

Rosa Karvonen

OPTIMIZATION OF NANOCELLULOSE HYDROGEL FOR MOUSE INTESTINAL ORGANOID CULTIVATION

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ABSTRACT

Rosa Karvonen: Optimization of nanocellulose hydrogel for mouse intestinal organoid cultivation
Master's thesis
Tampere University
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Supervisors: PhD Jenni Leppiniemi, MSc Mikko Oittinen, MSc Piia Mikkonen
Reviewers: Professor Heli Skottman, PhD Jenni Leppiniemi, MSc Piia Mikkonen
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Background and objectives of the study: Nowadays, biomedical research aims to build three-dimensional (3D) organ models to mimic better complex tissues and their functions. Organoids are self-organizing organ-like structures with near-native microanatomy. Organoids are a potential platform for drug screening and disease modelling. Conventionally in cell culturing animal-derived basement membrane extract mimics the extracellular matrix (ECM), which surrounds the cells and provides them with essential biochemical and mechanical cues. However, animal-derived materials suffer from reproducibility problems that limit their use in medical applications. Engineered matrices are promising alternatives to animal-derived materials. Nanocellulose hydrogels have shown their potential to mimic the native ECM with their high biocompatibility and tunable properties. In this thesis avidin-conjugated nanofibrillar cellulose hydrogel, GrowDex-A (GDxA), was studied as a 3D culturing platform for murine intestinal organoids. This thesis aimed to functionalize the GDxA with selected biotinylated proteins or peptides, and to optimize the culturing conditions for intestinal organoid cultivation.

Materials and methods: For GDxA functionalization, selected ECM proteins, vitronectin (VN), and laminin (LN) were biotinylated to bind them to GDxA via avidin-biotin interaction. In addition, biotinylated cyclic RGD (BcRGD) peptide and non-biotinylated laminin were used to functionalize GDxA. Intestinal crypts were cultured in GDxA functionalized with several protein combinations and concentrations. Functionalized GDxA was also supplemented with collagen (COL). Intestinal crypts were cultured in hydrogels with varying GDxA-COL ratios, and delayed crypt addition to premade GDxA-BcRGD-COL hydrogels was also studied. Several variables such as pH of COL, use of inhibitor, and incubation times in different steps of hydrogel preparation were investigated to find the optimal culture conditions. Bright-field microscopy and Live/Dead staining were used to characterize the organoid viability.

Results: Biotinylation was done successfully for VN and LN. A hydrogel containing 0.18 % (w/v) GDxA functionalized with 1.54 µg/ml BcRGD and supplemented with 1.4 mg/ml COL supported the intestinal organoid formation and was used in further experiments. When other GDxA-COL ratios were studied, cystic organoids formed only if hydrogel rolled into a thick mattress-like structure. When delayed crypt addition to premade GDxA-BcRGD-COL hydrogels was studied, cell growth was observed in every culture condition with differing COL pH levels and incubation times. The Live/Dead assay showed that during the first three culture days organoid growth in the GDxA-BcRGD-COL hydrogel seemed to be slower but otherwise similar to organoid growth in the animal-derived reference hydrogel.

Conclusions: Organoid formation was observed under many culturing conditions in GDxA-BcRGD-COL hydrogel, but more optimization is needed to produce GDxA-BcRGD-COL hydrogel reproducibly for intestinal organoid cultivation. More in-depth information on the genetics of the formed organoids is needed to confirm the similarity in growth between GDxA-BcRGD-COL and reference hydrogels. GDxA may have the potential as an alternative for animal-derived matrices for organoid cultivation when it is functionalized with BcRGD and supplemented with COL.

Keywords: intestinal organoid, nanocellulose, GrowDex-A, hydrogel, 3D cell culturing

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TIIVISTELMÄ

Rosa Karvonen: Nanoselluloosahydrogeelin optimointi hiiren suoliorganoidien kasvatukseen
Pro Gradu
Tampereen yliopisto
Bioteknologian tutkinto-ohjelma
Ohjaajat: FT Jenni Leppiniemi, FM Mikko Oittinen, FM Piia Mikkonen
Tarkastajat: Professori Heli Skottman, FT Jenni Leppiniemi, FM Piia Mikkonen
Huhtikuu 2022

Tutkimuksen tausta ja tavoitteet: Nykyään biolääketieteellisessä tutkimuksessa pyritään valmistamaan kolmiulotteisia (3D) elinmalleja, jotta monimutkaisia kudoksia ja niiden toimintoja voidaan jäljitellä tarkemmin. Organoidit ovat itsemuodostuvia elinten kaltaisia solurakenteita, joita voidaan hyödyntää esimerkiksi lääkeainetutkimuksessa sekä sairauksien mallintamisessa. Perinteisesti soluviljelyssä käytetään eläinperäistä tyvikalvouutetta, joka ympäröi soluja ja tarjoaa niille tärkeitä biokemiallisia sekä mekaanisia signaaleja soluväliaineen tapaan. Eläinperäisten tuotteiden käyttö kuitenkin aiheuttaa toistettavuusongelmia, mikä rajoittaa niiden käyttöä lääketieteellisissä sovelluksissa. Bioyhteensopivuutensa sekä muokattavuutensa ansiosta nanoselluloosahydrogeelit kykenevät jäljittelemään soluväliainetta ja ovat siten lupaavia eläinperäisten solukasvualustojen korvaajia. Tässä Pro Gradu -tutkielmassa avidiinikonjugoitua nanoselluloosakuitujen muodostamaa hydrogeeliä, GrowDex-A:ta (GDxA), tutkittiin hiiren suoliorganoidien viljelyalustana. Tämän tutkimuksen tavoitteena oli funktionalisoida GDxA valituilla biotinyloiduilla proteiineilla tai peptideillä ja optimoida viljelyolosuhteet suoliorganoidien kasvatukseen.

Materiaalit ja menetelmät: GDxA:n funktionalisointia varten soluväliaineen proteiineja, vitronektiinia (VN) ja laminiinia (LN) biotinyloitiin niiden sitomiseksi GDxA:han avidiini-biotiini vuorovaikutuksen avulla. Lisäksi biotinyloitua syklistä RGD-peptidiä (biotinyloivat cyclis RGD, BcRGD) ja biotinyloimatonta laminiinia käytettiin GDxA:n funktionalisoinnissa. Ohutsuolen kryptien kasvatuksessa käytettiin eri proteiiniyhdistelmillä sekä pitoisuuksilla funktionalisoitua GDxA:ta. GDxA:n joukkoon lisättiin myös kollageenia (collagen, COL), ja kryptia viljeltiin hydrogeeleissä, joiden GDxA-COL -suhde vaihteli. Lisäksi kryptien lisäystä edellisenä päivänä valmistettuihin GDxA-BcRGD-COL hydrogeeleihin tutkittiin. Optimaalisten kasvuolosuhteiden löytämiseksi useita muutujia, kuten COL:n pH:ta, inhibiittorin käyttöä ja inkubaatioaikoja hydrogeelin valmistuksen eri vaiheissa tutkittiin. Organoidien elinkelpoisuuden määrittämiseen käytettiin kirkaskenttämikroskopiaa ja Live/Dead-väryästä.

Tulokset: Proteiinien biotinylointi tehtiin onnistuneesti VN:lle sekä LN:lle. Organoidien muodostumista tuki hydrogeeli, joka sisälsi 0,18 % (w/v) GDxA:ta ja 1,54 µg/ml BcRGD:tä sekä 1,4 mg/ml COL:a, ja sitä käytettiin myöhemmissä tutkimuksissa. Muissa GDxA-COL -suhteissa suurirakenteisia organoideja muodostui vain, jos hydrogeeli oli rullautunut paksuksi patjaksi. Sellaisissa GDxA-BcRGD-COL hydrogeeleissä joihin kryptat lisättiin jälkikäteen, solukasvua havaittiin kaikissa olosuhteissa erilaisista COL:n pH arvoista sekä inkubaatioajoista huolimatta. Live/Dead väryä osoitti, että ensimmäisinä viljelypäivinä organoidien kasvu GDxA-BcRGD-COL hydrogeelissä oli hitaampaa mutta muuten samanlaista kuin eläinperäisessä kontrollihydrogeelissä.

Johtopäätökset: Organoidien muodostumista havaittiin monissa viljelyolosuhteissa GDxA-BcRGD-COL-hydrogeelissä, mutta lisää optimointia tarvitaan GDxA-BcRGD-COL-hydrogeelin toistettavaan valmistamiseen suoliorganoidien viljelyä varten. Syvällisempää tietoa organoidien genetiikasta tarvitaan GDxA-BcRGD-COL:ssa ja kontrolliviljelmässä kasvatettujen organoidien samankaltaisuuden todentamiseksi. GDxA voi olla potentiaalinen vaihtoehto eläinperäisille solukasvualustoille, kun se on funktionalisoitu BcRGD:llä ja täydennetty COL:lla.

Avainsanat: suoliorganoidi, nanoselluloosa, GrowDex-A, hydrogeeli, 3D-soluviljely

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck -ohjelmalla.

PREFACE

This master's thesis was performed in the Protein Dynamics and Intestinal Signaling, and the Epigenetics research groups at the Faculty of Medicine and Health Technology at Tampere University. The thesis was done in collaboration with UPM Biomedicals. Firstly, I would like to thank group leaders, Vesa Hytönen and Keijo Viiri for giving me an opportunity to work as a part of their groups and for helping me with my project. Also, I would like to thank the research groups and the NanOrganoid project group for helping me with various problems inside and outside the laboratory. The greatest gratitude I want to point to my supervisor Jenni Leppiniemi. You always had time to help, advise and support me throughout the whole project. Great thank you is also pointed to my other supervisor Mikko Oittinen, for guiding and encouraging me. I am also very thankful to my third supervisor, Piia Mikkonen who introduced me to the project and made this possible.

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ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
ASC	Adult stem cell
BCA	Bicinchoninic acid
BCM	Basal culture medium
BFN	Biotinylated fibronectin
BcRGD peptide	Biotinylated cyclic arginine-glycine-aspartic peptide
BLN	Biotinylated laminin
BSA	Bovine serum albumin
BVN	Biotinylated vitronectin
Ca-AM	Calcein-AM
CaCl ₂	Calcium chloride
CNC	Cellulose nanocrystals
CNF	Cellulose nanofibers
COL	Collagen
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EHS	Engelbreth–Holm–Swarm
ENR	Crypt culture medium
ESC	Embryonic stem cell
FN	Fibronectin
GDxA	GrowDex-A
HA	Hyaluronic acid
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iPSC	Induced pluripotent stem cell
LN	Laminin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PEG	Poly-ethylene glycol
PI	Propidium Iodide
PSC	Pluripotent stem cell
RGD peptide	Arginine-glycine-aspartic peptide
ROCK	Rho-associated protein kinase
RT	Room temperature
Real-time qPCR	Real-time quantitative polymerase chain reaction
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TPON	TEMPO-periodate-oxidized nanocellulose
VN	Vitronectin

1. INTRODUCTION

Tissue engineering and biomedical research have shifted toward building more accurate tissue and organ models for better treatment and diagnostic methods. Traditional models rely on monolayer cell cultures and ethically concerning *in vivo* animal models, which both differ greatly from native human tissues. Emerging three-dimensional (3D) tissue models can replicate human physiology and functions from the molecular level even to the whole organism level. (Liu et al., 2019) Organoids are 3D cell culture models which self-organize from stem cells and represent the structural and functional characteristics of some specific organ. Organoids may provide an accurate tool for various biomedical applications such as drug screening and disease modelling. (Clevers, 2016; Kratochvil et al., 2019; Kretzschmar & Clevers, 2016)

Many current organoid cultures rely on an animal-derived basement membrane extract as a culture scaffold. Due to the animal-based origin of these matrices, they have lot-to-lot variability and reproducibility problems that limit their use in therapeutic applications. Therefore, well-defined matrices for organoid cultivation are urgently needed. The culture scaffold must resemble the extracellular matrix (ECM) and provide the essential biochemical and mechanical cues to the organoids. (Gjorevski et al., 2016; Magno et al., 2020) Engineered matrices are promising alternatives to animal-based options. They provide tunable matrices which can be reliably used in medical applications. (Gjorevski et al., 2016; Kratochvil et al., 2019) Various alternative materials with positive results have been studied but to date, animal-based materials have not been completely replaced in organoid culturing (Curvello, Kerr, et al., 2021; Liu et al., 2019).

Nanocellulose hydrogels are natural, biocompatible, and non-toxic cell culture scaffolds that can mimic the ECM (Curvello et al., 2019). UPM Biomedical has recently launched an avidin-conjugated nanofibrillar cellulose hydrogel, GrowDex-A (GDxA). Once biotinylated, ECM proteins and peptides can be incorporated into the GDxA through conjugated avidin. (Leppiniemi et al., 2021) Rather than providing a passive cell culture environment, the functionalized GDxA provides biochemical cues to the cells and guides the organoid formation. In this thesis, GDxA hydrogel was examined and optimized as a culturing matrix for murine intestinal organoids. ECM proteins were bound to biotin and attached to GDxA. GDxA was functionalized with various proteins or peptides in different concentrations. Several culturing conditions were studied, and the growth of organoids was characterized to examine the viability of intestinal organoids in GDxA hydrogel.

2. LITERATURE REVIEW

2.1 Extracellular matrix

ECM is a noncellular complex network of macromolecules surrounding cells in all tissues and organs. ECM is mostly composed of water, fibrous proteins, proteoglycans, glycoproteins, and glycosaminoglycans, which are secreted locally and assembled into a mesh in close contact with cells. (Magno et al., 2020) The microenvironment around the cells during tissue morphogenesis affects the final composition of proteins and polysaccharides in each tissue and organ. Each tissue has its individual composition of these components which affect the functions and mechanical properties of the tissue. (Dudaryeva et al., 2021) ECM is a dynamic structure that is constantly remodelled by cells while they proliferate and migrate (Kratochvil et al., 2019). ECM offers sufficient mechanical support for cells but at the same time, it enables the diffusion of nutrients, transfer of metabolic waste, and transport of gases (Curvello et al., 2019). Through its unique characteristics, ECM creates the biochemical and mechanical properties of each tissue (Alberts et al., 2015; Frantz et al., 2010).

Fibrillar ECM proteins and glycoproteins provide adhesion sites for cells to anchor to the surrounding matrix (Magno et al., 2020). For cells, it is necessary to make cell-matrix interactions because the cell functions are maintained by the internal factors, but also through the connections with surrounding ECM (Dudaryeva et al., 2021; Liu et al., 2019). Interactions between ECM and cells contribute to transmitting signals and leading to the activation of signaling cascades. ECM can also store and release growth factors and important signaling molecules. (Heo et al., 2022) ECM regulates various cell functions such as cell survival, differentiation, growth, and migration. These functions are monitored through biochemical cues such as cytokines but also with biophysical cues such as niche elasticity and topography. (Dudaryeva et al., 2021; Kratochvil et al., 2019) ECM signals and the surrounding microenvironment have a robust relationship between cell functions and morphogenesis (Dudaryeva et al., 2021). Stem cells reside in a specialized instructive microenvironment called niche. The niche provides all necessary factors for stem cells to regulate their functions and to keep them in a stem cell state. ECM has a major role in keeping the niche environment controlled between cells and their surroundings. (Barker, 2014)

2.1.1 *ECM proteins*

Biopolymers in ECM contain multiple cell adhesion sites which enable cell attachment between cells and ECM (Kratochvil et al., 2019). Cell adhesion is mediated by integrins which bind the protein's cell adhesive site to the cell surface receptor (Heo et al., 2022; Kratochvil et al., 2019). One of the main cell adhesive sites in ECM proteins is the arginine-glycine-aspartic (RGD) sequence found in various proteins (Caliari & Burdick, 2016).

Laminin (LN), collagen (COL), fibronectin (FN), and vitronectin (VN) are common and important proteins in ECM, all possessing the RGD sequence in their structure (Curvello et al., 2019; Frantz et al., 2010; Magno et al., 2020). These proteins form a large part of the ECM matrix and take part in assembling the network of proteins in ECM. These proteins also form crucial links between cells and the surrounding matrix. (Alberts et al., 2015) COL, LN, and FN form a hydrated gel in the tissues (Frantz et al., 2010). FN has a large part in organizing the ECM and with VN they have a crucial role in maintaining the cell attachment (Bachmann et al., 2020; Frantz et al., 2010). COL is a major component in ECM providing structure and strength to tissues. COL type I is the most abundant COL type, and it has various roles in cell maintenance such as cell adhesion, supporting chemotaxis, and directing tissue development. (Curvello, Alves, et al., 2021; Frantz et al., 2010) For that reason, COL is an attractive material as a cell culturing platform (Caliari & Burdick, 2016; Jee et al., 2019). LN is the main component in animal-derived basement membrane extract, and LN forms a network of proteins through bonding with COL, FN, and other proteins. It is known to reinforce the mechanical properties and promote epithelial cell adhesion and differentiation. (Alberts et al., 2015; Broguiere et al., 2018; Curvello, Kerr, et al., 2021) A schematic of ECM and its major biopolymer classes are shown in Figure 1.

Various proteins and adhesive peptides have been vastly used in 3D cell culturing for mimicking the natural ECM to improve cell growth and enable essential interactions with cells and the matrix (Kratochvil et al., 2019). Adhesive sites, mainly RGD peptides are fundamental elements in these cultures, inducing high cell attachment and differentiation (Curvello, Kerr, et al., 2021). Protein concentration, spacing, presentation, patterning, and timing of ligand presentation influence the cultured cells (Kratochvil et al., 2019).

