TECHNISCHE UNIVERSITÄT WIEN

Diploma Thesis

Influence of human placenta substrate on endothelial network formation in different types of hydrogels

Carried out for the purpose of obtaining the degree of Diplom-Inginieur (Dipl.-Ing.) submitted at TU Wien, Institute of Applied Synthetic Chemistry

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Kurzfassung

Gewebe und Organe benötigen eine ausreichende Nährstoff- sowie Sauerstoffversorgung, welche durch das vaskuläre Gefäßsystem ermöglicht wird. Daher spielt die Ausbildung von vaskulären Strukturen eine entscheidende Rolle im Bereich des Tissue Engineerings. Ein Bereich des Tissue Engineerings befasst sich mit der Entwicklung von Biotinten. Diese bestehen aus Zellen und Materialen, die der extrazellulären Matrix (ECM) ähneln, und werden mittels 3D-Druck zu geeigneten Materialien verarbeitet, die die Vaskularisierung von Geweben fördern sollen.

In dieser Arbeit werden verschiedene Materialien untersucht, welche als potenzielle Biotinte eingesetzt werden können. Diese Materialien, sollen der extrazellulären Matrix ähneln, und Zellproliferation, -migration und -netzwerkausbildung unterstützen. Zwei Arten von Biomaterialien wurden in dieser Arbeit untersucht. Zum einen kamen Gelatine-basierte Hydrogele zum Einsatz, die mithilfe eines Photoinitiators quervernetzt werden und sich dadurch besonders für den Einsatz in Lithographie-basierten Bioprintern eignen. Zum anderen wurden Fibrinogen-basierte Hydrogele analysiert, die bereits vielfach in der Medizin zum Einsatz kommen. In beiden Arten von Materialien wurde humanes Plazentrasubstrat (hpS) inkorporiert, das aus verschiedenen Proteinen besteht, und die Vaskularisierung fördern.

Im Rahmen dieser Arbeit wurden die Eigenschaften und Anwendungsmöglichkeiten von hpS sowie dessen Einfluss auf das Verhalten von HUVEC (human umbilical vein endothelial cells) untersucht. Zusätzlich wurden die Einzelbestandteile von Gelatine-basierten Hydrogele auf Toxizität getestet. In nachfolgenden Experimenten wurden verschiedene Gelatine-basierte Hydrogele in 2D-Analysen miteinander verglichen, um vielversprechende Kandidaten auszuwählen, die anschließend mit Fibrinogen-basierten Hydrogelen in 3D-Experimenten beurteilt wurden.

Abstract

Vascular networks supply organs and tissues with essential nutrients and oxygen. Therefore, creation of vascular networks is important for the generation of viable tissues and organs in vitro for tissue engineering. The formation of vascular networks plays a crucial role in tissue engineering. One aspect of tissue engineering focuses on the development of bioinks. These bioinks are composed of cells and ECM-like materials, which support tissue vascularization.

In this thesis, various materials that could be used as potential bioinks are investigated. These materials mimic the extracellular matrix and support cell proliferation, migration and network formation. Two different types of materials are analyzed within this thesis: gelatine-based and fibrinogen-based hydrogels. Gelatin-based hydrogels are crosslinked using a photo initiator and are suitable for lithography-based-3D-bioprinting. In both types of biomaterial human placenta substrate (hpS) is incorporated, which is known for pro-vascularizing properties.

The study examined the properties and potential applications of hpS, as well its impact on endothelial network formation in HUVEC (human umbilical vein endothelial cells). Additionally, the individual components of gelatin-based hydrogels are tested for cell toxicity. Subsequent experiments compared different gelatin-based hydrogels in 2D assays to identify promising candidates, which are analyzed in 3D experiments like fibrinogen-based hydrogels.

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Introduction

In 1954, Joseph Murray was the first person who successfully performed a kidney transplantation between identical twins.[1] In 1990, he was awarded the Nobel Prize with Dr. Edward Donnall Thomas for his important scientific contribution to transplantation medicine.[2] In addition to the risks and side effects of a transplant, the search for a suitable donor is challenging. In Austria, for example, there were 829 people on the waiting list for organ donation at the end of 2021 and a total of 662 organs were transplanted in the same year.[3]

In comparison, regenerative medicine (RM) is concerned with the growth or healing of organs and tissues with the help of therapeutic cells. The basic idea here is to repair or even re-grow damaged tissue or organs using various cell technologies.[4] Such phenomena have already been observed in nature. For example, mexican axolotls can regrow legs after they have been cut off.[5] It is assumed that mammals also possess these abilities in the embryonic stage, but that this ability is lost during development.[6]

In the last three decades RM and tissue engineering gained importance, but it was probably already known in Greek mythology that certain parts of the human body can regenerate - Prometheus, who stole fire for mankind, was tied to a stone as punishment for this, where part of his liver was eaten by a bird every day, but it grew back the next day.[7] Human tissue can also regenerate or heal, but this ability decreases with age.[8]

RM finds broad application to various divisions of medicine, like dental medicine and orthopedics. Chronic and incurable diseases or damage could be treated with RM and thus enhance quality of life. Starting from diseases like Diabetes mellitus and Morbus Parkinson but also curing irreparable injuries of bone marrow. [9,10]

Therapies such as tissue engineering, gene and stem cell therapy are some of the possibilities offered by RM. However, RM can be used not only for clinical applications, but also in research and pre-clinical applications like organ-on-a-chip technology.[11,12]

Gene therapy makes it possible to treat diseases that are based on genetic disorders. Genetic material is introduced into the patient *in vivo* or *ex vivo*. One of the possible methods is gene replacement therapy. Here, genetically intact DNA/RNA sections are introduced into the target cell using a viral or non-viral vector, thus enabling the production of an intact gene product. The damaged gene is not removed or repaired, but the malfunction of the original gene is compensated for by an intact gene. In contrast, genome editing involves the targeted repair of damaged genes. This is done using so-called “gene scissors” - probably the best-known tool for this is the CRISPR/Cas system, for which Emmanuelle Charpentier and Jennifer A. Doudna were awarded the Nobel Prize in Chemistry in 2020. The method is based on using gene

scissors to cut out a specific section of DNA in the genome and replace it with an intact sequence, thereby preserving the intact gene product. [13–15]

Stem cell therapy has become important in the past decades. So-called stem cells are used in this form of therapy. Stem cells are cells that are not or not fully differentiated. This means that they can divide and remain stem cells or differentiate into more specialized cells. The potential of cells to differentiate into certain body cells is utilized in stem cell therapy, which can be used for example in Morbus Crohn treatment.[16,17]

Although organ-on-a-chip technologies are not used directly in clinical applications, they can nevertheless be regarded as a subgroup of RM. In the past, cellular mechanisms were usually only investigated in 2D cell culture models before being subsequently analyzed in vivo. Some difficulties have been encountered, as 2D models cannot represent every interaction taking place in a living organism. For this reason, the development of such organs on chips is a good way of modelling interactions that take place in vivo. Organs-on-a-chip are frequently used in the development and investigation of drugs.[11,18]

The methods listed above offer a great opportunity for patients with a wide range of diseases and injuries, but only just over 30 gene and cell therapy products are currently approved by the FDA. Gene and cell therapeutics authorized for the market are mainly used for the treatment of rare hereditary diseases or aggressive cancer. [19] The high research and development costs of gene and cell therapeutics leads to enormous production costs up to several hundred million to billions of US dollars.[20]

Tissue engineering aims to replace or repair damaged organs or tissue. The focus here is on the development of suitable biomaterials consisting of a scaffold, cells and bioactive molecules and signals– also known as the tissue engineering triad.[21]

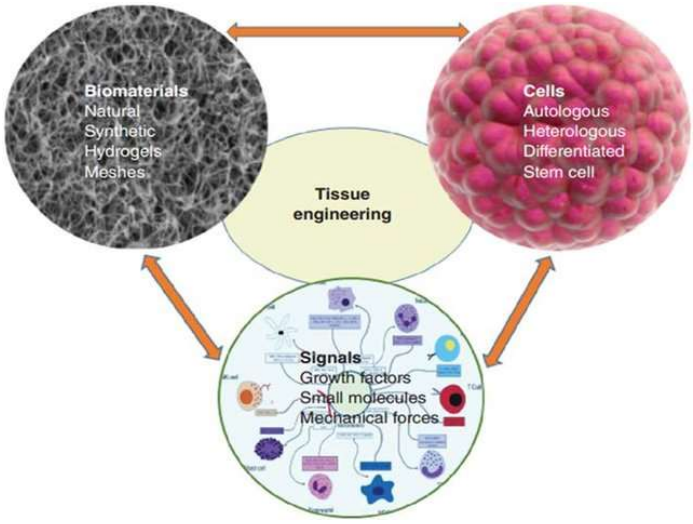

Figure 1: Tissue engineering triad.
All tissue engineered principles are based on three main components: cells representing the living component, biomaterials as scaffolds where cells are embedded and signals and bio-active molecules which support growth and maintenance of the biological system. Illustration from literature [22]

As expected, this is a very complex subject area and requires many disciplines from the natural sciences, medicine and technology. The Vacanti mouse experiment is a good example to describe the complexity of tissue engineering. The aim of this experiment was to develop an artificial ear. The scaffold was shaped from PGA/PLA (polyglycolic acid / polylactic acid) and then colonized with chondrocytes. The artificial ear was then cultivated for a week under physiological conditions until it was finally transplanted onto the back of a mouse (see Figure 2). After 12 weeks, the artificial ear was then examined and it was found that the cells had distributed evenly on the scaffold and components of the extracellular matrix (ECM) had already been produced, but that the nutrient and oxygen supply to the ear could not be guaranteed as no vascular structures had been formed in the transplant.[23,24] This experiment emphasizes once again the need for sufficient and intact vascular system.

