developing machine
- Discard antibody solution
- Rinse 3x15 (10) min with TBS-T (10-20 mL) and put on the roller at 4°C
- Remove last rinse, add at least 4 mL of secondary antibody (e.g. anti Rb HRP conjugated). Run on roller for 1 hour.
- Discard antibody solution. Rinse 3 x 15 (10) min with TBS-T (10-20 mL) at RT on shaker

Developing blot using SuperSignal West Pico PLUS Chemiluminescent substrate:

- Be sure developing machine is available.
- With lights off, mix equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution. Use 0.1 ml working solution per cm^2 of membrane. The working solution is stable for 8 hours at RT.
- Open drawer of machine and carefully put the clear plastic wrap on the tray and place the membrane on it. Add the chemiluminescence substrate, close the plastic wrap and remove bubbles with the roller
- Close drawer
- Log onto computer and open ImageLab 5.1 program Select new protocol Gel imaging:
    a. choose “calorimetric” to visualize ladder Select membrane size and for positioning the gel on the tray
    b. choose “chemiluminescent” choose “high resolution” for quantification
- Click to see membrane, change zoom to fit whole membrane.
- Click run. Adjust exposure time as needed, run again.
    a. Set exposure time if known protein (e.g. actin): 1 sec intervals for 10 seconds. Otherwise 5 sec intervals for 60 sec.

Note: Choose exposure time the lowest for publication (without overexposed pixels) or with few overexposed pixels for quantitative data.

- Save pictures to USB, select “export” and choose picture format.
- Log off, put membrane back in tube, clean machine surface.
- Run on roller 10 minutes (TBST), then remove, place in plastic wrap, label and store in refrigerator.

Stripping protocol

- Put the membrane in stripping buffer 4 mL/membrane in a 50 mL tube.
- Incubate membrane for 5-10 min in RT on a roller.
- Discard the buffer and refill with 4ml stripping buffer on roller for 10 min.
- Wash with 5-10 mL TBS for 2 x 10 minutes on a roller.
- Wash with 10 mL TBS-T for 2 x 5min on roller.
- Incubate with 20 mL blocking buffer (TBS-T + dry milk) for 2 h or overnight
- Continue with antibody incubation protocol

STEP	ITEM	Cat#	Quantity	Company
Protein extraction	RIPA lysis and extraction buffer	89900	100 ml	ThermoFisher
Sample purification and concentration	Pierce SDS-PAGE Sample Prep Kit	89888		ThermoFisher
Protein quantification	Pierce™ Rapid Gold BCA Protein Assay Kit	A53226	250 ml	ThermoFisher
Western blot	Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X)	78441	24 x 100 µL	ThermoFisher
	NuPAGE™ LDS Sample Buffer (4X) non reducing	NP0007	10 mL	ThermoFisher
	NuPAGE™ Sample Reducing Agent (10X)	NP0004	250 µL	ThermoFisher
	NuPAGE™ MOPS SDS Running Buffer (20X)	NP0001	500 ml	ThermoFisher
	NuPAGE™ Antioxidant	NP0005	15 ml	ThermoFisher
	NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 12-well	NP0322BOX	10 gels (1 box)	ThermoFisher
Immunoblot		NP00061	1 L	ThermoFisher
	iBlot™ Transfer Stack, PVDF, mini	IB401002	10 sets	ThermoFisher
Detection	Immobilon Western Chemiluminescent HRP Substrate	WBKLS0500	2 x 250 mL	Merck/Millipore
Stripping	Restore™ PLUS Western Blot Stripping Buffer	46430	500 ml	ThermoFisher

SDS-PAGE sample prep kit

Resin preparation and sample addition:

- Vortex the PAGE-prep Protein Binding Resin to evenly disperse the resin into a slurry
- Cut a pipette tip to transfer 20 µl of resin slurry into the center of a spin cup
- Based on protein concentration, add 1-300 µl of sample containing 1 – 70 µg total protein to the resin
- Add a volume of 100% DMSO equal to the sample volume added in the previous step. Cap tube and briefly vortex
- Incubate sample for 3 to 5 min at RT with occasional mixing to ensure maximum protein adsorption to the resin
- Centrifuge sample at 2000 x g for 2 min. Discard flow-through and blot collection tube on a paper towel.
- Reinsert spin cup into the same collection tube

Washing:

- Prepare solution by mixing 6 ml of DMSO with 6 ml of water (enough for 20 samples). Store wash solution at RT.
- Wash 1: Add 300 µl of wash solution to the resin. Cap tube and vortex until a homogeneous suspension is obtained.
- Centrifuge sample at 2000 x g for 2 min. Discard flow-through and blot collection tube on a paper towel.
- Wash 2: Add 300 µl of wash solution to the resin. Cap tube and vortex until a homogeneous suspension is obtained.
- Centrifuge sample at 2000 x g for 2 min. Discard flow-through and blot collection tube on a paper towel.
- Elution:
- Transfer the spin cup to a new collection tube and add 50 µl of Elution buffer to the resin. Cap tube and briefly vortex until a homogeneous suspension is obtained.
- Incubate sample at 60°C for 5 min. After incubation, briefly vortex the tube.
- Centrifuge sample at 2000 x g for 2 min. Discard spin cup and resin. Retain the collection tube containing eluate. **Note:** if desired perform a second elution to recover additional protein. This may result in excessive sample dilution.
- Store samples at -80°C or use it immediately to determine protein concentration.