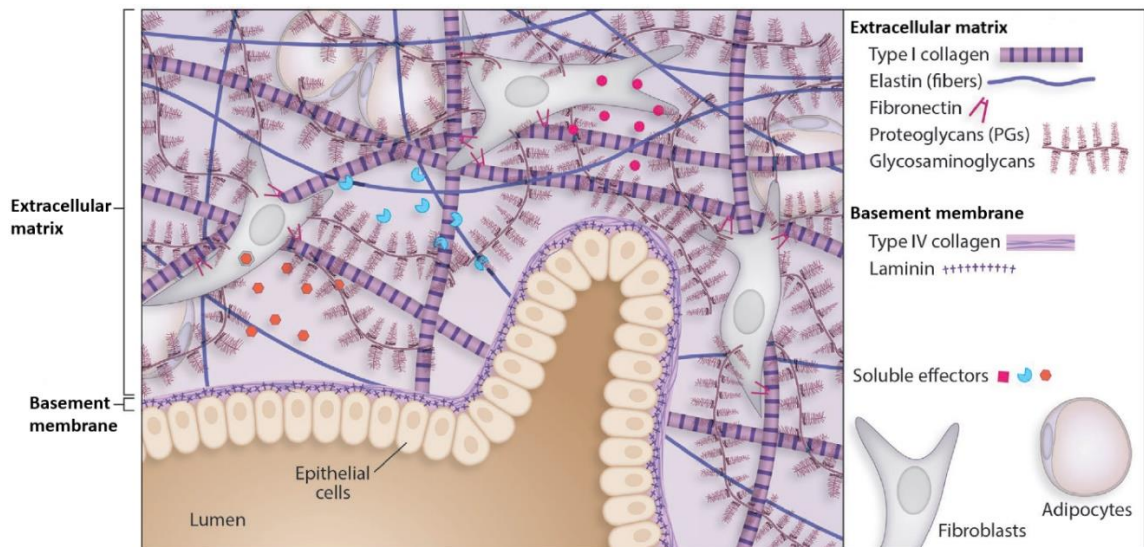


Figure 1. Major ECM components and their assembly in ECM and the basement membrane. Modified from Magno et al. (2020).

2.2 Organoids

First organoids were discovered in 2009 when the Clevers group embedded single intestinal stem cells into a substitute of ECM and discovered that crypt-like structures began to form similarly as in the original intestinal tissue (Sato et al., 2009; Yi et al., 2021). The group was able to identify the crucial ECM parameters for the organoid formation and to culture organoids in a medium with well-defined stem cell niche factors (Sato et al., 2009). Organoids are defined as self-organizing and self-renewing 3D *in vitro* grown organ-like structures with near-native microanatomy (Almeqdadi et al., 2019; Kretschmar & Clevers, 2016). Organoids can be initiated from healthy or cancerous primary tissue or generated from embryonic, adult, pluripotent, or induced pluripotent stem cells (ESC, ASC, PSC, or iPSC, respectively) by embedding the cells to ECM substitute and by providing them needed signals (Clevers, 2016; Kratochvil et al., 2019; Kretschmar & Clevers, 2016).

The formation of organoids relies on spontaneous morphogenesis that requires a single cell reorganization into complex and organized structures, which function as a specific organ. The stem cells undergo cycles of proliferation, differentiation, and self-organization while forming into organoids. (Kratochvil et al., 2019) The process involves numerous mechanisms such as physical rearrangement, biochemical and biophysical factors, and cell-matrix interactions (Kratochvil et al., 2019; Liu et al., 2019). Even though the stem cell self-renewal and differentiation are controlled with a specific amount of offered

cytokines and growth factors, the control over the self-organizing process is still limited because of its complex nature (Liu et al., 2019).

Several organoid types have been established in past years by varying the culturing environment and providing differing growth factor cocktails to the cells (Clevers, 2016). Established organoids include for example liver, kidney, lung, brain tissues, and mammary glands (Kratochvil et al., 2019; Kretzschmar & Clevers, 2016). Organoids resemble their native counterparts in multicellular architecture and functional features. As such, the establishment of long-term organotypic cultures provides a powerful tool between a two-dimensional (2D) cell culture and animal models for a wide range of biomedical applications. Organoids can replace traditional 2D and *in vivo* animal models in disease modeling and drug screening. (Gjorevski et al., 2016; Kratochvil et al., 2019; Kretzschmar & Clevers, 2016) Simplistic 2D models have often significant differences in gene expression, epigenetics, and cell functions compared to native tissues (Liu et al., 2019). Patient-specific organoids are possible to be generated from iPS cells and expansions of primary biopsies. Patient-specific organoids enable a more precise investigation of organ pathophysiology and potential treatments. (Kratochvil et al., 2019) Organoids are also a great tool for toxicology studies and developmental biology (Curvello, Kerr, et al., 2021).

2.2.1 *Intestinal organoids*

The epithelium of the small intestine is divided into crypts and villi (Fig. 2A). At the bottom of the crypts, Paneth cells are surrounding the intestinal epithelial Lgr5⁺ stem cells that generate all cell types of the intestinal epithelial. (Barker, 2014; Sato et al., 2009) Signals from the surrounding epithelium maintain the homeostasis in the crypts and stemness of Lgr5⁺ cells (Barker, 2014). Enterocytes, goblet cells, and enteroendocrine cells develop from Lgr5⁺ and start migrating from the bottom of the crypt to the tip of the villus (Barker, 2014; Sato et al., 2009). Environmental changes during the migration from the crypt to villus control the differentiation into specific cell types (Barker, 2014). When mature cells reach the tip of the villus, cells are lost by apoptosis in a rapid self-regeneration phase. The migration time of the cells is around five days. (Sato et al., 2009)

When Sato et al. provided a culture medium supplemented with epidermal growth factor (EGF), Noggin, and R-spondin to the single mouse Lgr5⁺ stem cells, cells started to form round, cystic structures. These structures formed crypt-like buddings which further developed into intestinal organoids with distinct crypt-villus structures seen *in vivo*. All intestinal epithelial cell types could be found in such organoid structures. (Kretzschmar &

Clevers, 2016; Sato et al., 2009) When the intestinal epithelium is cultured as an organoid, the budding crypts are on the basolateral side and the villus structures are inside the organoid on the apical side (Fig. 2B) (Montenegro-Miranda et al., 2020).

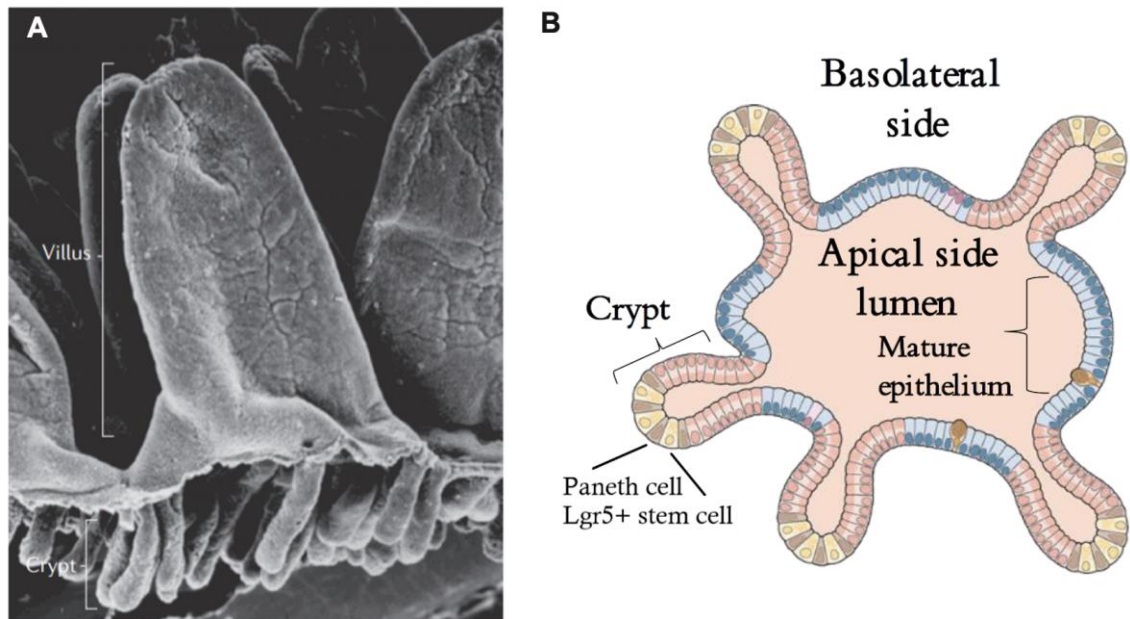


Figure 2. Crypt-villus structures of intestinal tissue and intestinal organoid. A) In intestinal tissue the crypts are invaginated into the submucosa and villus structures point towards the intestinal lumen. B) In intestinal organoids the stem cell containing crypts bud outward from the central structure and maturing epithelial cells migrate toward the lumen inside the organoid. Modified from Barker (2014) and Montenegro-Miranda et al. (2020).

At the beginning of the intestinal organoid culture, single intestinal epithelial stem cells or intestinal crypts are embedded into ECM resembling matrix. In the absence of mesenchymal cells that normally occur in the intestine, the R-spondin, EGF, and Noggin recapitulate the niche microenvironment of native intestinal tissue and support the stem cell state in the crypts. (Clevers, 2016; Sato et al., 2009) Intestinal crypts are vulnerable to single-cell disintegration, and usually these detached cells undergo apoptosis (Randell & Fulcher, 2012). Sato et al. used a Rho-associated protein kinase (ROCK) inhibitor in the organoid culture, and it significantly decreased cell death and prevented single-cell disintegration (Sato et al., 2009). When cultured in proper conditions, Lgr5⁺ stem cells undergo cycles of self-renewal, differentiation, and morphogenesis during organoid formation. In seven days, the organoids have formed large and complex 3D structures that contain several outward-pointing buds, which include all normal intestinal epithelial cell types. Due to the self-renewal and apoptosis in the tip of the villi, more individual cells start to appear around the organoid as the days of culture go by. (Brogiere et al., 2018; Gjorevski et al., 2016; Sato & Clevers, 2015) A schematic view of organoid culturing and formation of organoids is shown in Figure 3.

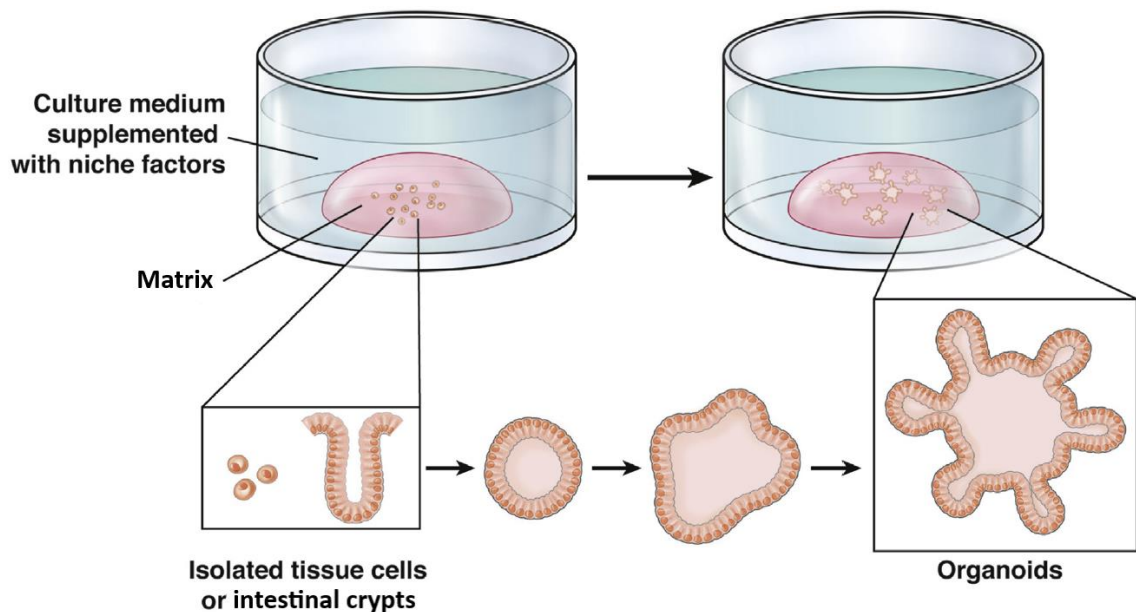


Figure 3. Intestinal organoids are embedded into the matrix as stem cells or crypts. The niche factors are provided for them in the culture medium. Organoids start to form outward budding crypt-like structures, and the formation of large cystic organoids takes about seven days. Modified from Fujii et al. (2019).

Intestinal organoids enable an indefinite supply of intestinal epithelium because organoids can be mechanically or enzymatically dissociated and passaged weekly for years while staying genetically and phenotypically stable (Broguiere et al., 2018; Clevers, 2016; Gjorevski et al., 2016; Sato & Clevers, 2015). Intestinal organoids are a robust tool for the investigation of intestinal functions and the development of intestinal tissue. Intestinal organoids can work as patient-specific models for genetic epithelial diseases and as a source for transplants. (Gjorevski et al., 2016; Sato & Clevers, 2015)

2.3 Hydrogels as a cell culture scaffold

Cell culturing has conventionally relied on 2D models, but they cannot possess all characteristics of the native 3D environment such as matrix stiffness and specific niche factors (Dudaryeva et al., 2021). A flat 2D culture system can also cause abnormalities in cell functions and cell growth (Caliari & Burdick, 2016; Magno et al., 2020). When the stem cell niche is recreated *in vitro*, the design should consider the biochemical and mechanical factors the cells have in their native tissue (Heo et al., 2022). *In vivo*, cells receive signals from all three dimensions and that is why more complex 3D culture models are needed (Caliari & Burdick, 2016). For organoids, a 3D culture environment is required because organoids are naturally self-organizing into 3D structures (Clevers, 2016). Hydrogels work as an alternative to ECM, and they can be tuned to match the

microenvironment needed for each tissue or organ model (Dudaryeva et al., 2021; Liu et al., 2019).

Hydrogels are complex 3D networks of polymers that can absorb a large amount of water. Hydrogels can be categorized into natural, synthetic, and hybrid hydrogels according to their composition. Hydrogels made from natural polymers such as COL, fibrin, or alginate are biomimetic and resemble the natural ECM. COL and fibrin have naturally cell adhesive ligands in their structures. (Caliari & Burdick, 2016; Liu et al., 2019) Synthetic polymers such as polyethylene glycol (PEG) are on the other hand bioinert, but they are more tunable materials due to their defined composition. For example, the mechanical properties of synthetic hydrogels are easy to adjust when natural polymers tend to have low stiffness. (Caliari & Burdick, 2016) Hydrogels can also be enzymatically or covalently crosslinked to alter the matrix of polymers (Liu et al., 2019). Over past decades the hydrogels have also been incorporated with proteins, peptides, and other molecules to resemble better the native ECM (Gjorevski et al., 2016; Liu et al., 2019). One hydrogel material alone cannot match the ECM perfectly due to its complexity, but different combinations of polymers can be blended to enhance the advantages of both materials (Caliari & Burdick, 2016; Liu et al., 2019).

Important parameters of hydrogel for 3D cell culturing are mechanical strength, porosity, elasticity, biodegradability, and ease of functionalization (Thiele et al., 2014). Properties of the hydrogels are dependent on the polymer type, gelation method, and fabrication technology. The hydrogel can be modified by varying for example its stiffness, topography, and chemical reactivity. By varying these factors, also cellular behaviours can be altered. In cell culture, the hydration efficiency and hydrogel porosity allow appropriate osmotic pressure and adequate transport of substances to the cells. (Liu et al., 2019) Hydrogels allow the exchange of nutrients, gases, metabolic wastes, cytokines, and growth factors between cells and matrix due to the interconnected network of pores (Bhattacharya et al., 2012; Liu et al., 2019). Hydrogels can also mimic the ECM microenvironment that cells have *in vivo* with biochemical and biophysical cues which affect the behaviour of cell growth and interactions (Caliari & Burdick, 2016; Liu et al., 2019). In Figure 4, tunable physicochemical properties and their effect on the cells are illustrated. Hydrogels are promising scaffolds for 3D culture systems such as tissue engineering and diagnostics devices due to their exceptional permeability, biocompatibility, and tunability (Caliari & Burdick, 2016; Liu et al., 2019).