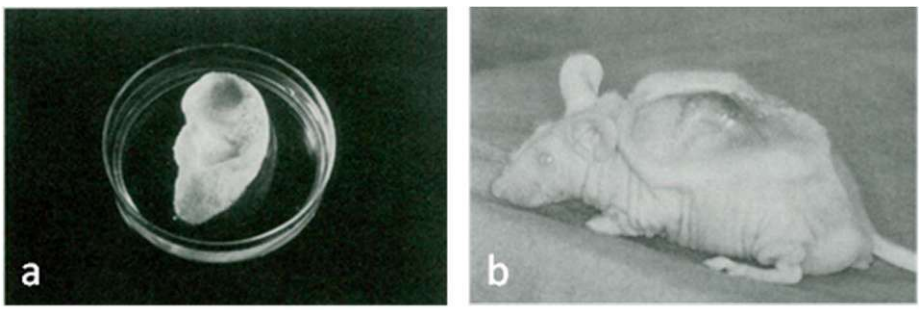

Figure 2: Vacanti Experiment
(a) Artificially moulded ear before transplantation (b) Vacanti mouse with ear on back. Illustration from literature[25]

Structure and Development of Blood Vessels

The vascular system ensures that tissues and organs are supplied with sufficient oxygen and nutrients. In an adult human, blood vessels in total can reach a length of up to 100 000 kilometers. [26] The circulatory system is separated into two blood systems (arterial, venous) and a lymphatic system. The vascular system is made up of different types of vessels, which are divided into three major groups: arteries, veins and capillaries. Arteries and veins are large vessels and have a diameter of up to several centimeters. Their main function is to transport blood over long distances in the body, whereas capillaries are small blood vessels with a diameter of a few µm. Capillaries are responsible for the exchange of nutrients and oxygen and nutrient supply to all tissues and organs.[27–29] All tissues and organs are dependent on sufficient oxygen and nutrient supply, while oxygen can diffuse approximately 100-200 µm through cells. [30] Blood vessels are made up of three layers: the tunica intima, tunica media and tunica adventitia (Figure 3). Tunica intima consists of an endothelium (monolayer of endothelial cells) and a basal lamina and acts as a thrombogenic barrier. Tunica media consists of multiple layers of smooth muscle cells (SMC) which synthesize essential molecules of the ECM like collagen. Tunica adventitia is the outermost layer of blood vessels and consists of connective tissues with incorporated fibroblasts. The composition and thickness of the layers depends on the type of vessel and its function. Arteries, for example, usually have a thicker tunica media, while capillaries have neither tunica media nor tunica adventitia, but consists of an endothelium and a basal lamina. [31]

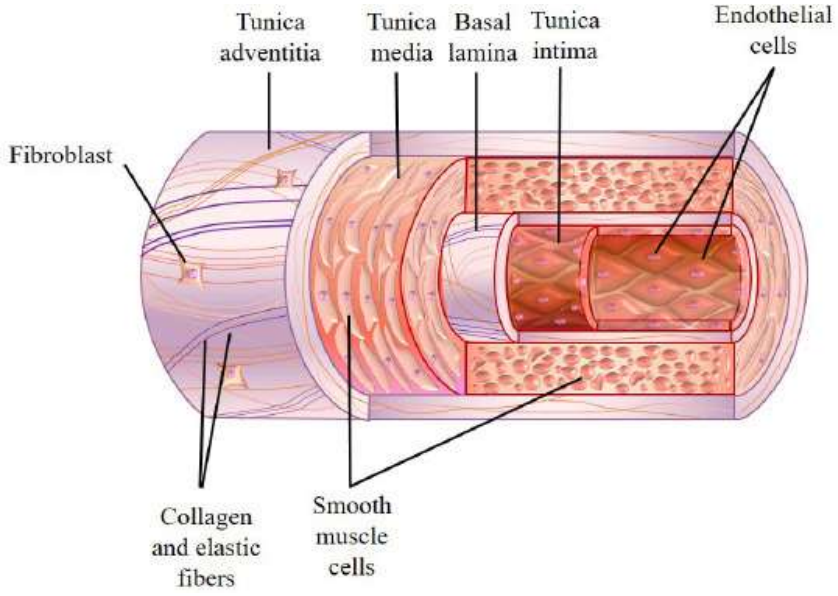

Figure 3: Schematic illustration of a blood vessels. Illustration from literature [31]

Blood vessels can be formed in two ways. The process in which the blood vessels develop *de novo* is known as vascularization, if vessels are formed from existing vessels, for example through sprouting, this process is called angiogenesis.[32] A

distinction is made between two types of angiogenesis: sprouting and intussusceptive angiogenesis.[33,34]

Intussusceptive angiogenesis is defined by the movement of opposing endothelial cells within a vascular lumen towards each other and forming a so-called intussusceptive pillar, which subsequently leads to the formation of new vessels (Figure 4B).[33,34]

The alternative, sprouting angiogenesis, underlies another process. Endothelial cells can sense a VEGF gradient and are stimulated to break through the basal lamina and migrate along the VEGF gradient led by a so-called tip cell. This tip cell forms filopodia that can detect this gradient and leads the tip of the new sprout. The endothelial cells behind it are called stalk cells. They proliferate, stabilize and form the vascular lumen of the new sprout (Figure 4A).[35,36]

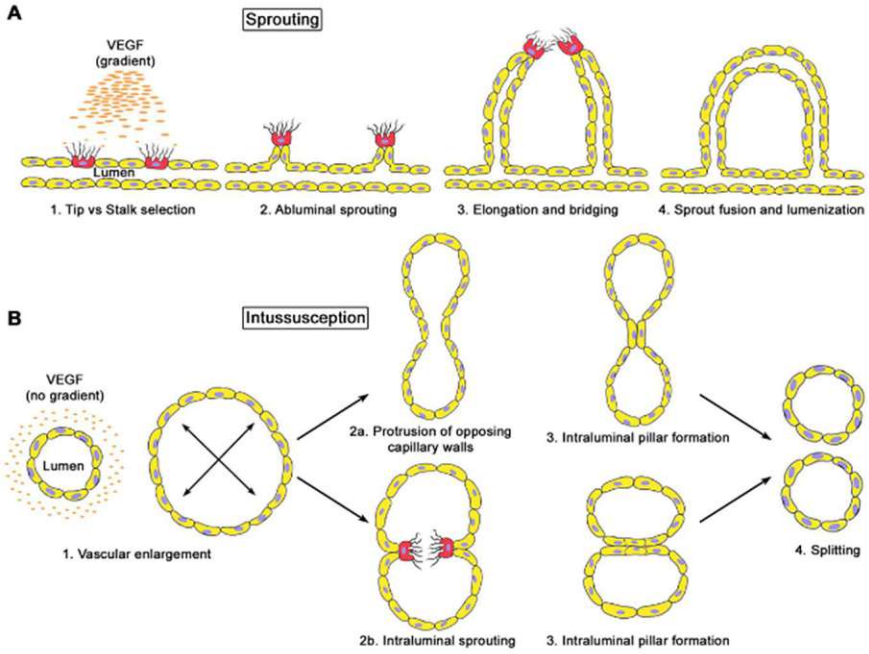

Figure 4: Sprouting vs. Intussusception
Illustration from literature[37]

Oxygen plays a crucial role in the development of new blood vessels. An excess of oxygen (accumulation of reactive oxygen species -ROS) as well as a lack of it can damage tissue and organs. For this reason, oxygen must be strictly regulated. HIF (hypoxia induced factor) plays a crucial role in this process. HIF is a heterodimeric protein that is built up from an α-subunit (three isoforms HIF-1α, HIF-2α and HIF-3α) and a β-subunit.[38–40]

HIF-α is always present, but if tissues are sufficiently supplied with oxygen (normoxia) HIF-α is hydroxylated by prolyl hydroxylases (PHD) and FIH-1 (factor inhibiting HIF-1) binds to HIF-α, which leads to the binding of von Hippel-Lindau protein (VHL) to the

complex. This causes ubiquitination of HIF-α and subsequently results in degradation in the proteosome. [39,41]

In a hypoxic state PHD is inhibited and VHL cannot bind to HIF-α, so that HIF-α accumulates and is translocated into the nucleus, where it dimerizes with HIF-β and becomes transcriptionally activated. The active transcription factor binds to HRE (hypoxia response elements) and induces the transcription of important angiogenic factors such as VEGF.[39,40,42,43]

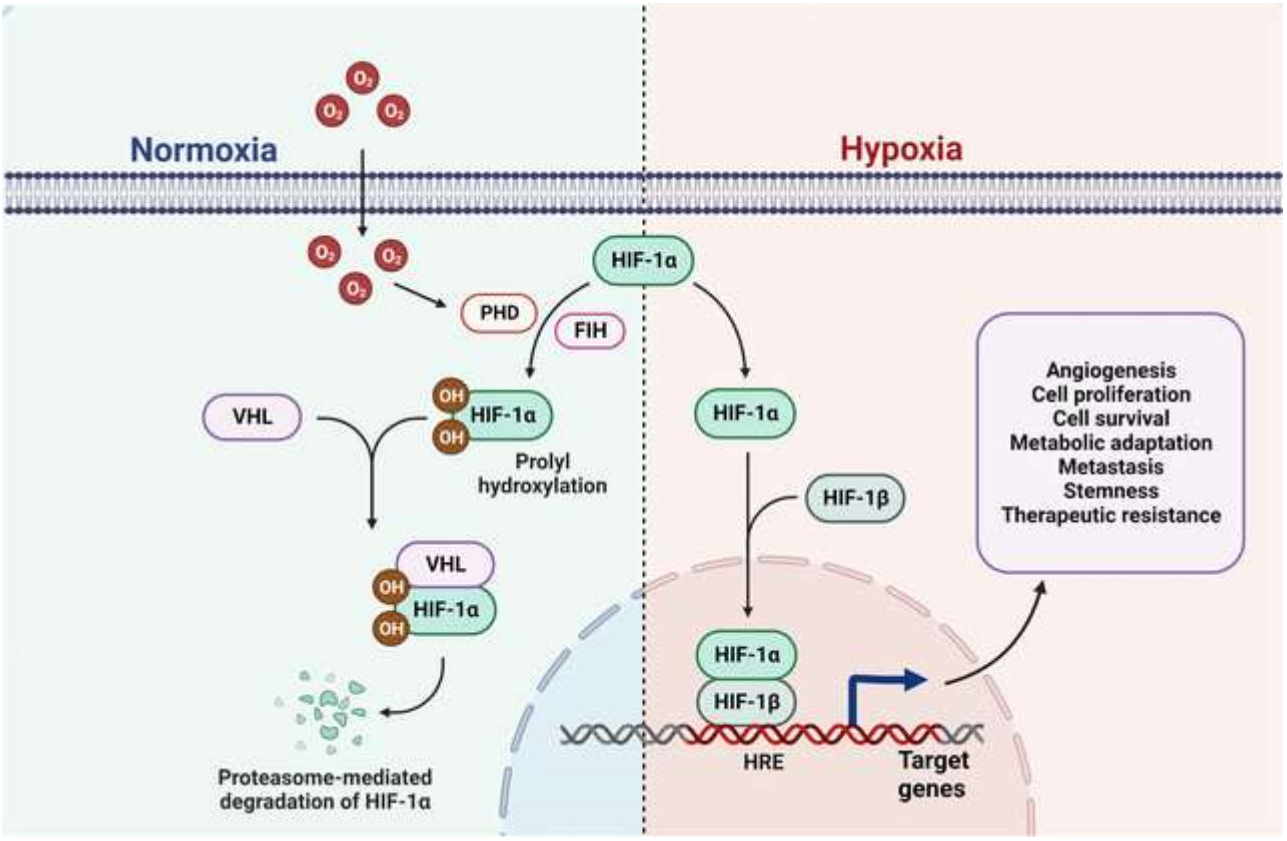

Figure 5: Regulation of HIF.

Illustration from literature[44]

Extracellular Matrix

All biomaterials in tissue engineering have something in common – they mimic the extracellular matrix (ECM). The ECM can be found in all animal organisms and is essential for every organism. The composition of the ECM varies enormously in tissues and organs. The ECM protects organs and tissues from mechanical forces, but it also plays a crucial role in the regulation of various functions such as cell proliferation, migration and development of cells. The ECM itself is formed by surrounding cells, for example fibroblasts act as a key factor in formation of many ECM components in connective tissue.[45,46]

Although the types of macromolecules in the ECM are constant, the exact composition of these types varies greatly between different tissues. Common representatives of the ECM are glycosaminoglycans (GAG), proteoglycans, collagens, elastin, laminin and fibronectin. Collagen is the most common protein of the human body and a major component of ECM.[47–49] Collagens can be divided into different subtypes according to their structure and their function. GAGs are very long polysaccharide chains of disaccharides and have a strong negative charge. Due to their structure and negative charge, they occupy a very large space within the ECM and attract cations from the environment, resulting in the absorption of large amount of water within the matrix. This property of ECM supports mechanical stability against compressive forces. A well-known example of a GAG is hyaluronic acid. Anti-aging, skin repair, wound healing and tissue regeneration display just a few biomedical applications of hyaluronic acid. Due to its anti-inflammatory properties and natural presence, it became a well-studied biomaterial for regenerative medicine. [50–54]

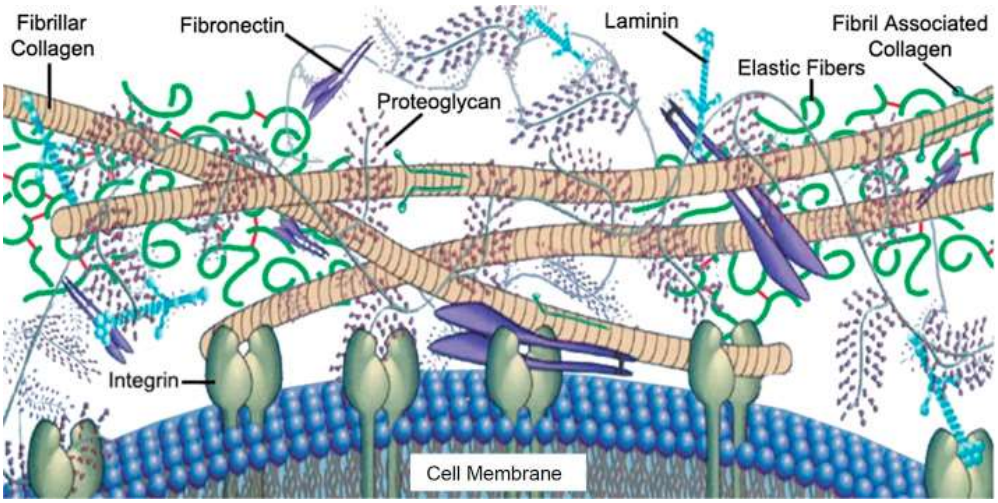

Figure 6: Schematic figure of ECM. Illustration from literature [55]

RGD (arginine-glycine-aspartate) motifs can be found in various proteins of the ECM, like in laminins or in collagens. They play a crucial role in cell adhesion, which is important for proliferation and migration of cells. RGD motifs were first time discovered in fibronectin, but in lots of other ECM proteins this highly conserved motif can be found. RGD motifs interact with integrins of the cells and regulate cell behavior. RGD motifs have been often incorporated in biomaterials, supporting cell growth and proliferation. This makes RGD motifs as a common used tool in RM [45,46,55,56]

Hydrogels in Tissue Engineering

Hydrogels are hydrophilic materials which can absorb large amounts of water without dissolving or changing their 3D structures. In everyday use hydrogels can be found in contact lenses or in diapers as superabsorbent. Since they can absorb a lot of water, they are interesting for biomedical applications or agriculture.[57]

For clinical use various requirements must be fulfilled. For example, hydrogels must be bioactive, biodegradable and replace the damaged or missing tissue. For this, it must be biocompatible, which means that it shows no cytotoxic effects on human body, and at the same time establishes an environment in which proliferation, migration and growth of cells is supported. Since the human body consists of various cell types and from these resulting organs and tissues, hydrogels must fulfill all requirements for specific tissue types and their location, e.g. in comparison, cartilage in knees experience different mechanical forces than the liver. All these requirements are great challenges to overcome during the development of a suitable hydrogel. The structure of ECM is widely used as a potential template for hydrogels. [57–59]

Hydrogels can be separated into three different classes: natural, synthetic and hybrid hydrogels. Natural hydrogels are isolated from natural materials. Common materials are collagen or fibrin. Synthetic materials origin from non-natural origin like polyethylene glycol (PEG) or polyvinyl alcohol (PVA) and hybrid hydrogels are combinations of different types of hydrogels with desired characteristics. [59,60]

Synthetic materials are relatively cheap and can be fabricated in large quantities and different shapes, but they are often less biocompatible and need chemical modification. In comparison, natural materials have better biocompatibility but are more expensive and their collection is more complex. Since natural polymers are often derived from animals, ethical aspects must be considered. [61–63]

Table 1: examples for synthetic and natural polymers [61–63]

Synthetic Polymers	Natural Polymers
polyethylene glycol (PEG)	hyaluronic acid
polyvinyl alcohol (PVA).	gelatin
polylactic acid (PLA)	collagen
polycaprolactone (PCL)	chitosan
polyacrylic acid (PAA)	alignate

Hydrogels are polymers that can absorb large amounts of water, but whose polymer networks remain intact in a swollen state. Depending on the type of monomers of hydrogels, there are different types of crosslinking possibilities. A distinction can be made between chemical and physical crosslinking. Crosslinked hydrogels are typically characterized by reversible interactions like ionic and hydrophilic/hydrophobic interactions. A major advantage of physical crosslinking is that no addition of potential harmful reagents is necessary. [64,65]

Chemically induced crosslinking is mostly irreversible and establishes stable covalent bonds. A common method is photopolymerization, which will be used as well in this project. Here, using light of a specific wavelength results in free radicals formed using a photo initiator (PI), which subsequently initiates chain polymerization (see Figure 7).[66]

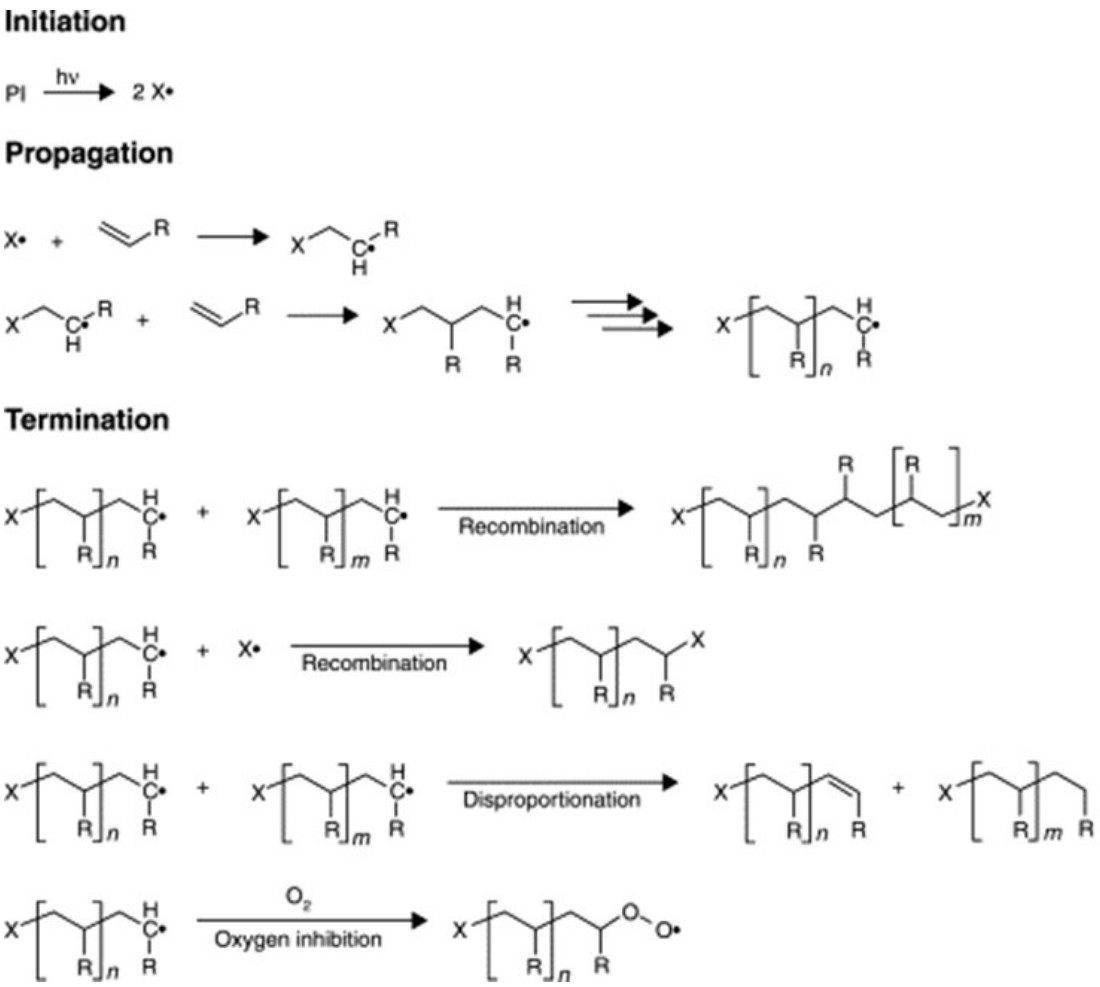

Figure 7: Principle of radical photopolymerization
Illustration from literature [67]

Radical photopolymerization is very popular in bioprinting, because the monomers used are mostly biocompatible and the polymerization rate is high. Well known photoinitiators are Irgacure 2959 (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) and LAP (lithium phenyl-2,4,6-trimethylbenzoylphosphinate). Both are classified as biocompatible, but nevertheless during photopolymerization cytotoxic metabolites can be built. [68,69]

Biofabrication

As mentioned before bioprinting became very popular in the past decade. According to that potential materials are necessary. Biomaterial inks consist of all necessary components to build up a stable biocompatible scaffold, but do not include viable material, the cells, in their formulation. In contrast, bioinks contain already cells in their formulation and are part of the printing process.[70] Scaffolds should be biocompatible, supporting cell proliferation and migration while also being degradable.[71]

Additive manufacturing technologies are used in TE to produce tissues in a controlled manner. A common strategy is based on the principle of Two-Photon Absorption (2PA).

The principle was first discussed by Maria Goeppert Mayer in 1931. However, as there was no suitable light source at that time, the principle was experimentally proven 30 years later. Utilizing an ultra-short-pulsed laser beam generates a high density of photons. Due to the high density of photons, two photons are absorbed almost simultaneously.[72–74] Two-Photon-Polymerization (2PP) bioprinting is based on the principle of 2PA. Due to the non-linear process, polymerization only takes place at the focal point of the laser, resulting to precisely controlled polymerization of biomaterials producing high-resolution structures. [75,76]