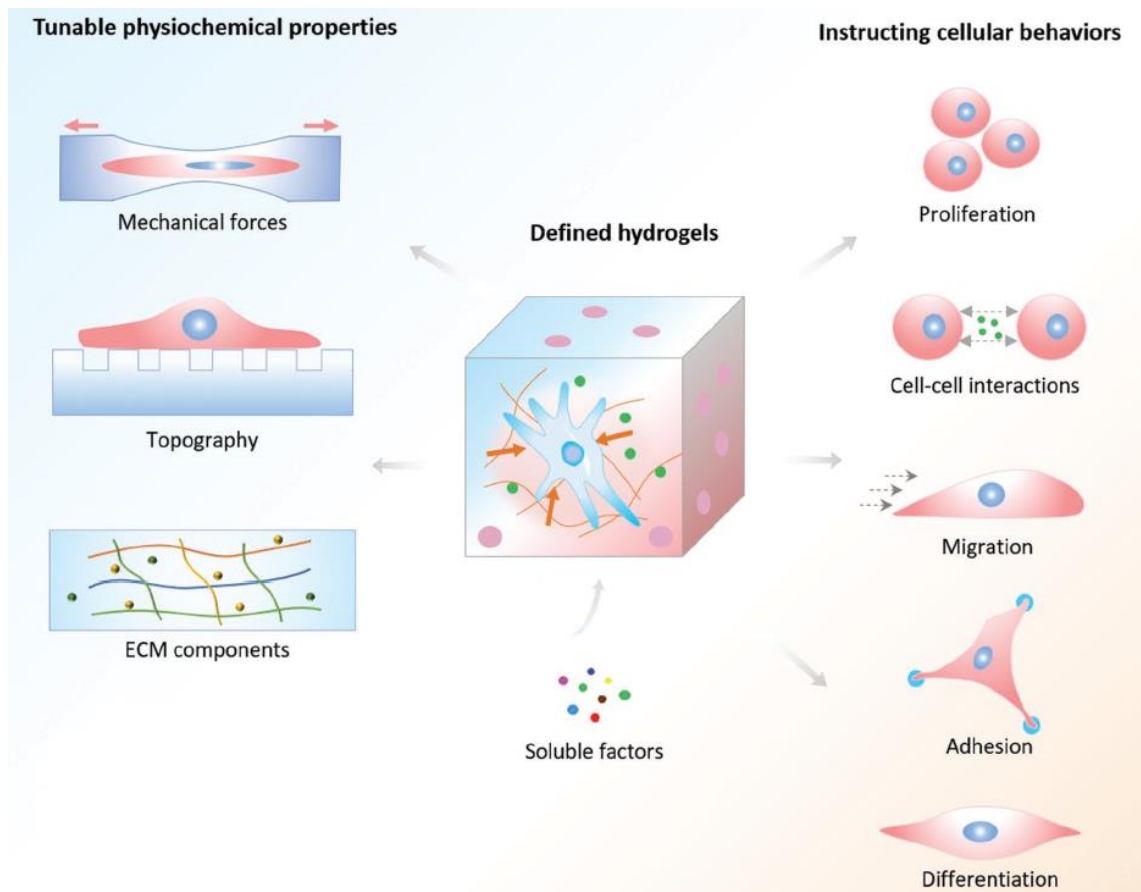


Figure 4. In defined hydrogels, the mechanical forces, topography, and ECM components create the cellular microenvironment and instruct cell proliferation, cell-cell interactions, migration, adhesion, and differentiation. Modified from Liu et al. (2019).

2.4 Hydrogels in organoid culturing

Organoids set requirements for the surrounding matrix material. It must mimic conditions found in the tissue of origin to support the organoid formation. Intestinal organoids require mechanically supportive but still flexible 3D material that enables the manipulation of the material during organoid formation. (Curvello et al., 2019; Gjorevski & Lutolf, 2017) Organoids form a pressure towards the surrounding matrix while budding, and Broguiere et al. found out that the pressure organoids directed to the matrix vary in different organoid formation phases (Broguiere et al., 2018). Viscoelastic material behaviour enables gel softening and relieves compressive stress throughout different phases (Gjorevski & Lutolf, 2017). Furthermore, organoids require that the matrix material is coated or filled with adhesion proteins (Curvello et al., 2019). Intestinal organoids modify the matrix while forming into organoids and for that, it is crucial to have sufficient cell adhesions to matrix material (Kratochvil et al., 2019).

Due to the challenging requirements of intestinal organoids, the current organoid culturing is mainly relying on an animal-derived hydrogel, commercially known for example as

Matrigel (Gjorevski et al., 2016). Matrigel is secreted from Engelbreth–Holm–Swarm (EHS) murine sarcomas and it consists of a heterogeneous mixture of ECM proteoglycans, proteins, and growth factors (Hughes et al., 2010). Analyses have identified over 1500 unique peptides and proteins from Matrigel. Even though Matrigel works well in organoid culturing it has many drawbacks due to its origin. (Kratochvil et al., 2019) Thorough chemical characterization of the material is not possible and large batch-to-batch variations limit the cultivation reproducibility in the material. Heterogeneous composition limits the tunability of the material. Due to a huge protein concentration, the cell adhesion and functions cannot be controlled, which may cause unexpected influences in drug testing and make the results inaccurate. (Broguiere et al., 2018; Kratochvil et al., 2019) The mouse tumour origin of Matrigel limits the use of organoids as transplants or in clinical applications as the material has a risk of immunogen and pathogen transfer (Gjorevski et al., 2016; Kretzschmar & Clevers, 2016).

During the last decades, engineered matrices have emerged as an important alternative to conventional animal-derived culture matrices. Engineered matrices provide better tunability with minimum batch-to-batch variability due to their fully chemically defined nature. Therefore, various engineered materials are being examined for organoid cultivation. (Kratochvil et al., 2019) For example, hydrogels made from COL, fibrin, alginate, PEG, and hyaluronic acid (HA) have been used as an alternative for Matrigel (Bergenheim et al., 2020; Kozlowski et al., 2021).

2.4.1 *Animal-derived hydrogels*

COL has been extensively investigated in 3D cultures and has been approved for use in several applications (Curvello, Alves, et al., 2021; Heo et al., 2022; Jee et al., 2019). COL is a thermo-, and pH-responsive material; it is soluble when the pH is low and COL is cooled to 4-8°C, and it forms a solid hydrogel when its pH is neutralized, and COL is heated to +37°C. Temperature and pH changes during the gelation affect the organization of COL fibres in the hydrogel. (Curvello, Alves, et al., 2021; Doyle et al., 2015; Sung et al., 2009) Polymerization temperatures affect the fibre diameter and gel porosity, which then greatly affect the cell adhesion and viability (Doyle et al., 2015). Sachs et al. embedded intestinal organoids into floating COL hydrogels and the cystic organoids fused and formed a macroscopic hollow intestinal tube within two days (Sachs et al., 2017). COL hydrogels alone are weak, but mechanical properties can be tuned by blending COL with other polymers. Hydrogels containing COL and polymers offer required stiffness but also improve cell-surface interactions to support cell growth. (Curvello, Alves, et al., 2021)

Human fibrin is commonly used as a tissue sealant, and it has biochemical and mechanical properties suitable for organoid expansion. Broguiere et al. developed fibrin-based hydrogels functionalized with LN, which supported long-term epithelial organoid formation. They discovered that RGD adhesion domains, naturally occurring in fibrin, are crucial for intestinal stem cell proliferation and organoid formation. In the same study, they identified LN to be the major biological signaling factor in the matrix for organoid growth. Fibrin hydrogel functionalized with LN was suitable for other organoid types beyond intestinal organoids. (Broguiere et al., 2018)

2.4.2 Animal-free hydrogels

PEG-based hydrogels are widely used in tissue engineering due to their nontoxicity and favourable hydration ability (Liu et al., 2019). Gjorevski et al. reported that two different PEG gels are needed for intestinal organoid culturing. Nondegradable stiff PEG gel promotes intestinal stem cells to form crypts and hydrolysing soft PEG gel triggers the differentiation of cells into functional cell types of the intestine. PEG gels functionalized with FN, LN, COL IV, HA, perlecan, or RGD peptide enhanced organoid survival and proliferation. If only minimal biochemical signals are provided to the organoids, stiffness of the hydrogel is essential for organoid formation. If PEG hydrogels were functionalized only with RGD peptides, organoids formed only in gels with stiffness around 190 Pa. (Gjorevski et al., 2016) Cruz-Acuña et al. injected organoids into the intestinal mucosal wound of mice, after culturing intestinal organoids in defined four-armed maleimide-terminated poly PEG hydrogel functionalized with RGD. Delivery of intestinal organoids into colonic wound increased significantly the wound closure compared to an untreated wound. (Cruz-Acuña et al., 2017) This indicated that intestinal organoids have a potential for colonic wound repair (Liu et al., 2019).

Alginate, a seaweed-derived polysaccharide, has also been used as a hydrogel to support intestinal organoid formation. As a material, it is inert and tunable, but it does not possess cell adhesion sites which are considered relevant for organoid formation. (Heo et al., 2022) Capeling et al. hypothesized that intestinal organoids naturally create their niche and do not need any inherent cell recognition or signaling proteins. In their study, they used alginate as a biochemically minimal matrix which gave only mechanical support for the organoids. The stiffness of the alginate was matched to the PEG hydrogel used by Cruz-Acuña et al. (2017). Organoids derived in alginate hydrogel were highly similar structurally and genetically to organoids grown in Matrigel and underwent similar maturation in both cultures. (Capeling et al., 2019)

HA hydrogels have also been studied vastly in organoid culturing (Kozłowski et al., 2021). Hunt et al. designed a hydrogel which was a combination of modified hyaluronan and modified elastin-like protein which have been previously reported to be well tolerated materials *in vivo*. The group was able to grow and passage primary human intestinal tissue organoids in this biopolymer hydrogel. (Hunt et al., 2021)

2.5 Nanocellulose hydrogels in organoid culturing

Wood-based nanocellulose is a promising natural hydrogel material for 3D culture applications. Nanocellulose refers to shorter cellulose nanocrystals (CNC) having a diameter of 5 nm and a length of 20 to 100 nm, or longer cellulose nanofibers (CNF) having a diameter of 3 to 100 nm, and a length of several micrometers depending on the source of cellulose, pretreatment, and defibrillation process. (Curvello et al., 2019; Lin & Dufresne, 2014) In this thesis, the term nanocellulose refers to CNF, because CNF-based hydrogel is used in the study. Nanocellulose can be obtained with various methods but 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) mediated oxidation is the most popular method for extracting nanofibers from wood pulp. TEMPO-mediated oxidation introduces negatively charged carboxyl groups to the surface of the nanofibers. (Isogai et al., 2011) Charged carboxyl groups enable editing of the surface of the nanofibers and crosslinking of the material, which can alter the mechanical properties of the hydrogel (Curvello et al., 2019).

When mixed with water, CNFs form a hydrogel stabilized by weak interactions. Nanocellulose hydrogels have high water content (95 to 99.9 wt%) and good mechanical and thermal properties, but alone nanocellulose is a relatively inert material. However, the cellulose backbone can be modified to immobilize biomolecules such as peptides and functional groups. Nanocellulose is an appealing material for biomedical applications because of its biodegradability, biocompatibility, and flexibility. Non-toxicity is an important feature of hydrogels meant for clinical purposes, and nanocellulose has not been reported to have significant toxic effects. (Curvello et al., 2019) Nanocellulose hydrogels have already shown good results in various culturing applications (Curvello, Kast, et al., 2021; Krüger et al., 2020; Leppiniemi et al., 2021). A schematic view of producing nanocellulose hydrogel from wood is presented in Figure 5.

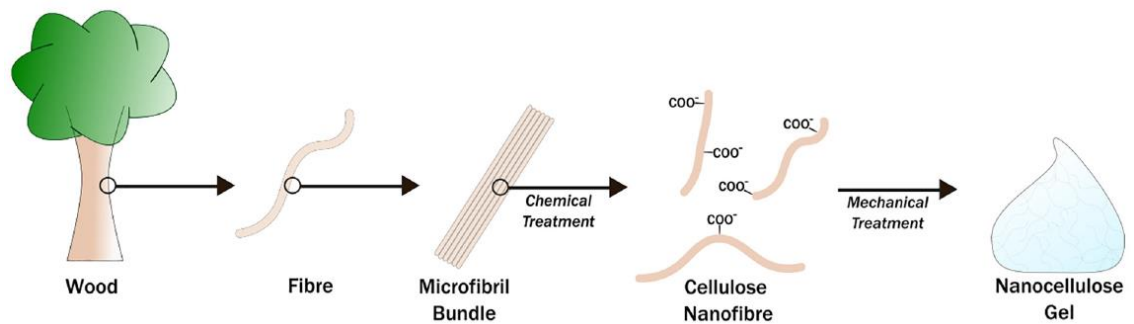


Figure 5. Nanocellulose gel production. Cellulose fibres are formed from microfibril bundles obtained from wood. By chemical and mechanical treatment, the cellulose nanofibers may be extracted, which form hydrogel with water. Modified from Curvello et al. (2019).

Curvello, Kerr, et al. cultivated small intestinal organoids within nanocellulose hydrogels conjugated with RGD peptide. They discovered that in plain nanocellulose hydrogel organoids turned into agglomerated clusters of debris in two days. Once they balanced the osmolality and the pH of the hydrogel with Glycine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) the organoid progressed into cystic structures similarly to organoids in Matrigel. Temperature responsive gelation of Matrigel was mimicked by ionic crosslinking of 0.1 wt% nanocellulose hydrogel with calcium chloride (CaCl_2). (Curvello, Kerr, et al., 2021)

The same group investigated cationic crosslinking of hydrogel to control the mechanical properties of nanocellulose with Ca^{2+} -, and Mg^{2+} -ions. They used TEMPO-oxidized nanocellulose and TEMPO-periodate-oxidized nanocellulose (TPON) where the latter has higher carboxyl content. Small intestinal organoids were cultured for four days in hydrogels functionalized with RGD peptide and crosslinked with Ca^{2+} -, or Mg^{2+} -ions. TPON-based hydrogel showed organoid growth comparable to Matrigel whereas TEMPO-based hydrogels exhibited less budding. (Curvello & Garnier, 2021)

Later Curvello, Alves, et al. produced multi composite hydrogel by mixing RGD peptide functionalized nanocellulose hydrogel with bovine skin type I COL. Blending of nanocellulose at a concentration of 0.1 wt% to 0.2 wt% COL increased the mechanical properties of hydrogel to match the properties of Matrigel. The mixture produces porous, viscoelastic, and thermo-responsive hydrogel with proper mechanical and biochemical properties for intestinal organoid growth. Crypts embedded in the nanocellulose-COL hydrogel started to form organoids after three days. Metabolic activity and cell viability were comparable to organoids grown in Matrigel. (Curvello, Alves, et al., 2021)

2.5.1 GDxA nanocellulose hydrogel

In recent years UPM Biomedicals has produced a CNF-based product family. Transparent nanofibrillar hydrogel product, GDxA is used in this thesis. In GDxA the nanocellulose is conjugated with avidin protein. (<https://www.upmbiomedicals.com/siteassets/documents/growdex-hydrogels-product-brochure-2021.pdf>, viewed 15.3.2022) The principle of the product relies on the high specificity and extraordinary affinity between avidin and biotin molecules (Laitinen et al., 2007). ECM proteins can be chemically attached with biotin i.e., biotinylated by using for example amine-reactive biotinylation reagents such as NHS-biotin. When biotinylated proteins are introduced with avidin-conjugated nanocellulose, avidin and biotin bind with extreme affinity, and biotinylated molecules become immobilized. (Leppiniemi et al., 2021) Bound proteins in the nanocellulose enable crucial cell-matrix interactions for organoid growth.

Compared to other materials, nanocellulose hydrogels are easy to use, because they can be handled at room temperature (RT) and do not require either crosslinking or gel polymerization. GDxA can be customized by binding different biotinylated molecules, including proteins, peptides, antibodies, or other bioactive molecules in varying concentrations to produce an optimal matrix for cell culture. Leppiniemi et al. demonstrated how GDxA functionalized with biotinylated FN and VN (BFN and BVN, respectively) increased mouse embryonic fibroblast proliferation significantly in protein concentration of 0.05 mg/ml. GDxA may provide a platform to develop well-defined ECM alternatives for 3D cultures, and such cultures could be reliably used for example in drug screening and disease modelling. (Leppiniemi et al., 2021) A schematic of GDxA functionalization and the resulting cell behaviour are presented in Figure 6.

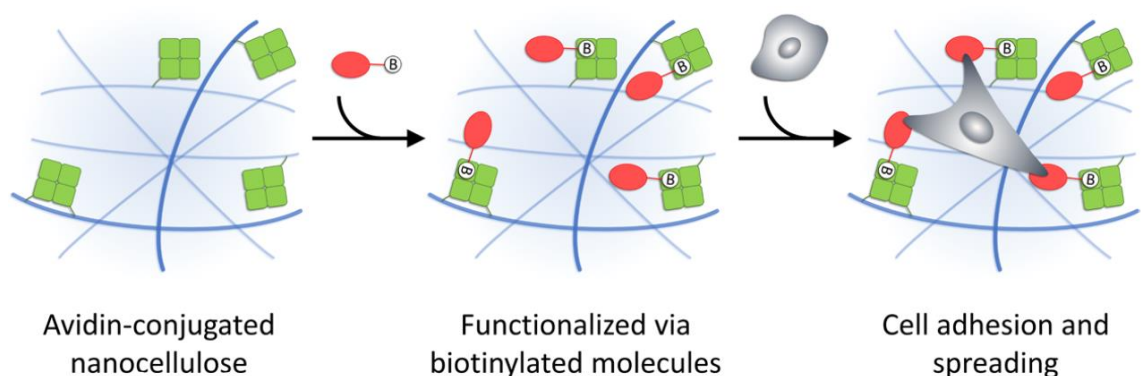


Figure 6. Conjugated avidins enable nanocellulose functionalization via biotinylated molecules. Biotinylated proteins provide adhesion sites for cells and support their adhesion and spreading. Modified from Leppiniemi et al. (2021).

3. OBJECTIVES

The aim of this study was to optimize GDxA hydrogel for the cultivation of mouse intestinal organoids. For this, selected ECM proteins were biotinylated and GDxA hydrogel was functionalized by binding the proteins to the hydrogel. Different combinations of biotinylated or non-biotinylated proteins and peptides were studied in the mouse intestinal organoid cultures in various concentrations. The growth of intestinal organoids in GDxA hydrogel was observed and characterized to see if the growth of organoids is similar to the reference culture. The detailed aims were:

1. Biotinylate selected ECM proteins.
2. Functionalize GDxA hydrogel with several proteins and peptides at different concentrations and combinations.
3. Characterize the growth of intestinal organoids in GDxA hydrogel.