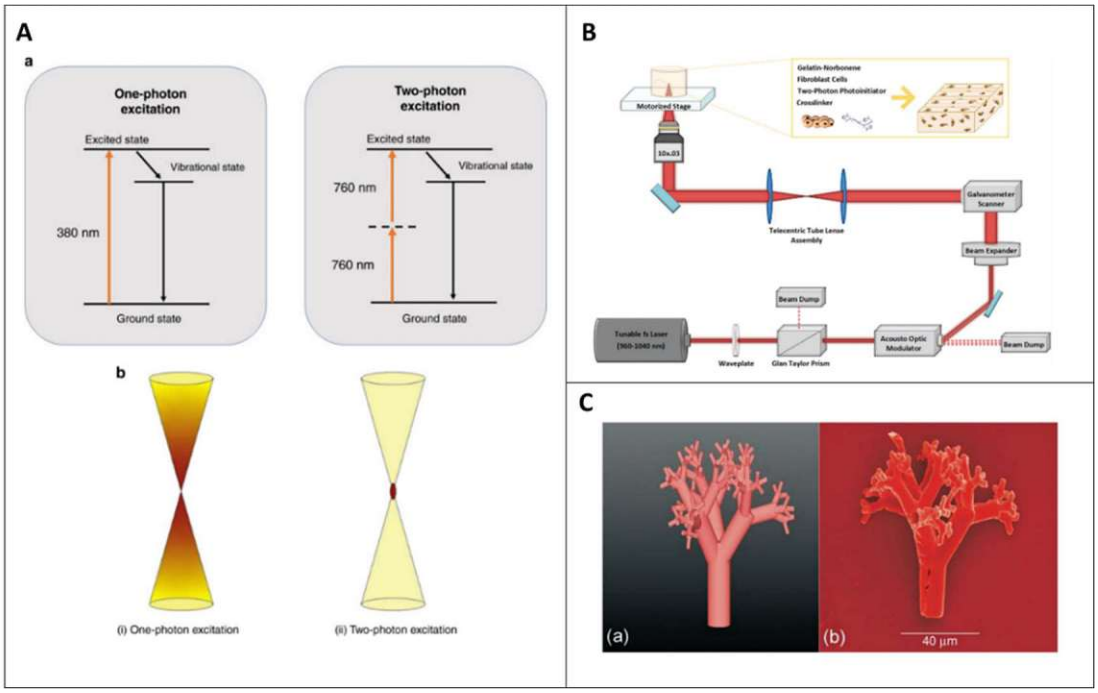

Figure 8: Principles of two-photon polymerization.
(A) comparison of one-photon excitation (linear) and two-photon excitation (non-linear); polymerization is restricted to the focal point in 2PP, resulting into high resolutions[75] (B) Schematic figure of two-photon polymerized additive manufacturing technique[77] (C) (left) CAD model of a vascular microcapillary for 2PP-Bioprinting (right) 2PP-printed vascular microcapillary[76] Illustrations from literature. [75 76 77]

2PP bioprinting offers an excellent opportunity for printing various materials with incorporated photoinitiators. Besides high-resolution, the method provides a highly reproducible procedure and using versatile materials, makes it advantageous for tissue engineering. However, usage of photoinitiators (PI) may have a negative effect on cells. Therefore, selection of an appropriate PI is necessary. The process time, the influence of irradiated PI and the metabolites formed during polymerization must be considered.[75,76,78]

Objective

Tissue engineering is concerned with artificial production and repair of organs and tissues. Sufficient nutrient and oxygen supply, as well as the removal of waste material, plays a key role in survival of biological materials. Therefore, vascularization of organs and tissues is a major challenge in tissue engineering.[79,80]

The aim of this thesis is to investigate the potential of human placenta substrate (hpS), which is believed to have pro-vascularizing properties like *Matrigel*. *Matrigel* is a commonly used material in angiogenesis assays, since it supports vascularization. Unlike *Matrigel*, which is derived from Engelberth-Holm-Swarm sarcoma of mice[81], hpS is isolated of human placenta, which is regarded as medical waste.[82]

HpS was provided by the cooperation partner *TCC (Trauma Care Consult)* to investigate application possibilities, properties of the material and impact on endothelial network formation when incorporated into different hydrogels.

For this purpose, HUVEC (human umbilical vein endothelial cells) are used, which form capillary structures under certain conditions such as co-cultivation with mesenchymal stem cells or cultivation on materials which mimic the extracellular matrix. In initial experiments, properties of hpS should be investigated, as well as their impact on network formation. Next, potential gelatin-based biomaterials with incorporated hpS should be manufactured in 2D, expecting promoting vascular structures. Appropriate candidates should be further examined in 3D experiments. Additionally, an already functioning system, HUVEC in co-culture with ASC in fibrin hydrogels, should be compared with monocultures of HUVEC in hpS-fibrin hydrogels.

State-of-the-Art

Regenerative medicine (RM) aims to repair or replace damaged tissues and organs. Sufficient nutrient and oxygen supply, but also the transportation of waste products, presents a major challenge in RM. To overcome this problem intense research in vascularization and angiogenesis was performed in the last years. The ECM is used as a reference point for designing potential biomaterials.

A very common material used in basic research is *Matrigel*. *Matrigel* is a commercially available material, sourced from Engelbreth-Holm-Swarm mouse sarcoma cells. The exact composition of *Matrigel* is not known yet, but it contains important biological macromolecules like laminin and collagen, but also cytokines and different growth factors, which play a crucial role in vascularization and angiogenesis. Since it is sourced from a mice tumor makes its application in clinics difficult, but as well the ethical aspects must be considered.[83] As with many other biologically extracted materials, there is a relatively high batch-to-batch variability that affects reproducibility. It would be advantageous to find synthetic alternatives to ensure a consistent quality standard and ensure the availability of the materials.[84]

Already in the 1980s, the potential of the human placenta in RM was investigated. The entire placenta can be seen as a source of diverse materials for RM. Extracts and cells can be isolated from it, and the amniotic membrane as well as placental tissue are also utilized in research and medicine.[85] Cord blood cells are of great interest because they are rich in hematopoietic stem cells, which can be used as an alternative to bone marrow in stem cell therapy.[86,87] A variety of extracts can be isolated from the placenta, including growth factors and a wide range of proteins. The most clinically used components of the placenta so far are the amniotic and chorionic membranes. These are particularly applied in surgery, such as in ophthalmology, for the treatment of corneal diseases.[88,89] Human amnion was as well used to treat chronic leg ulcers. The results suggested that components of the amnion have a pro-angiogenic effect that can be used in wound healing.[90] It contains many important proteins that promote angiogenesis. This includes angiogenin, which plays a key role in angiogenesis, but components of the extracellular matrix (ECM) such as laminin, type IV collagen and heparin sulphate have also been discovered in the human placenta.[91–93]This precious organ has therefore become increasingly important in RMin recent years. Marc C. Moor et al. showed, for example, that the implantation of a human umbilical vein scaffold incubated in human placenta matrix leads to the formation of new vessels in vivo in mice.[94]

The placenta is an organ that is only formed and needed during pregnancy. After pregnancy, it is regarded as a medical waste product, but the great biological potential of this supposed waste product was already recognized in the 20th century.[91] On average, the placenta reaches a size of 20 cm and weighs 500 g. It can be divided into three parts, a chorionic plate, which lies towards the fetus, a basal plate, which is adjacent to the mother and an intermediate space, which is known as the intervillous space. The placenta also provides the connection between mother and child. All gas and nutrient exchange take place via this organ.[95,96] The placental disc also contains many substances that are typical for the ECM. This includes, for example, collagen, which can be isolated and subsequently used for therapeutic applications. Furthermore, biologically active substances such as VEGF or FGF were found in the placenta, which are primarily used to support vascularization and proliferation of cells.[97,98]

According to the pro-vascularizing properties of human placenta J. Hackethal et. al focused their research on human placenta and its proteins. As mentioned above, the human placenta is crucial for the survival of the fetus and supplies it with all necessary nutrients and at the same time disposes waste material. It is by now known, that the human placenta as well the umbilical cord besides their big amount of pro angiogenic factors, contain stem cells, which are of great interest for regenerative medicine. The team of THT Biomaterials GmbH uses the great potential of the placenta and process parts of it to a biological active solution called human placenta substrate (hpS, Figure 9).[82]

The use of hpS has several advantages. First, it is isolated from a natural waste product, which contains valuable nutrients and biological active molecules. This reduces the use of animals as a source for different products and thus also avoids animal suffering. On the other hand, model organisms and associated proteins cannot be directly translated to humans; consider porcine insulin and recombinantly produced human insulin. Parts of the placenta are already being used clinically in wound healing and are well tolerated.[99]

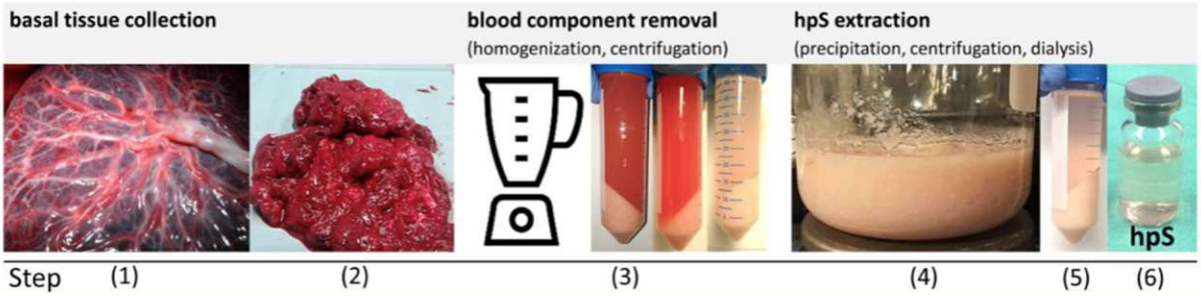

Figure 9: Processing of human placenta and extraction of hpS Illustration from literature.[100]

To produce hpS, the placenta's amniotic and chorionic membranes, as well as the umbilical cord, were removed. Additionally, clinically safe substances were used to obtain the hpS. Further analyses revealed that the substrate contains a wide range of proteins (Figure 10).[82]

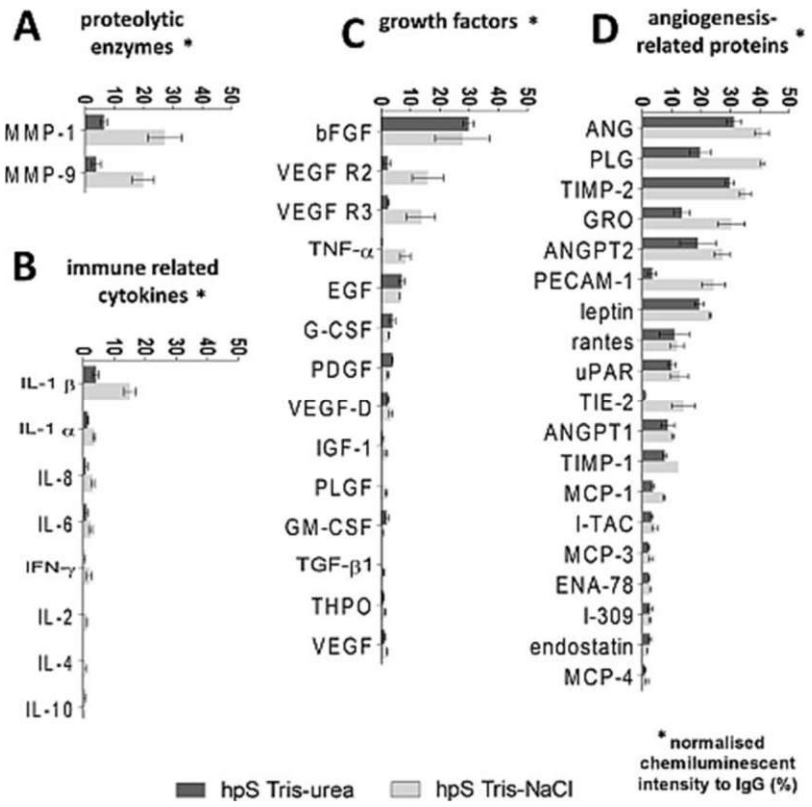

Figure 10: Component analysis of hpS
Illustration from literature [100]

Since hpS positively influences the network formation of endothelial cells, its use in various biomaterials could enhance vascularization. However, for practical application, hpS must be incorporated into hydrogels as the protein content is very low and hence no inherent crosslinking is possible.[101]

Gelatin-based hydrogels are widely used in the food industry and in RM. Gelatin-based scaffolds have broad applications in RM, including cartilage regeneration, drug delivery, and promoting neovascularization.[102] Gelatin consists of chains of repeating Gly-X-Y sequences, where X and Y are typically proline and hydroxyproline. The physical properties of gelatin can be influenced by factors such as temperature and pH. [103] Additionally, the properties can be modified through functional groups. A well-known example of this is gelatin methacryloyl (Gel-MA).[104] This material exhibits high biocompatibility and is also suitable for bioprinting applications.[105]

To produce gelatin-based hydrogels in combination with hpS, specialized methods are required. Glutaraldehyde is used for the crosslinking of protein-containing materials. This is achieved through the formation of Schiff bases with amino groups of the

proteins, resulting in crosslinking.[106] Furthermore, by incorporating functional groups into gelatin, thiol-ene chemistry can be utilized for crosslinking.[107]

Another material often used in biomedicine are fibrin constructs, like fibrin glue. Fibrin, derived from fibrinogen, is a natural component of human blood coagulation. Several studies have shown that endothelial cells, in combination with co-cultures such as adipose-derived stem cells (ASC), form network-like structures in fibrin hydrogels. As described by J. Hackethal et al., hpS also has a positive effect on network formation in endothelial cells such as HUVEC. Incorporating hpS into a fibrin matrix could result in a well-tolerated and promising bioink.[82]

As evident from current data, the possibilities for different biomaterials are almost limitless. By modifying components and production methods, potential bioinks can be developed that could become highly valuable in RM in the future.

General Part

Results and Discussion

1 HUVEC on Matrigel

HUVEC are very popular in research because they are easy to cultivate and come from a medical waste product - the umbilical cord – and the isolation of the cells has already been described in many papers.[108,109] If HUVEC are cultivated in an uncoated cell culture flask, they form their typical cobblestone structure (see Figure 11A). HUVEC are frequently used in in vitro studies of angiogenesis and vascularization. They can form network-like structures under certain conditions. For example, if they are cultivated on materials that mimic the ECM, like *Matrigel*, they form capillary structures (Figure 11B).

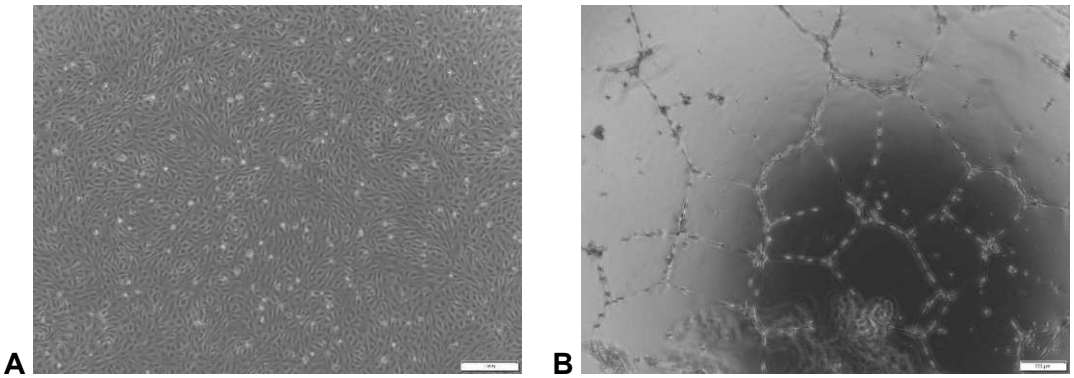

Figure 11: HUVEC cultured on different materials
(A) HUVEC cobblestone structure (B) HUVEC cultivated on Matrigel, formation of networks

Matrigel is a substance obtained from a murine tumor (Engelbreth-Holm-Swarm mouse tumor). The exact composition of this matrix has not yet been fully clarified, but it was discovered early that the protein composition is like those found in the basement membrane.[110]

Besides chemical and mechanical factors, like stiffness of a hydrogel, cell density also plays a crucial role in the formation of capillary structures. On softer hydrogels stronger cell-cell-adhesion than cell-hydrogel-adhesion was observed. This results in a slower migration of cells on softer gels and an optimal seeding density must be estimated, since with higher seeding density network formation is inhibited.[111]

To determine an optimal seeding density of HUVEC for 2D experiments, wells were coated with *Matrigel* and seeded with cell densities between 35,000 and 85,000 cells / cm² (Figure 12). As expected at lower seeding densities cells did not form networks

and at higher seeding density cells aggregated and built clusters of monolayers, which were interconnected to each other. According to the results, a seeding density of 45,000 to 48,000 cells / cm² was used for all further 2D experiments. These results were also supported by further analysis using Angitool.[112] The results obtained are comparable with those from previous studies and show that excessive cell densities inhibit vascularization and network formation.[111,113]

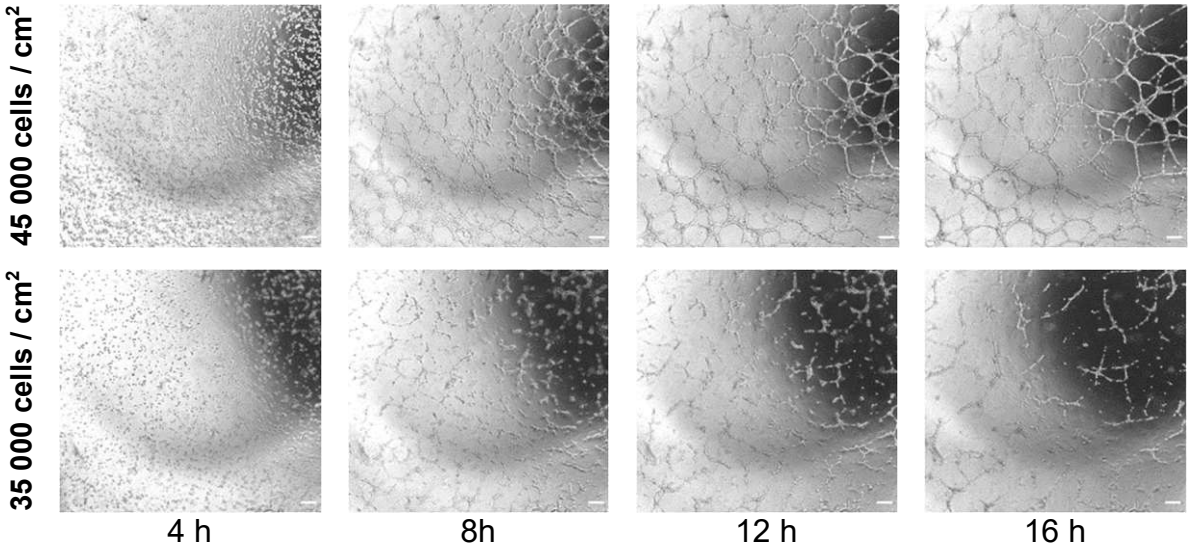

Figure 12: HUVEC over time.
Lower seeding density led to no / incomplete networks (35 000 cells / cm2). First networks can already be observed after 4 hours. Scale bar: 200 µm

2 HUVEC on human placenta substrate (hpS)

Human placenta substrate (hpS) is a substance obtained from the human placenta that is known to have pro-vascularizing properties. Like *Matrigel*, the material consists of important components of the basement membrane, including laminin-111, collagen IV and growth factors.[82]

As the provided hpS is a non-commercial product and no quality control testing was performed before, batch-to-batch variance was analyzed. Two different batches were provided by Johannes Hackethal, which were named hpS1 and hpS2 in the following chapter. A visual inspection revealed a difference in color and turbidity (Figure 13,iii). As the hpS originates from different donors and the substance is raw material that has not been fully purified, such color differences may occur. Occasionally, non-soluble particles were also detected within a batch in different aliquots. Based on the production of hpS, these particles were identified as residual placental tissue.[100] Turbidity might be explained by potential bacterial contaminations or as residual placental tissue and blood. The used hpS was sterilized for 30 minutes with UV-rays so that a contamination within the experiments could be avoided.

The lots were also analyzed within a cell culture-based assay in which the hpS-batch, incubation time of the coating with hpS and cell densities were varied. Clear differences in the network formation and morphology of the cells could be found between the two lots. While the hpS1 promotes network formation (Figure 13,i), it appears that the biological activity of the hpS2 has been lost or the substrate itself has a negative effect on the cells (Figure 13,ii). No networks could be observed, as well almost all cells detach from the surface and were present in aggregates. Performing live/dead staining led to complete detachment of the cells, even with experiments using hpS1, but in this case the network remained stable even in suspension. The results clearly show that there can be major differences between two different batches - while HUVEC networks formed in the hpS1-batch and the wells are comparable with each other, no consistent results could be achieved with the hpS2-batch. Cells aggregated very strongly and there was little or no network formation. For this reason, it is recommended to pool several lots in the future to minimize the variance between individual donors. Based on the results obtained, only the hpS1 was used for further experiments.

After the biological activity of the hpS1 was confirmed, different coating incubation times and seeding densities were as well investigated. Networks were formed in all samples regardless of whether the plates were previously incubated with hpS for 2 h or not. However, those samples in which the hpS was previously incubated for 2 h showed more stable networks, but regardless of the incubation period, the cells detached from the culture surface during further processing. It made no difference whether a cell count of 15,000 or 20,000 cells/well was used - in both cases, network formation could be observed.

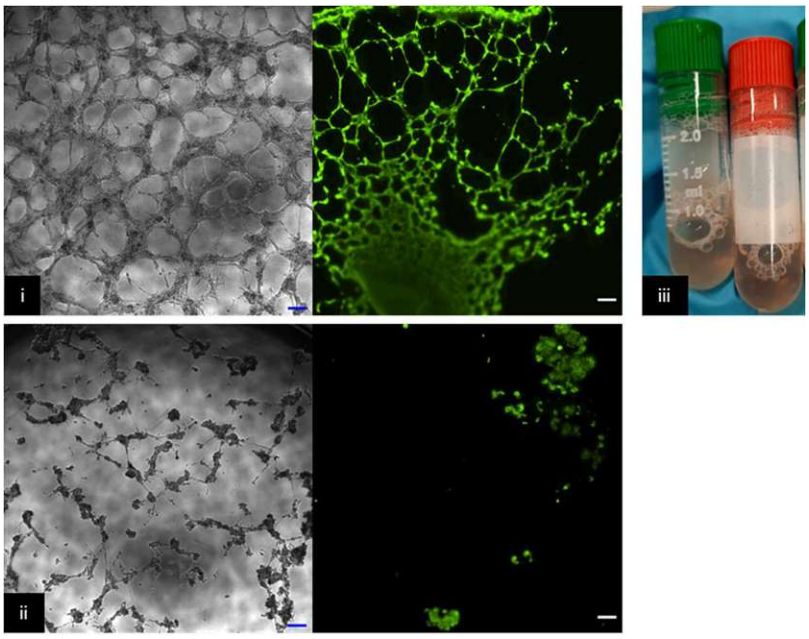

Figure 13: Different hpS-batches.
cultivation of HUVEC on two different hpS batches (i) hpS1-batch (ii) hpS2-batch. (iii) left "hpS2, right hpS1. Scale bar 200 µm

Unlike *Matrigel*, hpS remains liquid even at higher temperatures and does not gel. The main components of *Matrigel* are laminin, collagen IV, entactin and perlecan, which can also be found in the basement membrane. In addition, it contains a variety of growth factors such as transforming growth factor (TGF) or fibroblast growth factor.[114,115] Protein interactions within *Matrigel* lead to gelation at temperatures above 10 °C. As in *Matrigel*, collagen IV and laminin are major components of hpS.[100] The exact composition of hpS is not yet known, but it is considered, that the hpS composition affects the gelling process.