4. MATERIALS AND METHODS

4.1 Biotinylation of ECM proteins

Cultrex Mouse Laminin 1 (R&D Systems, Minneapolis, MN, USA) was dialyzed in phosphate buffered saline (PBS) for 24 hours by using a 10kDA Slide-A-Lyzer™ dialysis cassette (Thermo Fisher Scientific, Waltham, MA, USA) before biotinylation. VN was previously purified from human plasma and provided by the Protein Dynamics group (TAU). Biotinylation was performed by biotinylation reagent Ez-Link NHS-LC-biotin (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. NHS-LC-biotin was dissolved into dimethylsulfoxide (DMSO) in 10 mM concentration. For LN, a 40-fold molar excess of reagent was added to the solution and incubated for 1 hour at RT. The biotinylation was conducted similarly to VN, but a 7-fold molar excess of NHS-LC-biotin was used. After biotinylation, unattached biotins were removed by dialyzing biotinylated proteins in PBS for 48 hours. After dialysis, biotinylated proteins were sterile filtered with a 0.2 µm syringe filter (Sarstedt, Numbrecht, Germany).

Protein concentration of biotinylated proteins was determined by colorimetric method using Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for microplate procedure according to manufacturer's protocol. Both biotinylated proteins were diluted into a working range and pipetted into a microplate (Thermo Fisher Scientific, Waltham, MA, USA) as replicates. The working reagent was added to each sample and the microplate was mixed thoroughly with a shaker. After 30-minute incubation at +37°C, the absorbance at 562 nm was measured with plate reader EnVision (Perkin Elmer, MA, USA) at 562 nm. A dilution series of bovine serum albumin (BSA) was prepared and assayed alongside the biotinylated proteins. Concentrations of biotinylated proteins were determined based on the standard curve made from BSA dilution series results.

Biotinylation degree i.e., a mole-to-mole ratio of biotin per protein was determined by using 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay (Thermo Fisher Scientific, Waltham, MA, USA) and the procedure for microplate format was followed. Previously purified and lyophilized avidin was provided by the Protein Dynamics group. Protein concentration of avidin was determined for HABA/avidin solution. A HABA solution was made, and pH was adjusted with sodium hydroxide (NaOH, 34 mM). HABA/avidin solution was done according to instructions and sterile filtered. In addition to the undiluted

sample, a 1:2 diluted sample was made of biotinylated laminin (BLN) and BVN for the absorbance measurements. Along with the biotinylated protein samples, two control samples were made from D-Biotin (Fluka Chemie GmbH, Buchs, Switzerland) where one was known to saturate half of the binding sites of avidin, while the other saturates all the binding sites of avidin. First, the absorbance of HABA/avidin solution at 500 nm was measured. Then, the biotinylated protein was added to the well, the sample was thoroughly mixed with a shaker and the absorbance was measured again at 500 nm. Absorbance measurements were performed with EnVision plate reader and the measurement was done four times for each sample until the absorbance values remained constant.

4.2 Osmolality and pH balancing of GDxA

For this study, GDxA nanocellulose hydrogel was provided by UPM Biomedicals, Finland. All pipetting of GDxA was performed with low adhesion pipet tips (Thermo Fisher Scientific, Waltham, MA, USA) to reduce the loss of hydrogel. Osmolalities of all hydrogel components were measured with an osmometer (Osmomat 030, Gonotec, Logan, UT, USA). Based on these measurements (data not shown) osmolality of GDxA was matched to Matrigel with 150 mM Glycine (Sigma-Aldrich, St. Louis, MO, USA) and 15 mM HEPES (Lonza, Basel, Switzerland). Before mixing with biotinylated proteins, or crypts, the GDxA hydrogel was mixed with Glycine and HEPES mixture, and the pH was balanced to 7 with NaOH.

4.3 GDxA functionalized with biotinylated proteins

GDxA was functionalized with selected biotinylated proteins or peptides. During functionalization, balanced GDxA was mixed with the desired amount of biotinylated protein or peptide and incubated at RT at least for one hour. In all experiments with biotinylated proteins or peptides (Exp. 1-4), the GDxA concentration was 0.4 % (w/v). To obtain the desired dilution of balanced GDxA and biotinylated protein or peptide, the rest of the sample volume was filled with PBS, and later with basal culture medium [BCM, Dulbecco's modified Eagle medium (DMEM)/F12 (Gibco), containing 1:100 dilution 100x GlutaMax (Gibco), 10 mM HEPES (Gibco), 1:100 dilution 100x N-2 supplement (Gibco), 1:50 dilution 50x B27 (Gibco) all obtained from Thermo Fisher Scientific (Waltham, MA, USA), 1 mM N-acetyl-L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and Primocin 100 µg/ml (InvivoGen, Toulouse, France)] to increase the amount of nutrients in the hydrogel.

In addition to BLN and BVN, GDxA was functionalized with BFN, which was provided by the Protein Dynamics group. Also, biotinylated cyclic RGD (BcRGD, Peptide Institute, Inc., Osaka, Japan) was used in the organoid cultures alone, but also with non-biotinylated laminin. To obtain higher BLN concentrations in the hydrogels, the BLN was concentrated 10-fold by using a centrifugal concentrator (Vivaspin 500, Sartorius, Göttingen, Germany).

Crosslinking of GDxA hydrogel was studied by using CaCl₂ (Sigma-Aldrich, St. Louis, MO, USA) crosslinking solution (100 mM) with functionalized GDxA hydrogels. To crosslink the hydrogel, 5 µl of CaCl₂ was added to the bottom of the well plate and hydrogel was added on top of it. The hydrogel was held at RT for 5 minutes before adding crypt culture medium [ENR, BCM supplemented with 1 µg/ml Noggin (PeproTech, Rocky Hill, NJ, USA), 0.3 µg/ml R-spondin 1 (PeproTech, Rocky Hill, USA) and 0.05 µg/ml EGF (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)] on top of the hydrogel.

Supplementation of culture medium with 10 µM ROCK Y-27632 inhibitor (Cayman Chemical, Ann Arbor, MI, USA) and 10 µM CHIR99021 stemness booster (CHIR, Tocris Bioscience, Bristol, UK) were examined in organoid cultures. Used proteins and peptides, studied concentrations, and other specific details of the functionalized GDxA cultures are presented in Table 2 in chapter 5.2.

4.4 Functionalized GDxA supplemented with COL

In some experiments, rat tail COL I (3.52 mg/ml, Corning, Somerville, MA, USA) was supplemented with functionalized GDxA hydrogel. GDxA was first mixed with the desired amount of biotinylated protein or peptide. While keeping on cold, COL was neutralized by mixing nine parts of COL with one part of 10x PBS. The pH of the COL mixture was balanced by using NaOH. Functionalized GDxA and neutralized COL were mixed in the desired ratio while keeping on cold. A schematic view of sample preparation of functionalized GDxA-COL hydrogel and organoid culturing are shown in Figure 7.

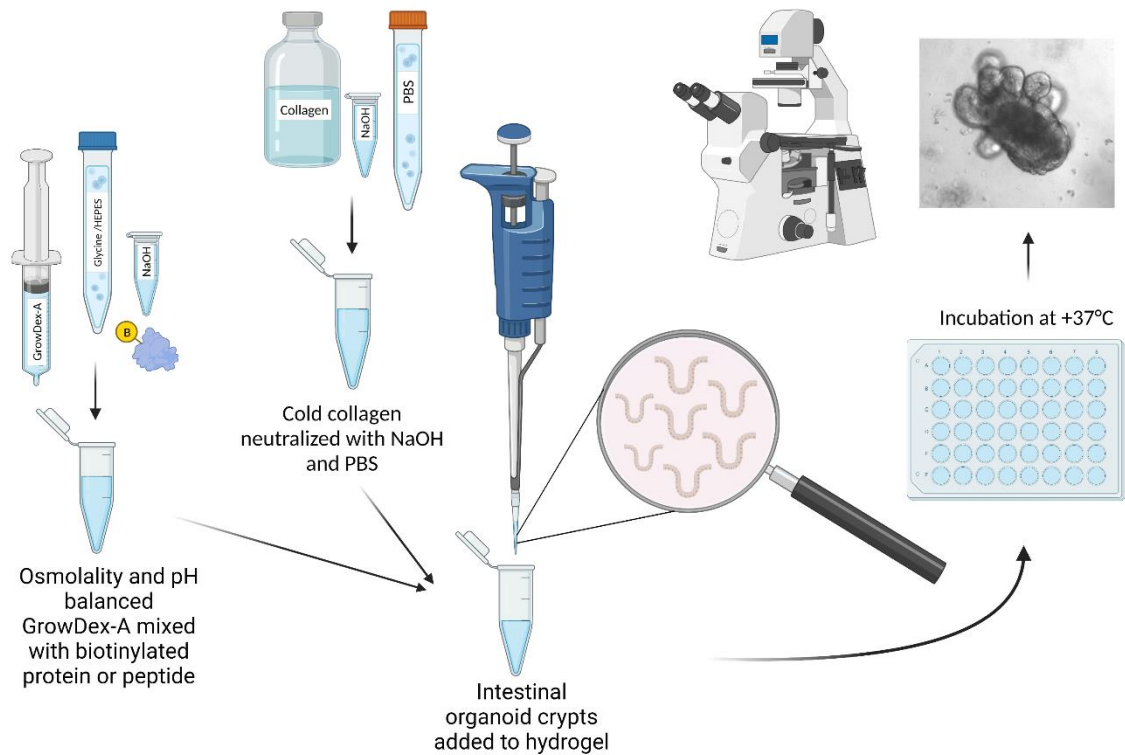


Figure 7. Preparation steps of intestinal organoid cultivation. Balanced GDxA is functionalized with biotinylated protein or peptide. COL is neutralized with NaOH and PBS and kept on cold. Functionalized GDxA and neutralized COL are mixed, and intestinal organoid crypts are added to the hydrogel. Finally, the hydrogel is pipetted to a well plate, the culture medium is added on top of the hydrogel, and the growth is observed regularly. Created with BioRender.com.

4.4.1 Optimization of GDxA-BcRGD-COL hydrogel preparation

During the optimization, examined variables were: 1) different ways to keep COL cold, 2) pH level of COL, 3) incubation time of neutralized COL before use, 4) GDxA-COL mixture incubation time on cold before adding crypts, 5) incubation time at RT after plating, 6) osmolality balancer, 7) use of ROCK inhibitor and 8) pipetting order of COL, GDxA, and crypts. All GDxA-BcRGD-COL optimization steps were executed with hydrogel containing 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g}/\text{ml}$ BcRGD, and supplemented with 1.4 mg/ml COL.

In the optimization phase, the samples were kept on a cold block or in an ice bath to assure the liquid form of COL. The COL was used at two pH levels ~ 6.8 with 15 mM NaOH or ~ 7.0 with 18.5 mM NaOH. When COL was neutralized, it was either incubated on cold for 60 minutes or it was mixed with GDxA and crypts right after neutralizing. Hydrogels were held on an ice bath precisely for 0 or 10 minutes after mixing GDxA and COL, before mixing the crypts into the hydrogel. After pipetting samples to well plates,

the plates were held at RT for 0, 10, 20, or 30 minutes before placing them into the incubator at +37°C. The pipetting order was also altered, when the GDxA was mixed with cell suspension before COL supplementation. Glycine and HEPES mixture was replaced with NaCl to balance the osmolality in a few experiments. In some experiments, the ROCK inhibitor was used in parallel samples for one day. Specific sample preparations and variables of experiments are described in more detail in Table 3 in the chapter 5.3.1.

4.4.2 COL hydrogels

Intestinal crypts were cultured in COL hydrogels as a reference to functionalized GDxA-COL hydrogels. While keeping on cold, COL was neutralized by mixing nine parts of COL with one part of 10x PBS. The pH of COL was balanced by using NaOH in two concentrations, 15 mM, and 18.5 mM. COL hydrogels were made in concentrations of 1.4 mg/ml and 2.0 mg/ml and the hydrogel was diluted with PBS or BCM. After plating the hydrogels, the well plate was held at RT for 0,10, or 20 minutes before placing it into an incubator at +37°C. Also, the cooling method, well plate size, and use of ROCK inhibitor were studied in COL cultures. All samples and their specific preparations are listed in more detail in Table 4 in chapter 5.3.2.

4.4.3 Varying GDxA-COL ratios

During the optimization, hydrogels with different ratios of functionalized GDxA and COL were studied. GDxA amounts were studied in a range between 0.1 %-0.4 % (w/v) and COL concentration ranged between 1.1-2.1 mg/ml. Different GDxA-COL ratios and variables used in the experiments are described in more detail in Table 5 in chapter 5.3.3.

4.4.4 GDxA-BcRGD-COL hydrogels with delayed organoid addition

Intestinal crypts were transferred to GDxA-BcRGD-COL hydrogels which were made on the previous day without adding intestinal crypts. In these hydrogels, the volume for cell suspension was replaced with plain BCM. Hydrogel base in these cultures was 0.18 % (w/v) GDxA functionalized with 1.54 µg/ml BcRGD and supplemented with 1.4 mg/ml COL. In these samples, COL had 15 mM or 18.5 mM NaOH concentration. Neutralized COL was incubated on an ice bath for 0, 30, or 60 minutes before mixing with GDxA. After pipetting hydrogels to a 24-well plate, samples were held at RT for 30 minutes before placing them into an incubator at +37°C for 40 minutes. After incubation, 350 µl

of BCM was added on top of each hydrogel to keep them moisturized. The next day intestinal crypts were added either 1) on top, or 2) inside the GDxA-BcRGD-COL hydrogel. Hydrogel variables used in experiments are described in more detail in Table 6 in chapter 5.3.4.

4.5 Intestinal organoid culturing and passaging

The study was approved by the Ethical Committee of Pirkanmaa Hospital District, (License number: ESAVI/21996/2021). A wild-type mouse cell line, C57BL/6 was used in this master's thesis, provided by the Intestinal Signaling and Epigenetics (TAU) research group.

Intestinal organoids in Matrigel (Corning, Bedford, MA, USA) were passaged every 4-7 days. For passaging Matrigel was mechanically disrupted, collected in a tube, and washed twice with cold PBS while centrifuging (5 min at 150 g at +4 °C) between washes. After two washes 1 ml of cold BCM was added on top of organoids and mechanical disruption of organoids to crypts was achieved by pipetting up and down around 10 times by a glass pipette with narrowed outlet. After final centrifugation (5 min at 150 g at +4 °C) BCM was removed, and crypts were divided in a division ratio of 1:3 or 1:4 depending on the visual assessment of the number of organoids in Matrigel culture. When resuspended in Matrigel-PBS mixture (7/3 ratio, referred to as Matrigel), 30 µl of mixture with crypts was pipetted into a 24-well plate and incubated at +37°C for 10-15 minutes for gelation, before adding 350 µl ENR culture medium on top of the hydrogel. For Live/Dead assay, 30 µl of Matrigel with crypts was pipetted to a 96-well glass-bottom plate (Cellvis, Mountain View, CA, USA) and after incubating 10-15 minutes at +37°C, 200 µl of ENR medium was added on top of the hydrogel. The culture medium was changed every second or third day and cultures were maintained at +37°C in 5 % CO₂ in a humidified incubator.

4.5.1 Organoid resuspension into GDxA and COL hydrogels

Crypts were resuspended from Matrigel organoid cultures into functionalized GDxA, functionalized GDxA-COL, or COL hydrogels. To these hydrogels, crypts were added at 10 % of the total sample volume. Crypts purified from Matrigel were resuspended in BCM in the needed volume. Crypt division ratio to hydrogels was 1:3 or 1:4 depending on the visual assessment of the number of organoids in Matrigel culture. Crypts were mixed with hydrogels by gently pipetting up and down.

After plating 50 μ l of functionalized GDxA hydrogel, 200 μ l for a 96-well plate and 350 μ l for a 24-well plate of ENR medium was added on top of the hydrogel. With functionalized GDxA-COL hydrogels 50 μ l of hydrogel was plated to a 24-well plate or a 96-well plate and incubated first 0-30 minutes at RT and then for 40 min at +37°C before adding ENR medium on top of the hydrogels. When using COL hydrogels, after plating 50 μ l of hydrogel to a 24-well plate or a 96-well plate, the hydrogels were incubated for 0-20 minutes at RT and then at +37°C for 40 minutes before adding the ENR medium on top of the hydrogels. For functionalized GDxA, functionalized GDxA-COL, and COL hydrogel cultures the medium was changed every or every second day. Organoid cultures in all conditions were maintained at +37°C in 5 % CO₂ in a humidified incubator.

With functionalized GDxA-BcRGD-COL hydrogels with delayed organoid addition, the crypts were mixed with BCM after isolating from Matrigel culture at a division rate of 1:3. 10 μ l of BCM with crypts was pipetted gently to the hydrogel, by placing the pipet tip into, or on top of the hydrogel. After pipetting crypts to the hydrogel, hydrogels were held for 30 minutes in an incubator at +37°C before adding 350 μ l of ENR medium on top of the hydrogels. The medium was changed every second or third day and cultures were maintained at +37°C in 5 % CO₂ in a humidified incubator.

Intestinal organoids grown in GDxA-BcRGD-COL hydrogel were passaged in experiment 5 on day 13. GDxA-BcRGD-COL hydrogel was mechanically disrupted, collected in a 15 ml tube, washed once with PBS, and centrifuged (5 min at 150 g at +4°C). 1ml of BCM was added on top of crypts and disrupted by pipetting up and down around 10 times by a glass pipette with a narrowed outlet. Crypts were centrifuged (5 min at 150 g at +4°C) and resuspended into Matrigel. 30 μ l of Matrigel with crypts was pipetted into a 24-well plate and incubated at +37°C for 10-15 minutes before adding 350 μ l of ENR medium on top of the hydrogel. The medium was changed every second or third day and the culture was maintained at +37°C in 5 % CO₂ in a humidified incubator.