The experiments demonstrate that hpS supports network formation, but the liquid state of the material leads to the collapse of these networks with even slight movements, thus complicating further handling. As displayed in previous results, hpS cannot be solely used as gel-forming material. However, due to its biological activity, it is a promising candidate for incorporation into various hydrogels supporting network formation and vascularization.

3 Influence of hydrogel components on cell viability and metabolic activity

Gelatin-methacryloyl (Gel-MA) is a semi-synthetic hydrogel, utilizing gelatin as a base structure modified with methacrylamide and methacrylate groups.[116] Based on promising findings in literature[117,118], Gel-MA hydrogels using LAP (0.1% (w/v)) as a cross-linker were produced and observed over an 18-hour period. HUVEC attached on the hydrogel after several hours; however, over time, the cells detached completely from the hydrogels and aggregated, regardless of whether the Gel-MA hydrogels contained hpS or not.

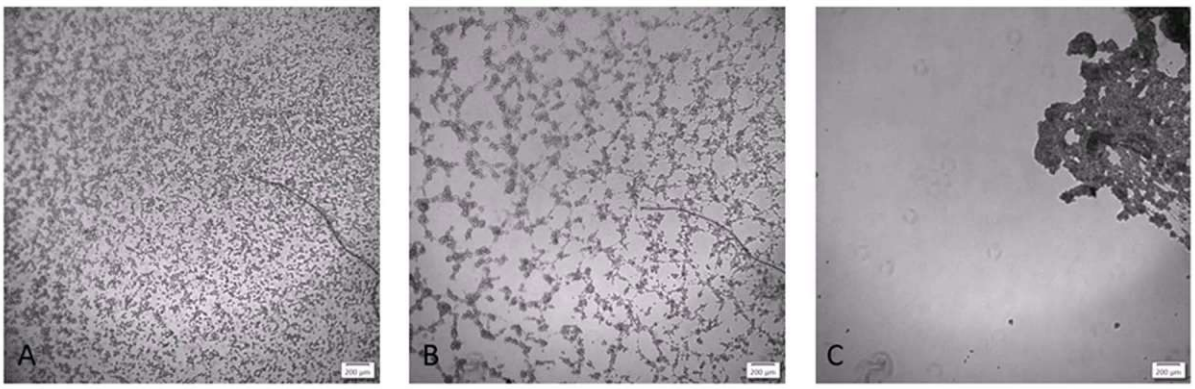

Figure 14: HUVEC on Gel-MA (5wt%, 0.1% LAP, 50% hpS)
(A) Directly after seeding with HUVECs. (B) 4 hours post seeding. (C) 8 hours post seeding.

The obtained results suggested that one or more components of the hydrogels may have a negative, if not cytotoxic, effect on the cells. Based on this, all components of the formulation were individually tested to investigate their influence on the cells using

Live/Dead staining and Tox-8 assays. The solvent (DPBS), the macromer (Gel-MA) in different concentrations, and the LAP photoinitiator (irradiated and non-irradiated) were investigated separately. These components were added individually in equivalent concentrations to those cultured HUVEC in the wells, considering the final volume of the medium as well.

DPBS was used as a diluent in all experiments and had been previously examined for its impact on cell viability and metabolic activity. As expected, DPBS had no negative effect on the cells, since it is a common buffer used in cell culture.

In the next step, three different concentrations of Gel-MA macromer and the PI itself (irradiated vs. non-irradiated) were tested. The varying macromer concentrations showed no negative impact on the cells compared to the control group. In all three cases, the relative metabolic activity, which can be correlated with viability, remained about 100%. Therefore, no further studies were conducted regarding the macromer concentration.

Xu et al. investigated the impact of LAP and Irgacure 2959 on cell viability, finding that LAP, compared to I2959, was less cytotoxic at higher concentrations[119]. Since the specific cells used in Xu et al. experiments were not precisely known, both photoinitiators were tested at different concentrations. Two versions of the PI solutions were prepared: in the first, the PI was dissolved in DPBS and added to HUVECs without radiation; in the second, the PI was irradiated according to the experimental plan for 2 minutes before being added to the cells. At least the impact of two different PIs were investigated – LAP and I2959. Non-irradiated LAP showed minimal impact (relative viability at approximately 80% regardless of concentration) on the cells. The irradiated LAP PI at a concentration of 0.1% (w/v) clearly demonstrated that all cells detached from the surface, with no living cells detected (Figure 13A). Additionally, the assessment of metabolic activity revealed that even at half the concentration (0.05% (w/v)), no metabolic activity of the cells can be measured. Only at a concentration of 0.025% (w/v) did the relative viability reach approximately 80%.

Irgacure 2959, even the non-irradiated, exhibited a cytotoxic effect at a concentration of 0.5 % (w/v), reducing relative viability to below 40%. Samples exposed to this concentration of PI showed no detectable metabolic activity. The next lower concentration of 0.25% (w/v) displayed relatively high metabolic activity (Figure 15B).

Selection of a suitable PI is essential when producing a bioink. Irgacure 2959 (I2959) and LAP (lithium phenyl-2,4,6-trimethylbenzoylphosphinate) belong to type I photoinitiators and are water-soluble. Both photoinitiators are considered as biocompatible and suitable for bioprinting.[69] Here, the results show clearly that PI concentration plays a crucial role in metabolic activity and cell survival. Comparisons of I2959 with LAP, showed that I2959 is less soluble in water and the duration of light exposure for stable gelation of Gel-MA hydrogels must be increased.[68]

To investigate the direct influence of the PI concentration on the stability of the hydrogel four Gel-MA hydrogels were produced using the previously tested LAP concentrations.

Cells were seeded and imaged after 24 hours. At the highest concentration, the same pattern was observed as in the previous experiment. Most cells detached, and those remaining on the gels formed large cell aggregates, loosely adhering to the gels. The lower concentrations did not negatively affect cell viability. However, the gel with a PI concentration of 0.01% (w/v) did not fully polymerize and did not form a stable hydrogel. Based on these results, LAP concentrations of 0.025% (w/v) were chosen for further experiments, as they exhibited no negative effect on cell viability and still formed a stable hydrogel.

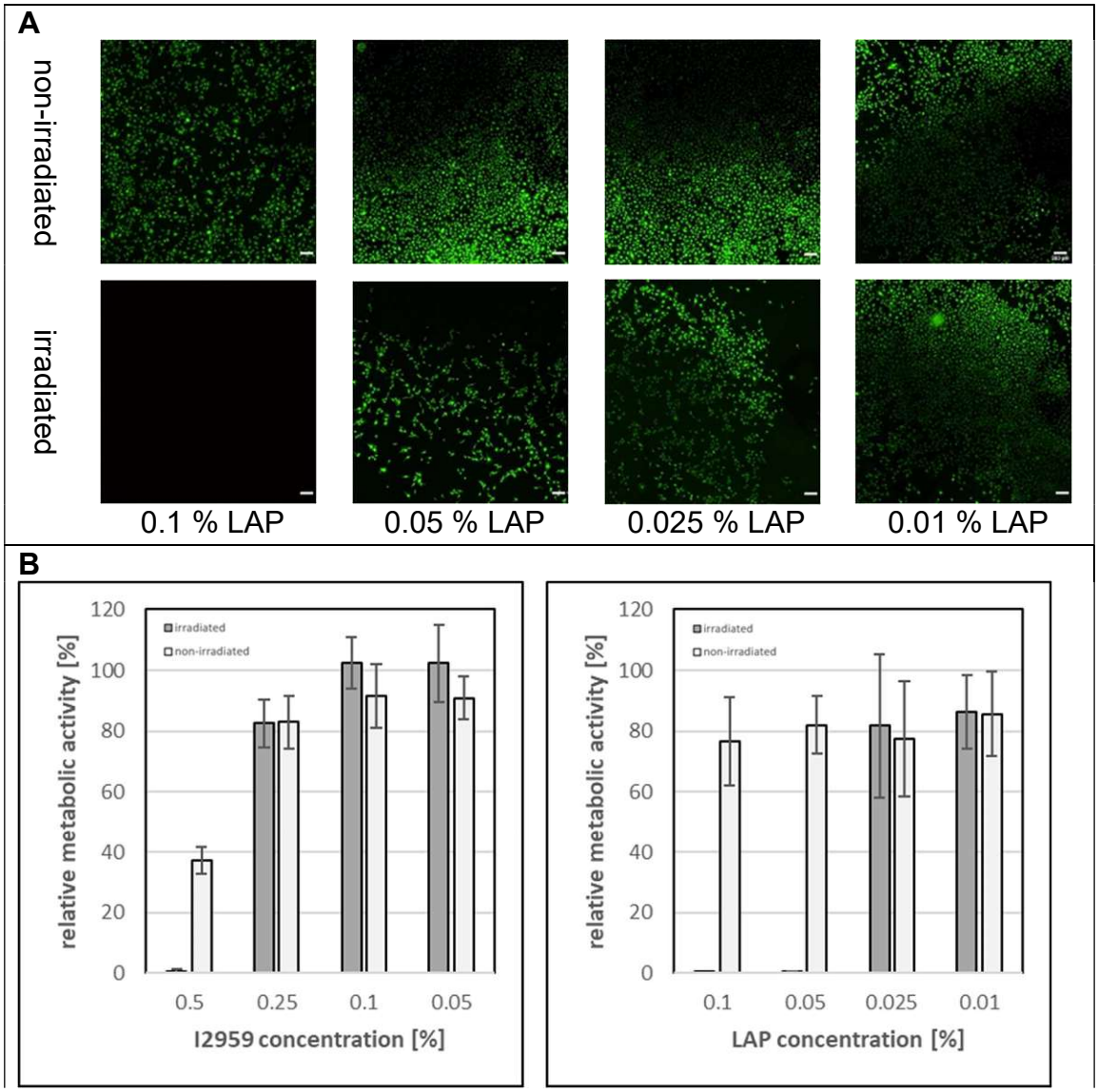

Figure 15: Investigation of individual component of Gel-MA hydrogel formulation.

(A) Live/Dead staining 2 days post seeding with different LAP concentrations (irradiated/non-irradiated). Scale bar 200 µM. (B) Results of Tox-8 Assay, metabolic activity in different PIs (Irgacure and LAP)

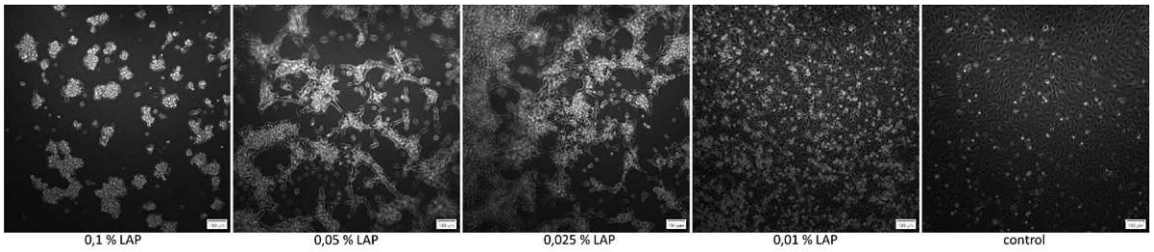

Figure 16: Gel-MA hydrogels with different LAP concentrations. Control on empty well without any coating.

4 Different gelatin-based hydrogels in 2D

Adding functional groups to gelatin-based hydrogels makes them a more versatile material in regenerative medicine. According to that different hydrogels were prepared. All formulations of the gels were prepared by Raffael Reichsöllner.[101] The prepared gels were prepared both with and without incorporated hpS to analyze the influence of hpS on network formation. Additionally, gels without incorporated hpS were coated with hpS to investigate the impact of hpS as external additive. An overview of all tested gels is displayed in Table 2. For all gels a final LAP concentration of 0.025 % (w/v) was used and the irradiation time was expanded to 3 minutes per gel.

Table 2: Overview of prepared hydrogels

	without hpS	incorporated hpS (50 vol%)	coated with hpS
Gel-MA 5 wt%	+	+	+
Gel-MA 10 wt%	+	+	+
Gel-MA 10 wt%, pre-cooled [1,]	+	+	+
Gel-MA 5wt% + PVA NB (DS=7%)/DTT (thiol ene =0.8) 5wt%	+[2,]	+	+[2,]
PVA-NB (DS=7%)/DTT (thiol ene =0.8) 10wt%	+	+	+
Gel-NB (DS=0.405 mol /g)/DTT +"Trap6M" (SFLLRNPNC) thiol ene =0.8 for DTT, thiol ene =0.2 for Trap6M)	+	-	-
Gel-NB (DS=0.405 mol /g)/DTT thiol ene =1) 10wt%	+	+	+
Gel-Norb (DS=0.207 mol /g)/DTT thiol ene =1) 10wt%	+	- [3,]	+
Gel-AGE (DS=1.508 mol /g)/DTT thiol ene =1) 20wt%	- [4,]	- [4,]	- [4,]

[1,] 40 minutes pre-cooling on 4 °C before UV-crosslinking
[2,] hydrogel polymerized, but detach from glas slide
[3,] no homogeneous formulation obtained after several hours of incubation at 37 °C, no hydrogel prepared
[4,] UV-irradiation extended to 6 min, no gelling

It has already been shown that HUVEC form capillary structures when co-cultured with mesenchymal stem cells (MSC) in Gel-Norb hydrogels (gelatin with norbornene groups), like those in *Matrigel*.[120] Based on the promising results in literature, this hydrogel was also examined in 2D experiments, but without using co-cultures to investigate the impact of hpS on network formation. Unfortunately, no homogenous formulation could be obtained, thus it was not selected for further experiments in 3D. HUVEC attached onto Gel-Norb hydrogels and built a monolayer, while on hpS-coated

Gel-Norb hydrogels networks can be observed, which would make them to a promising hydrogel, if an incorporation of hpS would be possible. Like other hydrogels, Gel-AGE and PVA NB (in combination with or without Gel-MA) have properties which are suitable for RM. Incorporation of RGD motifs for example support cell adhesion. TRAP6 (thrombin-receptor-agonist-peptide 6) can interact with platelets and subsequently activate them, which accelerate hemostasis. This makes gelatin-based hydrogels like Gel-NB functionalized with TRAP6 to promising candidates for RM.[101,121,122] As displayed in Table 2 many materials detached from glass side (Gel-MA + PVA NB) or even did not form a stable hydrogel (Gel-AGE). No cell attachment occurred in synthetic/hybrid hydrogels such as (Gel-MA)-PVA-NB. Hence, these hydrogels were also excluded from further tests. Gel-NB differs from Gel-Norb due to the presence of ionic carboxylate groups (Figure 17). Raffael Reichsöllner showed that hpS can be incorporated into Gel-Norb and Gel-NB hydrogels, but due to batch-to-batch variance no reproducible results could be achieved, and these hydrogels were as well excluded from further testing.[101]

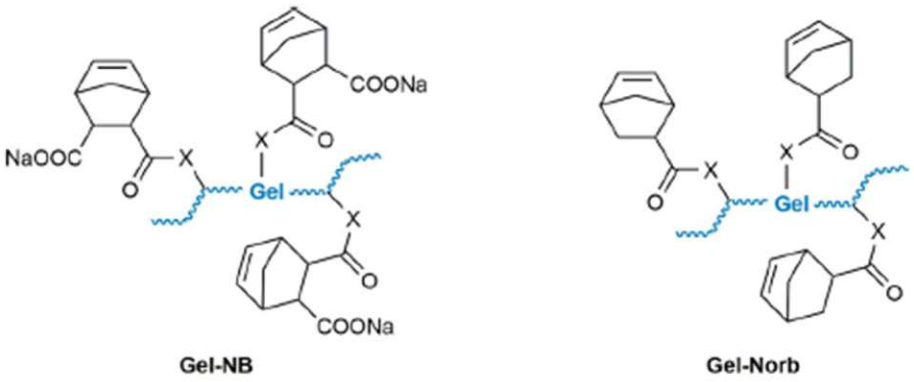

Figure 17: Structure of Gel-NB and Gel-Norb[101]

Since Gel-MA hydrogels achieved the most promising results, for further investigations the focus was placed on Gel-MA hydrogels. Gel-MA is a methacrylated gelatin, that is often used in biomedical applications. Gelatin is denatured collagen, which can be found in the ECM. Like many other ECM macromolecules collagen and gelatin have RGD motifs, which support the interaction of cells with the ECM. Biocompatibility and low costs in production, makes Gel-MA an attractive biomaterial.[123] If necessary, properties of Gel-MA hydrogels can be easily changed by varying macromer or PI concentration. As displayed in Figure 16 PI concentration influence gelation process and stability of the hydrogel.

Stiffness influences cell behavior such as migration and proliferation. For example, it has been shown that a Gel-MA substrate concentration of 10 % is ideal for the nerve regeneration of PC12 cells.[124] In another study, a stiffness gradient (increasing macromere concentration) was established within Gel-MA hydrogels where HUVEC

and MSC were incorporated. It was found that the proliferation of HUVEC reaches an optimum at a stiffness of 27.7 to 125.6 Pascal.[125] The stiffness of hydrogels can be further increased, for example by dual crosslinking. This involves initially inducing physical gelation by cooling at 4 °C and then producing a hydrogel using UV crosslinking.[101]

According to this, three distinct hydrogels were prepared: Gel-MA 5wt% - Gel-MA 10 wt% - and cooled Gel-MA 10 wt%, to investigate cell behavior. Figure 18 shows that there are clear differences between gels with/without incorporated hpS, but also at different stiffness degree. Regardless of whether hpS is incorporated or not, hydrogels with larger substrate content, and consequently greater stiffness, HUVEC tend to form a monolayer (Figure 18). This was as well observed with other endothelial cells on Gel-MA.[126] This suggests that HUVEC tend to proliferate with higher macromer concentration. One possible explanation is the larger amount of RGD motifs, which are known to support cell adhesion. Due to the confluency of the cells, it is difficult to guarantee that hpS has or does not have an impact on cells, but it shows clearly that stiffer hydrogels support cell attachment and growth.

In contrast, hpS in Gel-MA 5 wt% influences the cell behavior. Hydrogels without hpS exhibited numerous cell aggregates, and cell detachment from the gel surface could not be excluded. In hydrogels with incorporated hpS, HUVECs also formed aggregates, but these were interconnected by a network. This observation supports the hypothesis that hpS possesses pro-vascularizing properties.[82] The results obtained are also consistent with results from literature: softer gels, such as Gel-MA 5 wt% in this case, support network like structures, whereas stiffer gels promote the formation of monolayers.[127–129]

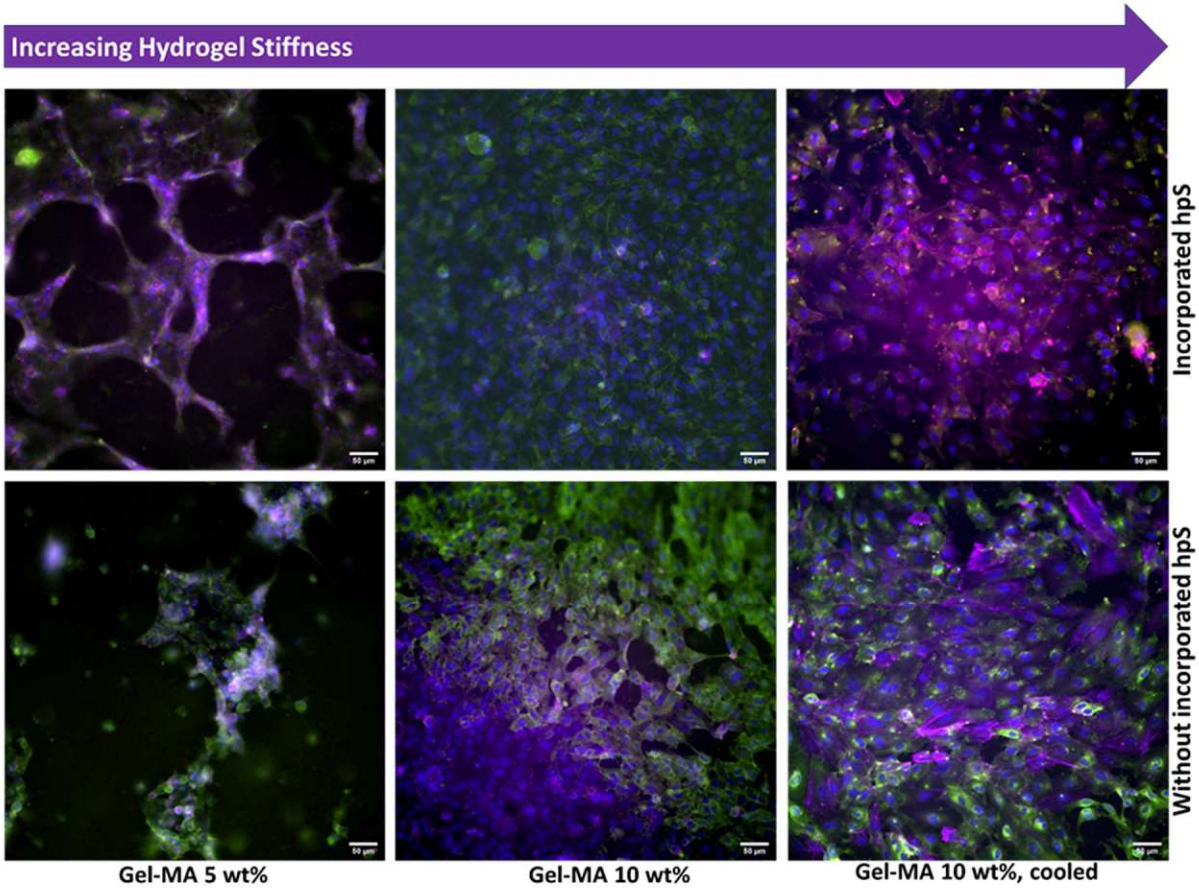

Figure 18: Impact of stiffness on cell behavior

HUVEC seeded and two days cultivated on Gel-MA hydrogels (with/without incorporated hpS) with increasing stiffness. Immunostains: DAPI-blue, F-actin-red, VE-cadherin-green

5 3D Gel-MA hydrogels

A major goal in RM is to establish a bioink suitable for biomedical applications. All prior experiments focused on the influence of components of the biomaterials, but also on their composition. As a next step, it is necessary to investigate cell behavior in 3D hydrogels. Since the previous results showed that Gel-MA 5 wt% yielded the most promising outcomes regarding network formation, it was selected for further experiments in 3D.