Due to the difference in well diameter between the 24-, and 96-well plates, the functionalized GDxA-COL hydrogel settles differently in the wells of 24-, and 96-well plates. In a 24-well plate, GDxA-COL hydrogel forms a “dome” in the centre of the well, whereas in a 96-well plate GDxA-COL hydrogel covers the whole well bottom and forms a disk (Fig. 8).

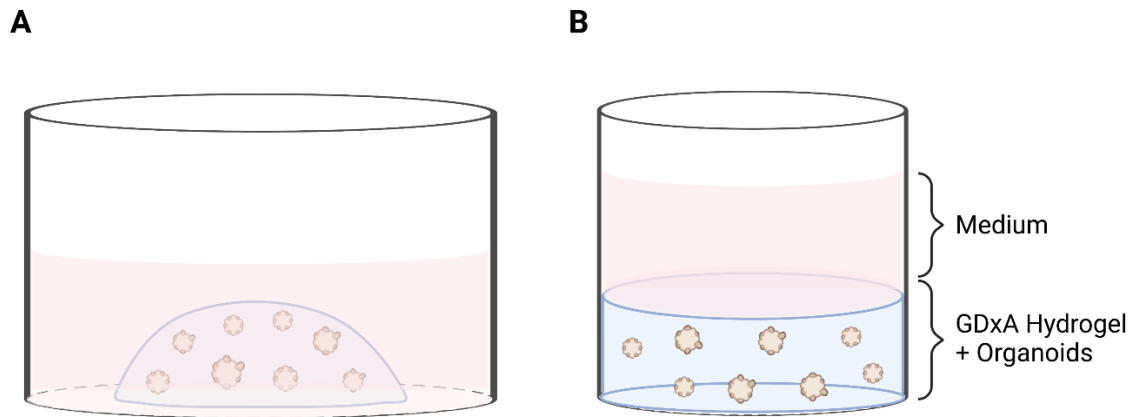


Figure 8. A functionalized GDxA-COL hydrogel with organoids A) in a 24-well plate as a dome and B) in a 96-well plate as a disk. Created with BioRender.com.

4.6 Characterization of organoid culture

Characterization methods were used to observe the viability and growth of intestinal organoids. Cultures were monitored with Primovert (Carl Zeiss AG, Oberkochen, Germany) microscope, and imaging during the experiments was executed with Axiovert 200M (Carl Zeiss AG, Oberkochen, Germany) microscope.

4.6.1 *Live/Dead staining*

A fluorescence cell viability assay was performed to visualize the live and dead cells in the organoid culture. Calcein-AM (Ca-AM, Invitrogen, Waltham, MA, USA) stains living cells, and Propidium Iodide (PI, Invitrogen, Waltham, MA, USA) stains dead cells. Ca-AM is detected as green and PI as red in fluorescence microscopy. Organoids used in the Live/Dead staining grew in a hydrogel composed of 0.18 % (w/v) GDxA, functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL (Exp. 13). For staining, the hydrogel amount was decreased to 30 μl and organoids were growing on a glass-bottom well plate to observe the organoids better via microscope. Cultures were stained and imaged daily on culturing days 1-5. As a reference, organoid cultures in Matrigel on a glass-bottom well plate were stained between culture days 1-5. For staining, the culture medium was removed, and the culture was washed twice with 150 μl of PBS before adding 100 μl of solution with 3 μM Ca-AM and 2 μM PI on top of the hydrogel. As a control, GDxA-BcRGD-COL hydrogel without organoids was stained with a solution containing 3 μM Ca-AM and 2 μM PI. Also, GDxA-BcRGD-COL hydrogels with organoids were stained separately either with 3 μM Ca-AM or with 2 μM PI as a control. Samples were incubated at +37°C in dark for 30 minutes before imaging with Axiovert 200M.

5. RESULTS

5.1 Biotinylation of ECM proteins

LN and VN were biotinylated for GDxA functionalization. After biotinylation, concentrations and biotinylation degrees of BLN and BVN were determined. BLN concentration decreased drastically from 1 mg/ml to 0.26 mg/ml during biotinylation. Biotinylation degrees of BLN and BVN were in the expected range. Due to the large size of LN (810 kDa), the desired biotinylation degree was higher than that of VN (75 kDa). Final protein concentrations of BLN and BVN and their biotinylation degrees are listed in Table 1.

Table 1. Concentrations and number of biotins per protein of BLN and BVN. Two dilutions of samples were used, and samples were run in replicates.

Protein	Concentration (mg/ml)	Number of biotins per protein
BLN	0.26	31.2
BVN	1.63	4.3

5.2 GDxA hydrogels functionalized with biotinylated proteins

Intestinal organoids were cultured in 0.4 % (w/v) GDxA hydrogels functionalized with biotinylated proteins or peptides. BVN, BFN, BLN, BcRGD, and a mixture of BcRGD and non-biotinylated LN were studied in cultures in different concentrations. A summary of functionalized GDxA samples and experimental details are listed in Table 2.

Table 2. A summary of experimental details of GDxA hydrogels functionalized with biotinylated proteins and peptides.

Experiment	Added protein or peptide	Concentration in culture	CaCl ₂ crosslinking	Balanced pH	ROCK and CHIR in culture (days)	Sample diluent	24-, or 96-well plate
1	BLN	0.09 mg/ml	Yes	No	0	PBS	24
	BLN	0.09 mg/ml	No	No	0	PBS	96
	BVN	0.3 mg/ml	Yes	No	0	PBS	24
	BVN	0.3 mg/ml	No	No	0	PBS	96
2	BVN	0.3 mg/ml	No	Yes	4	PBS	96
	BVN	0.3 mg/ml	No	Yes	0	PBS	96
	BFN	0.1 mg/ml	No	Yes	4	PBS	96
	BFN	0.1 mg/ml	No	Yes	0	PBS	96
3	BcRGD	3.4 µg/ml	No	Yes	0	BCM	96
	BcRGD	3.4 µg/ml	Yes	Yes	0	BCM	96
	BcRGD	3.4 µg/ml	No	Yes	3	BCM	96
	BcRGD	3.4 µg/ml	Yes	Yes	3	BCM	96
	BcRGD+	3.4 µg/ml	No	Yes	0	BCM	96
	LN	+0.02 mg/ml					
	BcRGD+	3.4 µg/ml	Yes	Yes	0	BCM	96
	LN	+0.02 mg/ml					
4	BcRGD+	3.4 µg/ml	No	Yes	3	BCM	96
	LN	+0.02 mg/ml					
	BcRGD+	3.4 µg/ml	Yes	Yes	3	BCM	96
	LN	+0.02 mg/ml					
4	BLN	0.71 mg/ml	No	Yes	3*	BCM	24

* Only ROCK inhibitor used in the culture medium

Intestinal crypts were cultured in GDxA hydrogel functionalized with 0.09 mg/ml BLN or 0.3 mg/ml BVN (Exp. 1). After three days crypts had disintegrated into individual cells and clusters of debris in all culture conditions. Hydrogels had mostly broken into smaller pieces in 24-well plates, which made medium changing difficult. The pH of GDxA hydrogel was not measured for the experiment, and therefore there was no certainty that the hydrogel had optimal pH for intestinal organoid cultivation.

In subsequent cultures GDxA was functionalized with 0.3 mg/ml BVN or 0.1 mg/ml BFN (Exp. 2). Cultures supplemented with ROCK and CHIR had some small crypts left after four days, but after a few days, crypts were no more observed. Intestinal crypts were also cultured in GDxA hydrogels functionalized with 3.4 µg/ml BcRGD and 3.4 µg/ml BcRGD + 0.02 mg/ml LN (Exp. 3). When cultures were supplemented with ROCK and CHIR with and without CaCl₂ crosslinking, crypts could be observed after three days (Fig.

9). However, on culture day five all cultures were full of disintegrated single cells. In cultures without ROCK, CHIR, or crosslinking, the crypts had disintegrated into a single cell already in three days. CaCl₂ crosslinking did not seem to stabilize the hydrogel to the bottom of a well plate, and it was left out of an upcoming experiment. CHIR was neither used in the subsequent experiments. When GDxA was functionalized with 0.71 mg/ml BLN (Exp. 4), only a few crypts could be observed from the culture after three days, and a few days later crypts disintegrated into single cells.

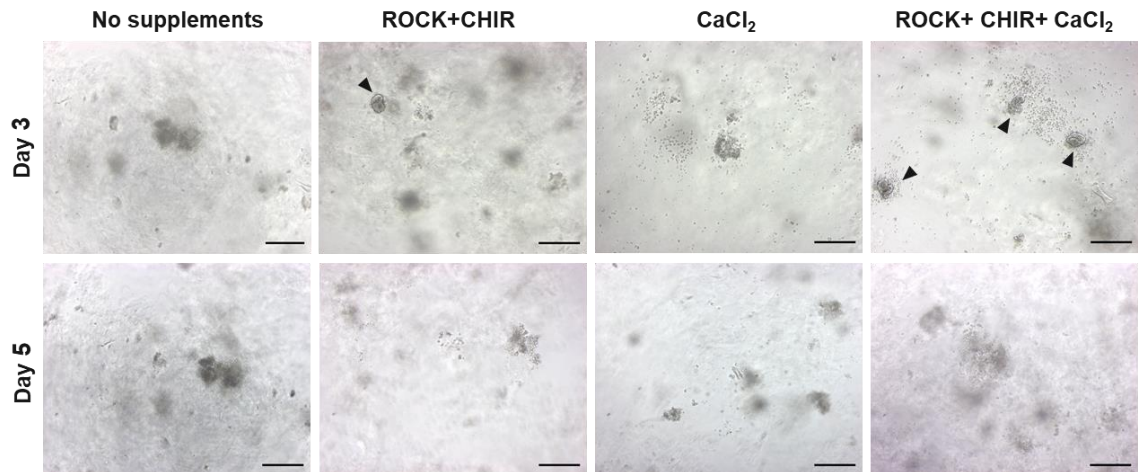


Figure 9. 0.4 % (w/v) GDxA hydrogel functionalized with 3.4 $\mu\text{g/ml}$ BcRGD + 0.02 mg/ml LN in different culture conditions (Exp. 3). Intestinal organoid culture with no supplements, culture supplemented with ROCK and CHIR, culture crosslinked with CaCl₂, and culture supplemented with ROCK, CHIR, and crosslinked with CaCl₂. Cultures are presented on culture days three and five. Intestinal crypts on culture day three are indicated by arrows. Scale bars 200 μm .

5.3 Functionalized GDxA hydrogel supplemented with COL

5.3.1 Optimization of GDxA-BcRGD-COL hydrogel preparation

Preparation of functionalized GDxA-BcRGD-COL hydrogel was optimized for intestinal organoid cultivation. All optimization steps with GDxA-BcRGD-COL hydrogel were conducted with a hydrogel composed of 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL (Exp. 5-13). BcRGD was chosen to be used as a functional ligand in the hydrogel because there were pipetting problems with whole biotinylated proteins. A summary of optimization steps and variables are presented in Table 3.

Table 3. Experiments and variables during optimization of functionalized GDxA-COL hydrogel preparation for intestinal organoid cultivation.

Experiment	Osmolality balancer	NaOH concentration in COL (mM)	Cooling method	Cooling time of COL before use (min)	First pipetted with GDxA	Cooling time of sample before plating (min)	Sample incubation time at RT after plating (min)	Well plate material	24-, or 96-well plate	ROCK inhibitor in culture (days)	Parallel sample without ROCK
5	Gly/HEP	15	Cold block	-	COL	Not meas.	Not known	Plastic	Both	1	Yes
6	Gly/HEP	15	Ice bath	-	COL	Not meas.	Not known	Plastic	96	0	-
7	Gly/HEP	15	Ice bath	-	COL	Not meas.	0	Plastic	96	0	-
	Gly/HEP	15	Ice bath	-	COL	Not meas.	10	Plastic	96	0	-
	Gly/HEP	15	Ice bath	-	COL	Not meas.	20	Plastic	96	0	-
8	Gly/HEP	18.5	Ice bath	-	COL	Not meas.	0	Plastic	96	0	-
	Gly/HEP	18.5	Ice bath	-	COL	Not meas.	10	Plastic	96	0	-
	Gly/HEP	18.5	Ice bath	-	COL	Not meas.	20	Plastic	96	0	-
	NaCl	18.5	Ice bath	-	COL	Not meas.	0	Plastic	96	0	-
	NaCl	18.5	Ice bath	-	COL	Not meas.	10	Plastic	96	0	-
9	Gly/HEP	15	Cold block	-	COL	10	0	Plastic	96	1	Yes
	Gly/HEP	15	Ice bath	-	COL	10	0	Plastic	96	1	Yes
	NaCl	15	Cold block	-	COL	10	0	Plastic	96	1	Yes
	NaCl	15	Ice bath	-	COL	10	0	Plastic	96	1	Yes
	Gly/HEP	15	Cold block	-	COL	0	0	Plastic	96	1	Yes
10	Gly/HEP	18.5	Ice bath	-	Crypts	Not meas.	20	Plastic	96	1	No
11	Gly/HEP	15	Ice bath	60	Crypts	Not meas.	30	Plastic	96	1	Yes
12	Gly/HEP	15	Ice bath	60	Crypts	Not meas.	30	Glass	96	1	No
13	Gly/HEP	15	Ice bath	90	Crypts	Not meas.	30	Glass	96	1	No

GDxA-BcRGD-COL hydrogel supported organoid formation when COL was neutralized with 15 mM NaOH, and COL was cooled on a cold block (Exp. 5). Neutralized COL was mixed with GDxA and after the addition of crypts, the hydrogel was pipetted into 24-, and 96-well plates and transferred to an incubator at +37°C before the addition of medium. After three days the crypts started growing and budding similarly to those in Matrigel cultures (Fig. 10). After five days the organoid growth seemed to accelerate and on culture day seven, large cystic organoids were formed. There was no visible difference between cultures with or without ROCK inhibitor. No difference was observed between formed organoids in 24-, or 96-well plates, but the hydrogels in the 24-well plate started to float and bent, which made the culture hard to repeat. On culture day 13, organoids from a 24-well plate and cultured without ROCK inhibitor were mechanically disrupted and passaged back to Matrigel to inspect if the organoids grown in functionalized GDxA-BcRGD-COL hydrogel are still viable. After passaging, the crypts started to grow similarly to crypts in the Matrigel reference (Fig. 10).

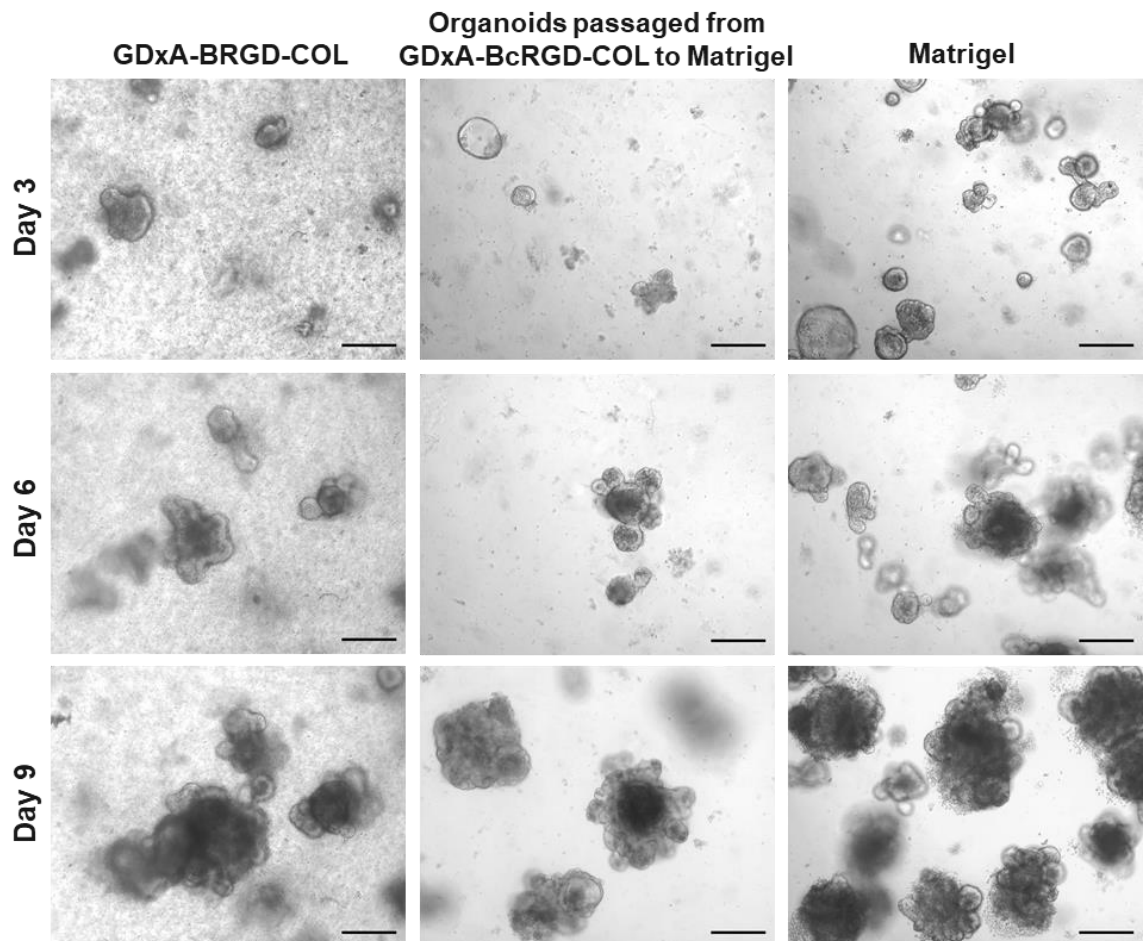


Figure 10. The organoid formation between culture days 3-9 in cultures with 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL hydrogel, passaged crypts from the above-mentioned GDxA-BcRGD-COL hydrogel to Matrigel, and Matrigel reference in 24-well plates (Exp.5). Scale bars 200 μm .

In the next GDxA-BcRGD-COL culture NaOH concentration of COL was held at 15 mM, but a cold block was replaced with an ice bath to avoid possible freezing during sample preparation (Exp. 6). ROCK inhibitor was not used in the culture because no clear benefit of its use was observed. To prevent the hydrogel bending, 96-well plates were used in the following experiments. After three days only a few crypts could be observed in the culture, and it was mostly filled with individual cells disintegrated from the crypts. On culture day five only cell debris was left.

In the next GDxA-BcRGD-COL cultures NaOH concentration of COL was increased to 18.5 mM (Exp. 7, 8, and 10). Samples were kept at RT for 0, 10, or 20 minutes after plating, to examine whether it affects the hydrogel structure and supports organoid formation. NaCl was also examined as a reagent to balance the osmolality alongside Glycine and HEPES mixture. In experiment 10, crypts were mixed with GDxA before adding COL to the mixture. Already on culture day two most of the crypts in all conditions had turned into agglomerated cell debris and some crypts had formed unusual, elongated shapes. There was no visible difference between samples with different preparation methods.

The concentration of NaOH in COL was decreased back to 15 mM, and both cold conditions, a cold block, and an ice bath were studied in the next GDxA-BcRGD-COL cultures (Exp. 9). NaCl was studied as an osmolality balancer alongside Glycine and HEPES mixture. Mixed GDxA and COL were held on cold for 0 or 10 minutes before adding the crypts to the culture, to examine whether the temperature of the GDxA-BcRGD-COL mixture during plating affected organoid formation. After crypts were mixed, samples were immediately pipetted to the 96-well plate and placed into the incubator at +37°C. In most samples, the crypts had fallen apart and were filled with agglomerated debris after two days. The sample which was made on a cold block and held 0 minutes on cold before mixing with crypts, had several budding crypts in it. On culture day nine a few crypts could be observed in the culture, but there were several smaller crypts beside each other rather than big uniform organoids as in Matrigel.

In following GDxA-BcRGD-COL cultures 15 mM NaOH concentration in COL was used (Exp. 11, 12, and 13). Neutralized COL was incubated on an ice bath for 60 minutes and then blended with a ready mixture of GDxA and crypts. Samples were incubated at RT for 30 minutes after plating. In experiment 11, crypts in the culture disintegrated into individual cells after five days, but crypts in a sample from experiment 12 started growing and budding after four days (Fig. 11). The crypts formed into cystic organoids similarly to those grown in the Matrigel reference.

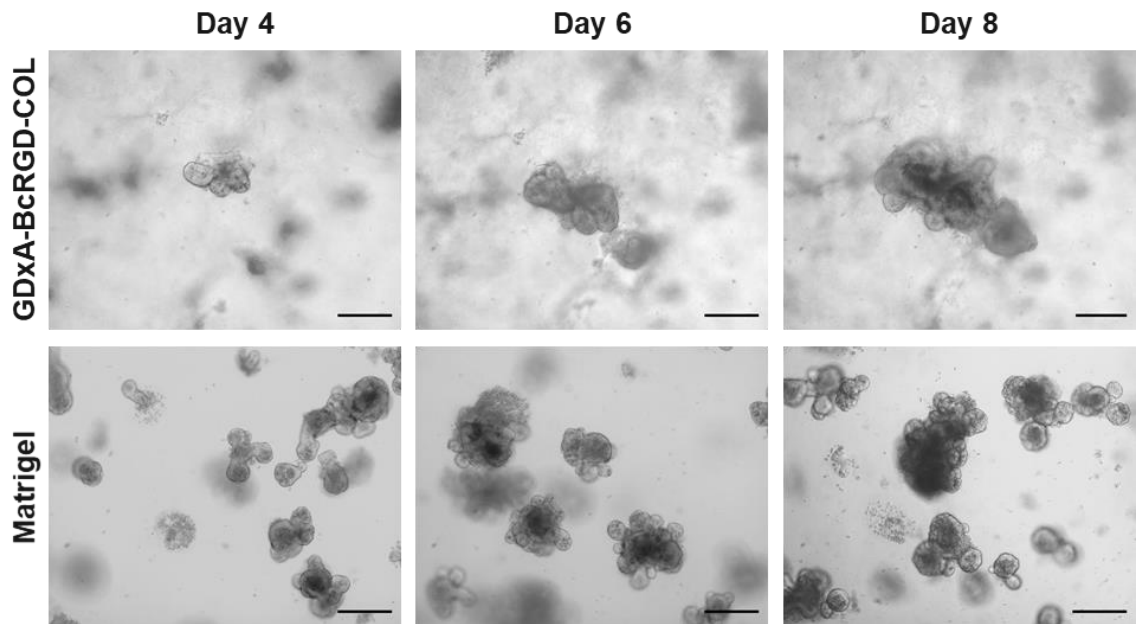


Figure 11. Organoid formation in 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL hydrogel and in Matrigel reference between culture days 4-8 (Exp. 12). Scale bars 200 μm .

In experiment 13, the hydrogel amount pipetted to the well plate was decreased from 50 μl to 30 μl , to get a thin hydrogel sheet of GDxA-BcRGD-COL hydrogel to the bottom of the well plate for Live/Dead assay. Intestinal organoid viability in GDxA-BcRGD-COL and Matrigel cultures was characterized by Live/Dead staining between culture days 1-5. After one day (Fig. 12A) living crypts were visible in both cultures. Diffused single apoptotic cells were observed in both cultures, probably related to the passaging process. Crypts in GDxA-BcRGD-COL culture had apoptotic cells inside and around them, which may indicate that crypts have started the disintegration process. After three days (Fig. 12B) living cystic organoids were detected in both cultures. A strong green signal was detected in the budding crypt structures and dead cells were observed inside the organoids in both cultures. Dead cells inside the organoids were probably related to the normal intestinal epithelium life cycle and rapid self-regeneration. More dead cells can be observed from GDxA-BcRGD-COL culture and only a few crypts started budding compared to Matrigel, where nearly all crypts formed organoids. After four days the Ca-AM fluorescence signal from GDxA-BcRGD-COL culture decreased and reliable results could not be obtained anymore, even though some crypts could be observed with the bright field microscopy.

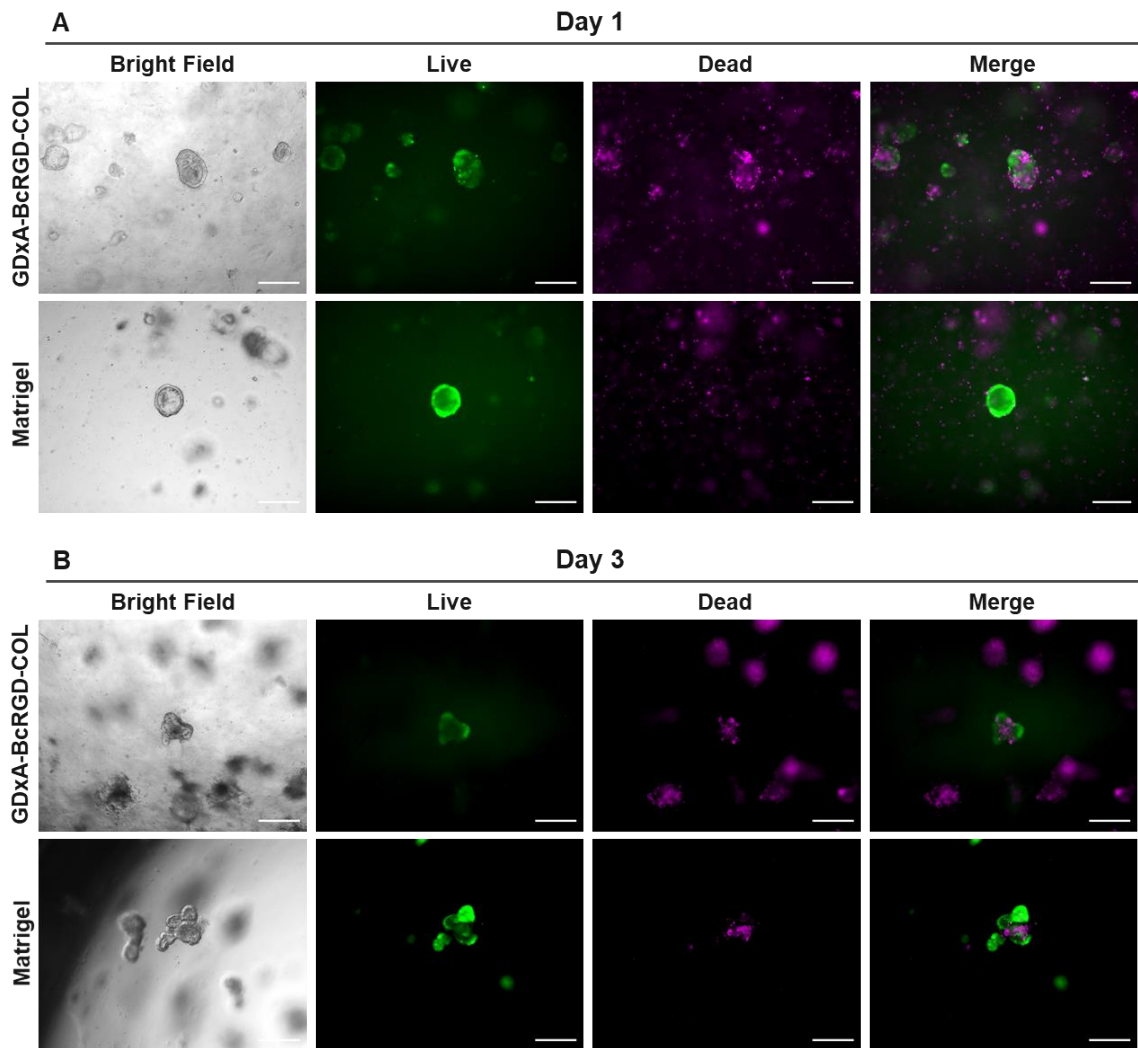


Figure 12. Intestinal organoid viability characterization in 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL hydrogel and in Matrigel reference (Exp. 13). Cultures stained with Calcein-AM (green, live cells) and PI (magenta, dead cells). Bright field, live, dead, and merge pictures provided from A) culture day one, and B) culture day three. Scale bars 200 μm .

5.3.2 COL hydrogels

COL hydrogels were also studied for intestinal organoid cultivation as a reference for functionalized GDxA-COL cultures. A summary of COL cultures and experimental details are presented in Table 4.

Table 4. Experimental details of the sample preparation of COL hydrogels.

Experiment	COL concentration (mg/ml)	NaOH concentration in COL (mM)	Cooling method	Sample diluent PBS or BCM	Sample incubation time at RT after plating (min)	24-, or 96-well plate	ROCK inhibitor in culture (days)
5	2.0	15	Cold block	Both	Not meas.	Both	1
	2.0	15	Cold block	Both	Not meas.	Both	0
6	2.0	15	Ice bath	BCM	Not meas.	96	0
7	1.4	15	Ice bath	BCM	0	96	0
	1.4	15	Ice bath	BCM	10	96	0
	1.4	15	Ice bath	BCM	20	96	0
10	1.4	18.5	Ice bath	BCM	20	96	1

A hydrogel with 2.0 mg/ml COL and 15 mM NaOH concentration supported the growth of organoids with or without ROCK inhibitor, but the organoid growth was different compared to organoid formation in Matrigel (Exp. 5). Crypts seem to attach to the bottom of the well plates and large cell containing mat-like structures were observed under the crypts. Uniform cystic organoids were not forming in COL hydrogels. Differences in the COL, functionalized GDxA-COL, and Matrigel hydrogels on culture day seven can be seen in Figure 13. When culture conditions were repeated, the crypts broke into individual cells in five days, and organoid growth was not observed (Exp. 6).

Day 7

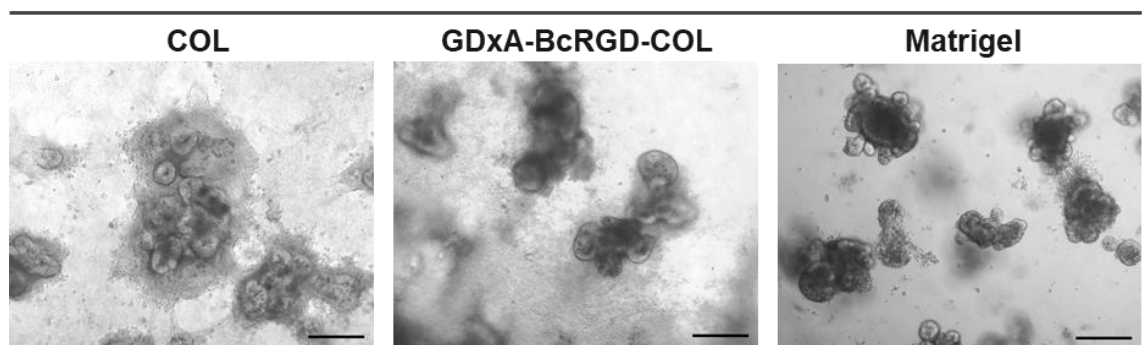


Figure 13. Growth of intestinal organoids in 2.0 mg/ml COL hydrogel, 0.18 % (w/v) GDxA functionalized with 1.54 μ g/ml BcRGD and supplemented with 1.4 mg/ml COL hydrogel and Matrigel in 24-well plates on culture day seven (Exp. 5). Scale bars 200 μ m.

COL concentration was matched to hydrogel used in the preparation optimization of GDxA-BcRGD-COL hydrogel, and in the next cultures 1.4 mg/ml COL concentration was used. After plating, samples were held at RT for 0, 10, or 20 minutes before placing into an incubator at +37°C (Exp. 7). In all conditions, the crypts disintegrated into cell debris after three days. In the next culture, NaOH concentration in 1.4 mg/ml COL was raised to 18.5 mM and the 24-well plate was held at RT for 20 minutes after plating, to mimic the GDxA-BcRGD-COL hydrogel preparation steps (Exp.10). After four days the hydrogel rolled into a thick mattress-like structure, and crypts started to fuse and form a line of organoids.

5.3.3 Hydrogels with varying GDxA and COL ratios

During the optimization of GDxA-BcRGD-COL hydrogel preparation, also different ratios of functionalized GDxA and COL were studied. A summary of studied GDxA-COL ratios and experimental details are presented in Table 5.

Table 5. Experiments and sample preparations of hydrogels with varying functionalized GDxA and COL ratios.

Experiment	GDxA amount (w/v)	COL amount (mg/ml)	Added protein or peptide	Concentration of protein or peptide ($\mu\text{g/ml}$)	NaOH concentration in COL (mM)	Cooling method	Cooling time of COL before use (min)	First pipetted with GDxA	Sample incubation time at RT after plating (min)	ROCK inhibitor in culture (days)	Parallel sample without ROCK	24-, or 96-well plate
4	0.2 %	2.1	BcRGD	1.12	15 mM	Ice bath		COL	Not meas.	3	Yes	24
	0.3 %	1.6	BcRGD	1.68	15 mM	Ice bath		COL	Not meas.	3	Yes	24
	0.4 %	1.1	BcRGD	2.25	15 mM	Ice bath		COL	Not meas.	3	Yes	24
5	0.1 %	2.0	BcRGD	0.84	15 mM	Cold block		COL	Not meas.	1	Yes	Both
	0.1 %	2.0	BFN	25	15 mM	Cold block		COL	Not meas.	1	Yes	Both
6	0.18 %	1.4	BFN	11	15 mM	Ice bath		COL	Not meas.	0	-	96
10	0.18 %	1.7	BcRGD	1.54	18.5 mM	Ice bath		Crypts	20	1	No	96
	0.18 %	2.0	BcRGD	1.54	18.5 mM	Ice bath		Crypts	20	1	No	96
11	0.25 %	1.4	BcRGD	2.11	15 mM	Ice bath	60	Crypts	30	1	Yes	96
	0.30 %	1.4	BcRGD	2.53	15 mM	Ice bath	60	Crypts	30	1	Yes	96

Varying GDxA and COL amounts were examined in the range between 0.18-0.4 % (w/v) and 1.1-2.1 mg/ml, respectively. Hydrogels were functionalized with BcRGD (Exp. 4, 10, and 11). On culture day three, in hydrogel with 0.3 % (w/v), GDxA functionalized with 1.68 $\mu\text{g/ml}$ BcRGD and supplemented with 1.6 mg/ml COL various crypts were observed in cultures with and without ROCK inhibitor. After eight days a few small crypts were still observed in the cultures, but no cystic organoids were formed. Crypts in other GDxA-COL ratios had mostly fallen apart after three days, regardless of the hydrogel preparation method or use of ROCK inhibitor. Hydrogel composed of 0.4 % (w/v) GDxA functionalized with 2.25 $\mu\text{g/ml}$ and supplemented with 1.1 mg/ml COL tore apart on culture day one during culture medium change.

Hydrogels made of 0.1 % (w/v) GDxA and 2.0 mg/ml COL were functionalized either with BFN or BcRGD (Exp. 5). Crypts formed organoids in GDxA-BcRGD-COL hydrogel when the culture was supplemented with ROCK inhibitor and cultured on a 24-well plate. Cystic organoids were forming in sections where the hydrogel had detached from the 24-well plate bottom and rolled into a thick mattress-like structure. In other culture conditions, the crypts seemed to disintegrate and form a cell containing mat-like structure with elongated shapes (Fig. 14).

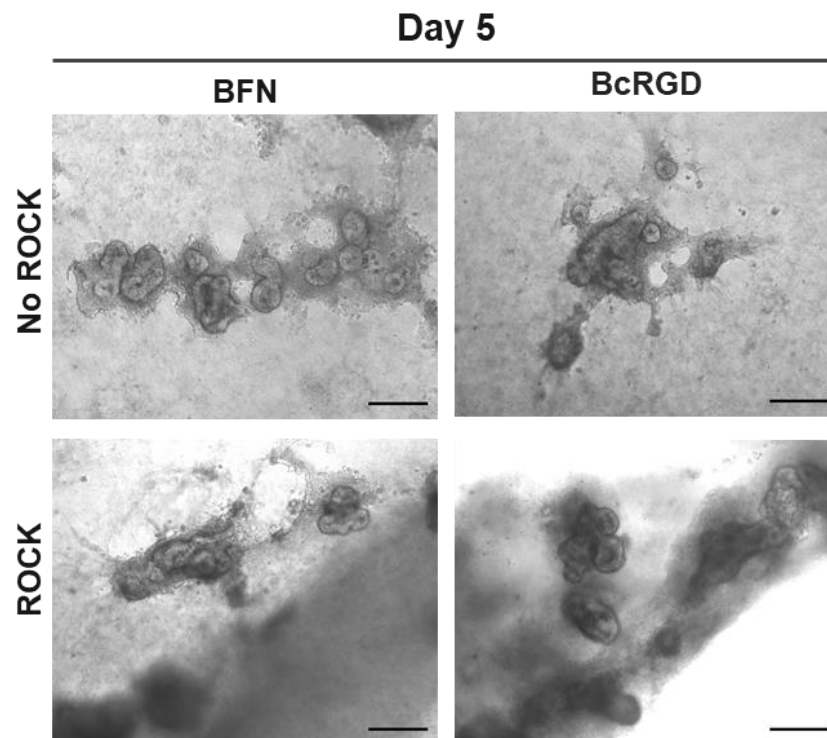


Figure 14. Hydrogel with 0.1 % (w/v) GDxA and 2.0 mg/ml COL functionalized with BFN or BcRGD on culture day five, with and without ROCK in a 24-well plate (Exp. 5). Scale bars 200 μm .

5.3.4 GDxA-BcRGD-COL hydrogels with delayed organoid addition

Intestinal crypts were transferred to hydrogels on 24-well plates which were made the day before and held at +37°C before adding the crypts either 1) inside or 2) on top of the hydrogel. These hydrogels were made of 0.18 % (w/v) GDxA functionalized with 1.54 µg/ml BcRGD and supplemented with 1.4 mg/ml COL. A summary of preparations of GDxA-BcRGD-COL hydrogels with delayed crypt addition is presented in Table 6.

Table 6. Sample preparation of GDxA-BcRGD-COL hydrogels with delayed organoid addition.

Experiment	NaOH concentration in COL (mM)	Cooling time of COL before use (min)	ROCK inhibitor in culture (days)	Crypts added to the hydrogel
14	18.5	30	0	Inside
15	15	0	1	Inside
	15	30	1	Inside
	15	60	1	Inside
	18.5	0	1	Inside
	18.5	30	1	Inside
	18.5	60	1	Inside
	15	0	1	On top
	15	30	1	On top
	15	60	1	On top
	18.5	0	1	On top
	18.5	30	1	On top
	18.5	60	1	On top

When crypts were pipetted inside the premade GDxA-BcRGD-COL hydrogel, they formed cystic organoids in hydrogel where the COL was balanced with 18.5 mM NaOH and cooled for 30 minutes before mixing with GDxA (Exp. 14). The organoid formation was seemingly similar to growth in the Matrigel reference (Fig. 15).

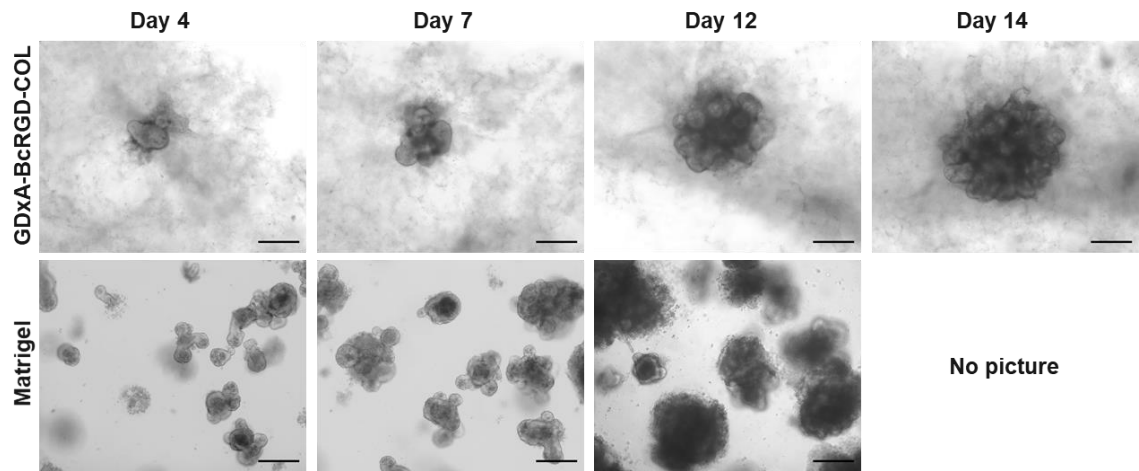


Figure 15. Organoid formation on culture days 4-14 in 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL hydrogel with delayed crypt addition (Exp. 14). Organoid culture in Matrigel on culture days between 4-12. Scale bars 200 μm .

In the next GDxA-BcRGD-COL cultures, COL was neutralized with 15 mM (Fig. 16A) or 18.5 mM (Fig. 16B) NaOH, and hydrogels were held for 0, 30, or 60 minutes at RT after plating. The next day crypts were added inside or on top of the hydrogels. Crypts started to grow in all cultures, and after 13 days crypts or organoids could be observed in nearly all cultures. The only culture where crypts could not be observed was hydrogel where COL was balanced with 15 mM NaOH, it was incubated at RT for 60 minutes and crypts were pipetted inside the hydrogel. In other culture conditions, the crypts seemed to form separate crypts rather than large cystic organoids. Overall, better growth was observed in cultures where crypts were pipetted on top of the hydrogel. Fewer crypts were growing in samples where the crypts were pipetted inside the hydrogel, possibly due to the sinking of the crypts to the bottom of the well plate after seeding. A lot of cell debris and elongated cell shapes were observed in all cultures, partly related to the long culturing time.

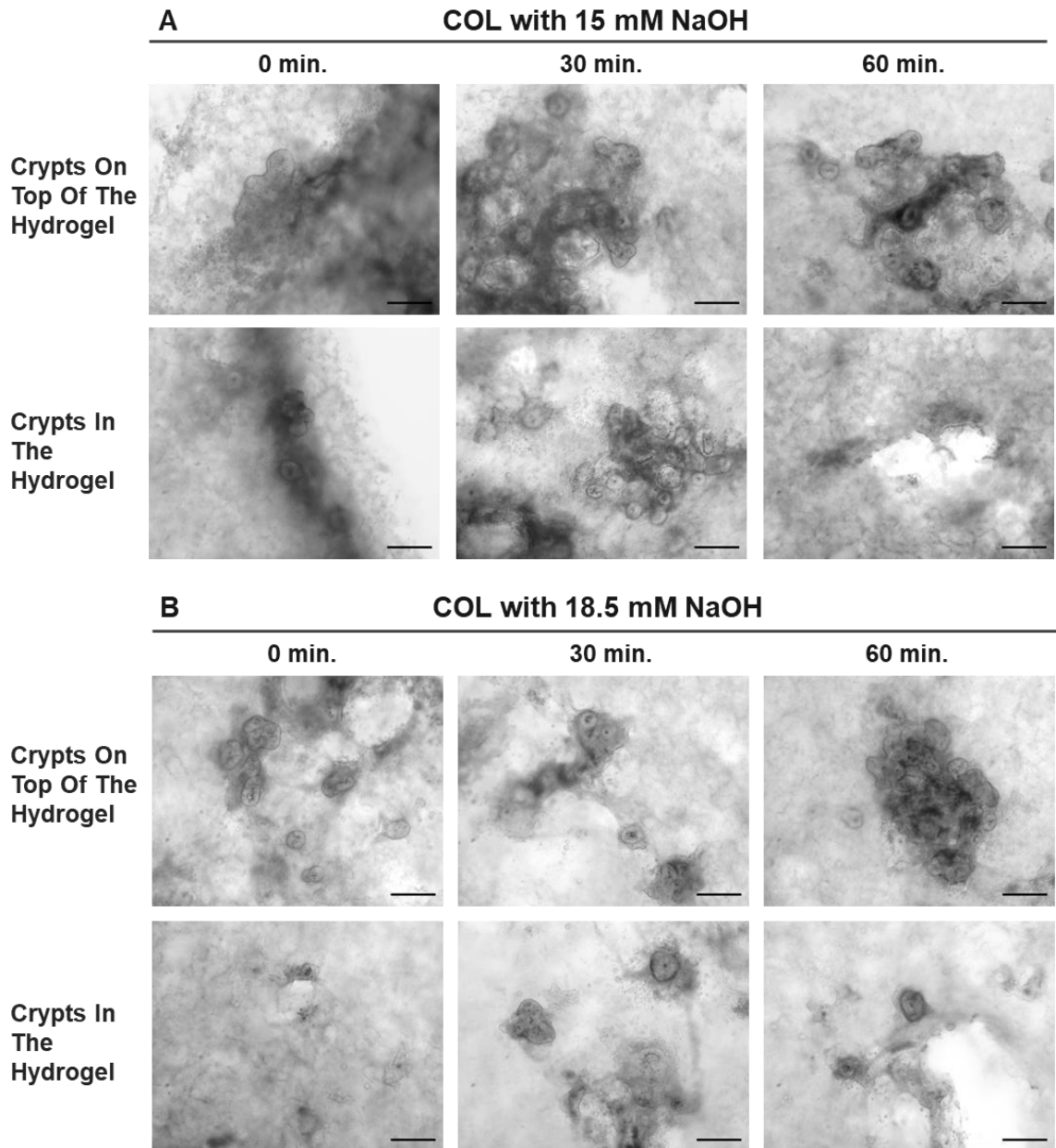


Figure 16. Organoid growth on culture day 13 in hydrogels with 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL (Exp. 15). Plated samples were held for 0, 30, or 60 minutes at RT before placing into the incubator at +37°C. The next day the crypts were added on top of, or into the hydrogels. COL was used in two NaOH concentrations, A) 15 mM or B) 18.5 mM. Scale bars 200 μm .

6. DISCUSSION

Organoids are promising models for many biomedical applications, including disease models, drug testing platforms, and replacements for animal models (Clevers, 2016; Kretzschmar & Clevers, 2016). Conventionally intestinal organoids are cultured in undefined animal-derived matrices which have batch-to-batch variability that leads to reproducibility problems and prevents their use in clinical applications (Kratochvil et al., 2019; Kretzschmar & Clevers, 2016). As a defined and biocompatible hydrogel material, nanocellulose, which can be functionalized with ECM proteins, has shown its capability to be supportive matrix material for intestinal organoid cultivation (Curvello et al., 2019; Curvello & Garnier, 2021). The aims of this thesis were to biotinylate selected ECM proteins, functionalize the avidin conjugated nanocellulose hydrogel, GDxA with different proteins or peptides, and to optimize the GDxA as a culturing platform for intestinal organoids, and characterize the organoid growth.

6.1 Biotinylation of ECM proteins

Hydrogels are functionalized with ECM proteins to mimic the natural growth conditions of organoids *in vivo*. ECM proteins in the hydrogel enable crucial cell-matrix interactions that affect various cell functions such as growth, differentiation, and migration. (Kratochvil et al., 2019). When biotinylated proteins are introduced to the GDxA, they are efficiently bound to nanocellulose via avidin-biotin interaction (Leppiniemi et al., 2021). After a simple biotinylation process, biotinylated ECM proteins can provide needed interactions for growing organoids in the nanocellulose.

VN and LN were chosen to be biotinylated and used in the organoid cultures due to their importance in cell adhesion (Frantz et al., 2010; Magno et al., 2020). Biotinylation of VN was successful, the protein concentration (1.64 mg/ml) and the biotinylation degree of 4.3 biotins per protein were in the expected range. The concentration of BLN (0.26 mg/ml) decreased drastically during the biotinylation process but the biotinylation degree of 31.2 biotins per protein was in the expected range. The low concentration of BLN may be explained by the fact that LN is a large protein capable of self-polymerizing. For example, Broguiere et al. kept the LN unmodified in their research because of its feature to assemble into an insoluble matrix (Broguiere et al., 2018). Possibly BLN has started to polymerize during dialysis processes, and it may have attached to the dialysis cassette wall.

6.2 GDxA hydrogels functionalized with biotinylated proteins

GDxA hydrogel was functionalized with biotinylated proteins or peptides and examined as a culture matrix for murine intestinal organoids. BVN, BFN, BLN, BcRGD, and a mixture of BcRGD and non-biotinylated LN were studied in these cultures in different concentrations. RGD sequence has been shown to be an important peptide motif for cell adhesion, and immobilized RGD peptide has been previously reported to be crucial for intestinal organoid growth (Broguiere et al., 2018; Curvello, Kerr, et al., 2021). Mixing GDxA with whole biotinylated proteins (BVN, BLN, BFN) was challenging and some hydrogel got stuck to the pipette tip regardless of the use of low adhesion pipette tips. For this reason, there were difficulties to keep the samples homogenous and repeatable. These large proteins may have adsorbed non-specifically to the negatively charged carboxyl groups on the surface of the nanofibers, which could affect the hydrogel properties. Because of the pipetting issues, BcRGD was mostly used in the following experiments when the hydrogel was supplemented with COL.

Functionalized GDxA hydrogel culture conditions alone did not seem to support intestinal organoid formation in studied protein concentration. The formation of intestinal crypts into cystic organoids was not observed even though the hydrogel was CaCl₂ crosslinked, ROCK and CHIR were added to the complete culture medium, or the diluent was changed from PBS to BCM. When the culture medium was supplemented with ROCK and CHIR with and without CaCl₂ crosslinking, the crypts survived a few days longer before disintegrating into single cells.

Organoid culturing results in functionalized GDxA hydrogels did not greatly differ between used biotinylated protein concentrations. Compared to other studies, the protein concentrations used in this study were largely lower (0.0034 mg/ml - 0.71 mg/ml) due to the low initial concentration of proteins. There is a possibility that used biotinylated protein concentrations in GDxA were not high enough to provide needed biochemical cues for organoid growth. For example, the lowest protein concentration for LN and COL Broguiere et al. used in their hydrogels was 0.5 mg/ml, and immobilized RGD peptide concentration in hydrogels used by Curvello, Kerr, et al. was 2 mM (Broguiere et al., 2018; Curvello, Kerr, et al., 2021). Previous works have reported that enrichment of LN up to 3 mg/ml benefited the formation of organoids (Curvello, Kerr, et al., 2021). Broguiere et al. studied that in fibrin hydrogel LN concentration of 0.5 mg/ml led to intestinal stem cell proliferation and cyst formation, but an LN concentration of 2 mg/ml was needed for organoid formation (Broguiere et al., 2018). Curvello, Kerr, et al. did not find any difference in intestinal organoid formation between nanocellulose hydrogels without

functionalization or functionalized with 0.5 mg/ml LN. Curvello, Kerr, et al. also criticized that the successful outcome of the studies with LN may be due to the generous amount of protein, also extracted from EHS mouse tumour (Curvello, Kerr, et al., 2021). In this thesis, only one GDxA hydrogel functionalized with BLN (0.71 mg/ml) reached the protein concentration that the other groups have used. However, the culture did not seem to support organoid formation, and crypts in the culture did not survive longer when compared to other used protein concentrations.

A few functionalized GDxA hydrogels detached from the bottom of the 24-well plate and fragmented into hydrogel pieces, usually during medium change, even though it was performed carefully. For this reason, CaCl₂ crosslinking was studied with functionalized GDxA hydrogels. CaCl₂ was added first to the bottom of the well before hydrogel addition. It was hypothesized to prevent the hydrogel fragmenting and to keep the GDxA hydrogel at the bottom of the well plate. Curvello, Kerr, et al. and Curvello and Garnier have successfully used Ca²⁺-mediated crosslinking for 0.1 (w/v) nanocellulose hydrogel to match its mechanical properties to Matrigel, and to achieve the needed viscoelastic modulus for intestinal organoid formation (Curvello, Kerr, et al., 2021; Curvello & Garnier, 2021). Fragmenting of the GDxA hydrogel occurred regardless of the CaCl₂ crosslinking and it seemed that the crosslinking did not work in used functionalized GDxA samples. The conjugated avidin in the GDxA may have affected to the crosslinking process. CaCl₂ is an ionic crosslinker of which Ca²⁺-ions crosslink carboxyl groups on the surface of the nanocellulose (Curvello et al., 2019). In GDxA hydrogel, some of these carboxyl groups are conjugated with avidin, which may affect the crosslinking efficiency of CaCl₂. Detachment from the well plate and fragmenting of GDxA hydrogel may also be due to the use of a 24-well plate. In a 24-well plate, the GDxA hydrogel is in a dome model and contacts only with the bottom of the well. In a 96-well plate, due to the smaller diameter of the well, the GDxA hydrogel is also in contact with the sides of the well, supporting it and making the hydrogel more stable.

6.3 Functionalized GDxA-COL hydrogels in organoid culturing

COL has been used in 3D cell culturing due to its good qualities such as thermo responsive nature (Curvello, Alves, et al., 2021; Heo et al., 2022). COL polymerization process is sensitive to pH and temperature changes, which makes it a tunable material (Doyle et al., 2015). Previously Curvello, Alves, et al. discovered that intestinal organoids grown in plain COL hydrogel have abnormal morphology and topography. In this thesis, the results were similar. If hydrogel stays at the bottom of the well plate, the crypts started to dis-

tegrate in a few days. It seems that COL alone does not support intestinal organoid formation, at least as a static culture. As a mixture with nanocellulose, COL has been used successfully in intestinal organoid culturing, which was also observed in this thesis (Curvello, Alves, et al., 2021).

6.3.1 Optimization of GDxA-BcRGD-COL hydrogel preparation

In this study, 0.18 % (w/v) GDxA hydrogel functionalized with 1.54 µg/ml BcRGD and supplemented with 1.4 mg/ml COL supported the intestinal organoid growth and formation. COL was neutralized with 15 mM NaOH, and the well plates were placed into the incubator at +37°C right after plating, but exact times were not measured (Exp. 5). After three days crypts embedded in the hydrogel started to grow and produce budding crypts similarly to the reference culture. Organoids formed large cystic 3D structures, but the growth was slower than in Matrigel culture. Cultures with or without ROCK inhibitor showed similar organoid growth in 96-, and 24-well plates. After 13 days organoids were passaged from GDxA-BcRGD-COL to Matrigel and continued organoid formation, which represented that the cells were vital after culturing in nanocellulose hydrogel. In the future, it would be desirable to investigate organoid passaging from nanocellulose hydrogel to similar hydrogel.

However, the repetition of the culture turned out to be difficult. In several experiments, the variables in the GDxA-BcRGD-COL hydrogel preparation were examined to find the optimal method to produce GDxA-BcRGD-COL hydrogel reproducibly. The pH and temperature changes during hydrogel processing are known to affect the COL polymerization, such as fibre thickness and mesh size, and the structure of the GDxA-BcRGD-COL hydrogel was altered by varying these features. Balancer of osmolality, pipetting order of GDxA, COL, and crypts, pH of COL, the use of ROCK inhibitor, and various incubation times in different steps of hydrogel preparation were investigated to find the optimal conditions for intestinal organoid growth. Crypts embedded in GDxA-BcRGD-COL hydrogel often disintegrated into single cells in a few days and organoid formation was not observed, probably due to insufficient biochemical or mechanical properties.

The organoid formation was again observed in GDxA-BcRGD-COL hydrogel in conditions where neutralized COL was incubated on an ice bath for 60 minutes before mixing with GDxA or cells, and a well plate was held at RT for 30 minutes before placing into the incubator at +37°C (Exp. 12). The hydrogel preparation differed from the first successful GDxA-BcRGD-COL culture (Exp. 5) where the COL was not incubated on an ice bath before use, and the well plates were not incubated at RT. Also, the sample cooling

method, well plate material, and pipetting order of GDxA, COL and crypts were different between experiments, but both GDxA-BcRGD-COL hydrogels had structures that could support organoid formation regardless of differing preparations. It must be noted that in experiment 12, ten parallel samples of hydrogel were prepared but crypts developed into organoids just in one well, while in experiment 5 the organoid formation was observed in all four prepared parallels.

Various studies emphasize the importance of both, biochemical and mechanical cues needed for intestinal organoid formation (Broguiere et al., 2018; Capeling et al., 2019; Cruz-Acuña et al., 2017; Gjorevski et al., 2016; Liu et al., 2019). Even though the same hydrogel composition, 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL was used during the optimization of GDxA-BcRGD-COL hydrogel preparation, the culturing results differed because of varying preparation methods. The mechanical properties of GDxA-BcRGD-COL varied between samples when different COL pH levels and incubation times during the sample preparations were used. The rheology of functionalized GDxA-COL hydrogels was not examined closely, and this could help to produce matrices mechanically closer to intestinal tissue or Matrigel. Further investigations concerning the mechanical properties of functionalized GDxA-COL hydrogels would be useful when producing an optimal GDxA-BcRGD-COL preparation method. The optimal culture condition for intestinal organoids is not straightforward and different studies suggest various cues to be crucial to organoid formation. Broguiere et al. underlie that RGD adhesion sites are necessary for intestinal organoid growth, but Capeling et al. proved that it was not needed when they cultured organoids in alginate hydrogel without biochemical cues (Broguiere et al., 2018; Capeling et al., 2019). On the other hand, Gjorevski et al. argue that when a generous number of proteins are provided, stiffness is not such an important factor for intestinal organoid formation (Gjorevski et al., 2016).

Live/Dead staining gave insight about the viability of crypts in GDxA-BcRGD-COL and Matrigel hydrogels. Staining on day one showed that the crypts were mostly filled with living cells in both cultures, but also dead cells were in close contact with the crypts. Dead cells were most likely single cells transferred to the hydrogel during passaging, but it may also be a sign of invasive apoptosis. On day three some dead cells, probably due to normal self-regeneration, could be observed inside the crypt in GDxA-BcRGD-COL culture. However, cells in the crypts were mainly alive and a slight budding could be observed. Similar effects happened to crypts in Matrigel, but crypts had already formed into cystic structures in three days. After four days, the Ca-AM signal from GDxA-BcRGD-COL hydrogel decreased even though a few budding crypts were observed with

bright field microscopy. Even though the crypt formation into organoids seemed similar in both cultures during the first three days, there were just a few budding crypts in GDxA-BcRGD-COL culture whereas nearly all crypts formed into cystic organoids in Matrigel culture. Also, after four days the budding crypts in GDxA-BcRGD-COL disintegrated into single cells. The GDxA-BcRGD-COL hydrogel for Live/Dead staining samples was done according to the preparation method used in the latest successful GDxA-BcRGD-COL hydrogel (Exp. 12), where the COL was incubated for 60 minutes before use, and after plating the hydrogel was incubated at RT for 30 minutes before placing to the incubator at +37°C. However, the hydrogel sample size for the Live/Dead assay was decreased from 50 μ l to 30 μ l to observe the organoid better, and the incubation time of COL before use sample was 90 minutes instead of 60 minutes. Decreased hydrogel amount may have an impact on organoid formation due to changes in mechanical properties or provided space in the hydrogel, which may not enable the formation of large cystic organoids. The longer incubation time of COL could have an impact on COL polymerization and mesh size in GDxA-BcRGD-COL that may affect organoid formation, if the crypts embedded in the hydrogel cannot form needed adhesions with the surrounding matrix.

6.3.2 GDxA-COL hydrogel ratios and delayed crypt addition

Different functionalized GDxA and COL ratios were also examined as a culture matrix for intestinal organoids. The organoid formation was not observed in samples where hydrogel stayed statically in the bottom of the well plate. However, when 0.1 % (w/v) GDxA functionalized with 0.84 μ g/ml BcRGD and supplemented with 2.0 mg/ml COL hydrogel in a 24-well plate had rolled up to a thick mattress-like structure, the crypts developed into cystic organoids. This may be because in these parts the hydrogel stiffness was different, nutrient uptake was better or the structure provided more protein adhesion sites. Curvello, Alves, et al. used 0.1 % (w/v) nanocellulose functionalized with 2 mM RGD peptide and supplemented with 2.0 mg/ml COL successfully in intestinal organoid culturing (Curvello, Alves, et al., 2021). In this thesis, the same hydrogel composition with lower RGD peptide concentration did not seem to support organoid formation when the hydrogel was attached to the well plate bottom. However, some differences occur also in the used nanocellulose hydrogels. Curvello, Alves, et al. used TEMPO-oxidized nanocellulose which requires covalent attachment of RGD peptides, whereas in this study, avidin conjugated nanocellulose was used which is functionalized with RGD peptides through avidin-biotin interaction. The GDxA is made from birch, and the nanocellulose used by Curvello, Alves, et al. is eucalyptus, which may affect the nanocellulose composition. (Curvello, Alves, et al., 2021)

Intestinal crypts were also transferred to GDxA-BcRGD-COL hydrogels which were prepared on the previous day. Hydrogel composition of these hydrogels was 0.18 % (w/v) GDxA with 1.54 $\mu\text{g/ml}$ BcRGD supplemented with 1.4 mg/ml COL. NaOH concentration of COL, COL incubation time before mixing with GDxA, and delayed pipetting of crypts either 1) inside, or 2) on top of hydrogel varied between samples. In experiment 14, the concentration of NaOH was 18.5 mM, and COL was incubated for 30 minutes before mixing with GDxA. On the next day, crypts were pipetted inside the GDxA-BcRGD-COL hydrogel. Added crypts attached to the hydrogel and started to form big cystic organoids like in the Matrigel. In the next experiment 18.5 mM and 15 mM NaOH concentrations in COL were used, and COL was incubated for 0, 30, or 60 minutes before mixing with GDxA. The next day crypts were added inside or on top of GDxA-BcRGD-COL hydrogel. Crypts started to grow in every condition but better in cultures where crypts were pipetted on top of the hydrogel. This may be since a bigger number of crypts did attach to the hydrogel when pipetted on top of the hydrogel. The morphology of formed structures was still different compared to organoids formed in Matrigel. More singular crypts seemed to form rather than big uniform organoids. Interestingly even though the same sample preparation to GDxA-BcRGD-COL hydrogel was performed for one sample in both experiments with delayed crypt addition, the growth of organoids between these samples was not similar. It may be that the crypts addition differed between samples and crypts attached to the GDxA-BcRGD-COL hydrogel differently, affecting the organoid formation. In addition to the inaccurate placing of organoids, when crypts were pipetted into the hydrogels, the pipet tip tore the GDxA-BcRGD-COL hydrogel, which could also affect the organoid growth. However, these results suggest that delayed organoid addition could be a possible way to grow intestinal organoids in GDxA-BcRGD-COL hydrogel, and the crypt addition should be studied further to make it a reproducible method.

The growth rate of intestinal organoids was slower in every successful functionalized GDxA-COL culture when compared to Matrigel. Budding of crypts can be observed in Matrigel culture in two days, while in GDxA-COL hydrogel a slight budding can be observed after 3-4 days, and it takes around seven days in GDxA-COL hydrogel to form into large cystic organoids. This may be due to drastic environmental change when crypts are transferred from protein-rich Matrigel to nanocellulose hydrogel functionalized usually with one biotinylated protein or peptide. Even though COL was added to the functionalized GDxA culture, the concentration and spectrum of proteins are decreased when compared to Matrigel culture, which is composed of over 1000 proteins. In many studies, the characterization of intestinal organoids is often done in the first three or four culturing days. For example, Curvello, Alves, et al., Curvello, Kerr, et al. and Curvello

and Garnier investigated the organoid viability, proliferation, and the success of the culture condition in just the first three culture days when intestinal crypts have not yet formed large cystic organoids (Curvello, Alves, et al., 2021; Curvello, Kerr, et al., 2021; Curvello & Garnier, 2021). However, in this study, the Live/Dead assay showed that in the first three days the organoid formation seemed promising and comparable to Matrigel culture, but a few days later the formed structures in GDxA-BcRGD-COL hydrogel disintegrated into single cells.

6.3.3 *Future aspects*

ROCK inhibitor and CHIR stemness booster are known to influence to the self-renewal and differentiation of intestinal stem cells (Almeqdadi et al., 2019). For example, Sato et al. used the ROCK inhibitor in the intestinal organoid culture to prevent single-cell disintegration (Sato et al., 2009). With functionalized GDxA hydrogels, supplementation of ROCK and CHIR in a culture medium seemed to help the crypts to survive in GDxA hydrogel for a few days longer compared to samples without them. With functionalized GDxA hydrogels ROCK and CHIR were held on cultures for three or four days, depending on the experiment. Later it was hypothesized that prolonged exposure to ROCK and CHIR could have some negative effects on the crypts, and the use of ROCK was decreased to one day. In this study, the organoid formation was successful in GDxA-BcRGD-COL cultures with and without ROCK inhibitor, and it seems that ROCK inhibitor is not essential for intestinal organoid formation. Even though the use of ROCK inhibitor may help the intestinal crypts to survive in their first culturing days, it did not seem to prevent disintegration. Because intestinal crypts are sensitive to single-cell disintegration, single intestinal epithelial stem cell cultures have been used in the organoid culturing to prevent the phenomena (Randell & Fulcher, 2012). For example, Sato et al., Brogiere et al., and Gjorevski et al. used single intestinal stem cells in organoid culturing, not intestinal crypts, which were used in this thesis (Brogiere et al., 2018; Gjorevski et al., 2016; Sato et al., 2009). In the future, single intestinal stem cell cultures could be studied with functionalized GDxA and functionalized GDxA-COL hydrogels.

Even though COL polymerization can be altered with varying pH and temperature of COL, those are difficult variables to keep constant, especially when slight changes have a big impact (Doyle et al., 2015). The viscous character of COL affected greatly its pipetting and handling properties and as an animal-based product, precise modification of its polymerization process is also challenging because the material may have unexpected behaviour. Pipetting issues with mixing GDxA and whole biotinylated proteins made it even harder to make homogenous functionalized GDxA-COL hydrogels reproducibly.

Curvello, Alves, et al. notify that COL and nanocellulose make various interactions with each other and are not under control and yet fully understood (Curvello, Alves, et al., 2021). These reasons may explain why it was difficult to accurately reproduce the cultures done in this thesis. Due to the large size of examined culture conditions, sometimes only one parallel environment was made. It does not comprehensively describe the culture's suitability for intestinal organoid cultivation. To properly evaluate studied culture conditions, each experiment should be repeated, which was just noted to be rather challenging due to the behaviour of large biotinylated proteins and COL. In the future rat tail COL could be replaced with Food and Drug Administration approved COL, or COL peptides to decrease animal-based materials in the hydrogel.

Further investigation of the formed intestinal organoids and their genetics is needed to gain more accurate understanding of the organoids cultured in nanocellulose hydrogel. Real-time quantitative polymerase chain reaction (real-time qPCR) samples of several organoid cultures were harvested for this thesis, but the intestinal organoids in the reference culture (Matrigel) died just before collecting them. Without organoids grown in Matrigel, there is no reference where to compare the gene expression levels. For this reason, the real-time qPCR could not be concluded during the thesis.

As described in this thesis, there are various potential substituents for Matrigel, and many research groups have reported positive outcomes with many materials, also with nanocellulose hydrogels. However, with nanocellulose hydrogels, more optimization work is still needed to replace all use of Matrigel in organoid culturing. Curvello, Kerr, et al. reported that before embedding the intestinal crypts to nanocellulose hydrogel, intestinal organoids had to be cultured first in Matrigel. They discovered that with their nanocellulose hydrogel, at least 20 % (v/v) of Matrigel supplementation in the hydrogel was needed before organoid culturing was successful in functionalized nanocellulose without Matrigel supplementation. In the future, the goal is to directly seed the dissected crypts from the intestine to nanocellulose hydrogel, with no need for Matrigel. (Curvello, Kerr, et al., 2021)

In this study, intestinal organoid formation was observed under many culture conditions in GDxA-BcRGD-COL hydrogel. GDxA nanocellulose hydrogel has great features needed for biomedical applications such as tunability and biocompatibility. It has shown its ability to support intestinal organoid culturing when functionalized with BcRGD and supplemented with COL. Research groups are still discussing which are the needed crucial factors for intestinal organoid formation, and more research is needed to understand the delicate interaction network between cells and their surroundings.

7. CONCLUSIONS

In this thesis, an avidin-conjugated nanofibrillar cellulose hydrogel, GDxA, was examined as a culturing matrix for murine intestinal organoid cultivation. The aims of this thesis were, 1) to biotinylate selected ECM proteins, 2) to functionalize GDxA hydrogel with different proteins and peptides to optimize the culturing conditions, and 3) to characterize the organoid growth in the functionalized GDxA culture.

The biotinylation of selected proteins was done successfully. A hydrogel containing 0.18 % (w/v) GDxA functionalized with 1.54 $\mu\text{g/ml}$ BcRGD and supplemented with 1.4 mg/ml COL supported organoid formation. The organoid formation was observed in GDxA-BcRGD-COL hydrogels where the crypts were added during hydrogel preparation, and in premade GDxA-BcRGD-COL hydrogels with delayed crypt addition. During the first three culture days, the organoid growth in GDxA-BcRGD-COL hydrogel seemed to be slower but otherwise similar to growth in Matrigel. The behavior of the whole biotinylated proteins and COL made the repetition of culture conditions challenging. Further optimization is required to prepare GDxA-BcRGD-COL hydrogel reproducibly for intestinal organoid cultivation. More in-depth information on the genetics of the formed organoids is needed to confirm the similarity of formed organoids between GDxA-BcRGD-COL and Matrigel cultures. Various materials have been investigated as an alternative for animal-derived matrices. GDxA may have the potential as an alternative for animal-based materials when functionalized with BcRGD and supplemented with COL.

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