Biologically active materials play a key role in tissue engineering and present one of the three pillars of regenerative medicine. In previous experiments, hpS was used as the biologically active component, containing important growth factors and elements of the ECM.[82] But previous research on vascularization showed that there is a possibility to initiate cell proliferation and network establishment by co-culturing with adipose derived stem cells (ASC). ASC support cell growth and secrete different pro-angiogenic factors like VEGF. [130–133]

In following experiments, the focus was placed on two biological active components, which support network formation – ASC and hpS. During the cultivation of the hydrogel constructs the cells migrated from the middle to the outer regions of the gel and dynamic structures in peripheral regions were observed. Immunohistochemical staining showed that these network-like structures were built by ASC and not HUVEC, because the used antibody (VE-Cadherin) is typically binding to endothelial cells like HUVEC. The two groups (Gel-MA with/without incorporated hpS, cultured with ASCs and HUVECs) do not exhibit remarkable differences. Cells migrated from the outer regions of the gel onto the well plate surface establishing a monolayer (Figure 19). Potential influence of insufficient nutrient supply was ruled out by further experiments with decreased gel "thickness". The cells were encapsulated in thin cylindrical hydrogel layers with a height of approximately 300-400 µm instead of a droplet hydrogel. Even then cell migration out of the gel occurred. The diffusion limit of oxygen in tissues is 100-200 µm.[134] By adjusting the thickness of the gels, the cells should have been supplied with sufficient oxygen, but cells still migrated out of the gel and settled on the culture surface of the well. This suggests that this phenomenon occurs due to another fundamental problem. Culturing HUVECs over a longer period (up to 18 days) led to re-migration of the cells into the gels. When nearly a confluency of 100 % was reached, cells started migrating back into the gel (only at 2D-level). Within the gel (3D), cells remained spherical without any connections to other cells and without live signal, assuming they were dead.

No significant networks (originating from HUVEC) could be detected in all samples, regardless of whether hpS was incorporated or not. As described above, the selection of a suitable PI can influence cell behavior. In addition to the general toxicity within the formulation, the formation of radicals and metabolites must also be considered, which can also influence cell viability. Nevertheless, no precise explanation for the migration and death of the cells can be found based on the results obtained. Since Gel-MA has previously been shown to be a reliable material in 2D experiments, additional investigations are needed to identify the underlying causes, but these are not part of this thesis.

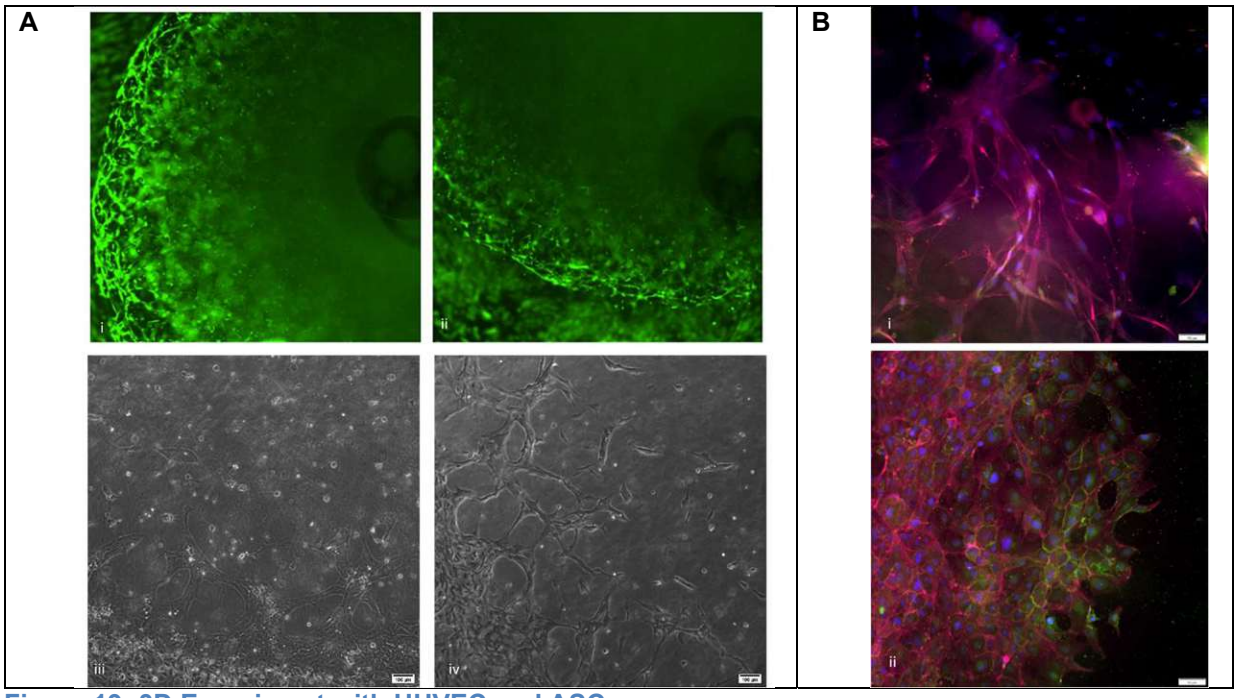

Figure 19: 3D Experiment with HUVEC and ASC.
(A) Live Staining with Calcein AM; 3 days post seeding. (B) Immunohistochemical staining; VECadherin (green), Phalloidin (red), DAPI (blue); 7 days post seeding. (A) i and iii: Gel-MA without incorporated hpS with HUVEC-ASC co-culture (A) ii and iiv: Gel-MA with incorporated hpS with HUVEC-ASC co-culture. (B) i: Gel-MA without incorporated hpS, Co-Culture of HUVECs with ASCs; HUVEC express VECadherin (green) while ASCs not. ii: Same sample, different spot. HUVEC migrated out of the gel and built monolayer onto glass slide

Cheng et al. developed a comparable experimental set-up in which HUVEC were embedded as a co-culture with ASC in a Gel-MA hydrogel (10 % w/v), cultured for 21 days and then implanted into a mouse. Their experiments demonstrated that this hydrogel is a promising candidate for future applications in vivo, since vascular networks can be observed in vitro but as well in vivo.[135]

This raises the question of why we observed different results. Cheng et al. used a different photoinitiator – VA-086. VA-086 is an azo polymerization PI that can be activated by blue-light. It is considered to be less toxic compared to alternatives like Irgacure 2959.[136,137]

6 3D Fibrin-hpS Hydrogels

An alternative to gelatine-based hydrogels are fibrin hydrogels, which are already widely used in traumatology. Fibrin plays an important role in tissue recovery and wound healing. Therefore, it is a common material used in trauma care, especially as fibrin glue.[138] The principle of fibrin glue is based on natural secondary hemostasis (Figure 20A). In case of injury a series of coagulation factors are activated via the intrinsic or extrinsic pathway. Genetic defects that affect the coagulation factors lead to diseases such as hemophilia.[139]

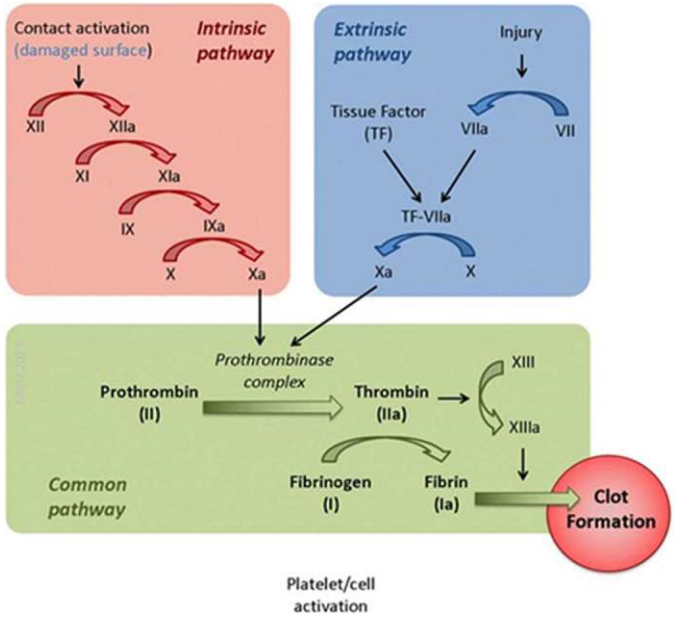

Figure 20: Principle of hemostasis - Coagulation Cascade. Illustration from literature[140]

The natural formation of fibrin hydrogels was used for further experiments. Fibrinogen is a naturally occurring glycoprotein composed of two D domains and one E domain. Thrombin cleaves fibrinopeptide A (FpA) and fibrinopeptide B (FpB) from fibrinogen, allowing interaction of E- and D-domains, leading to a formation of a fibrin polymer. Factor XIIIa then facilitates further cross-linking, resulting in the formation of a stable fibrin polymer.[138,141,142]

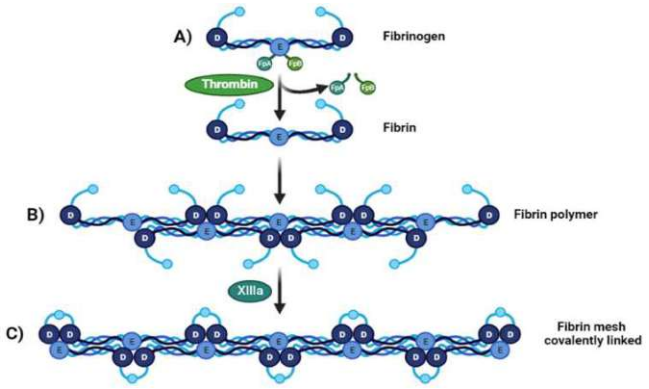

Figure 21: Schematical Figure of Fibrin Polymerization
Illustration from literature[141]

Fibrin exhibits excellent biocompatibility within the human body, making it a promising candidate for further applications as a biomaterial in tissue engineering beside fibrin glue. Additionally, fibrin scaffolds are naturally degradable during the healing process. In literature two approaches of possible applications of fibrin-based scaffolds are investigated. First, the modification of fibrin scaffolds resulting in organ specific properties. Second, mimic fibrin properties on synthetic materials, achieving hydrogels with ECM-properties, but reproducibility is given.[142]

As previously described, HUVEC can alter their behavior and form vascular structures. Previous studies showed that fibrin hydrogels support network formation of HUVEC when co-cultured with mesenchymal stem cells (MSC).[143,144] MSC can be found in adults and can differentiate into different types of cells like adipocytes or chondrocytes. According to this they play a crucial role in different divisions of RM like neural regeneration, wound regeneration and many more. [144,145]

Due to the versatile applications and excellent biocompatibility of fibrin in humans, this study investigated the interaction of fibrin-hpS hydrogels with endothelial cells. For all fibrin experiments GFP-labeled HUVEC were used, unless otherwise described. Initially, the influence of different compositions of fibrin hydrogels were investigated. Therefore, fibrinogen and hpS concentration was varied, and the impact of thrombin on hydrogel formation was investigated. As displayed in Figure 21 thrombin plays a crucial role in fibrin polymerization. It cleaves FpA and FpB from fibrinogen, producing fibrin monomers that are able to establish fibrin polymers. [141] Thrombin concentration affects not only the structure of the fibrin clot but also the time required to form a stable clot.[146] As thrombin concentration increases, time of polymerization reaction increases as well. In this study a thrombin concentration of 2 U/mL was used. In comparison, in fibrin glues, like TISSEEL, a concentration of 500 U/mL is used to achieve fast wound closure.

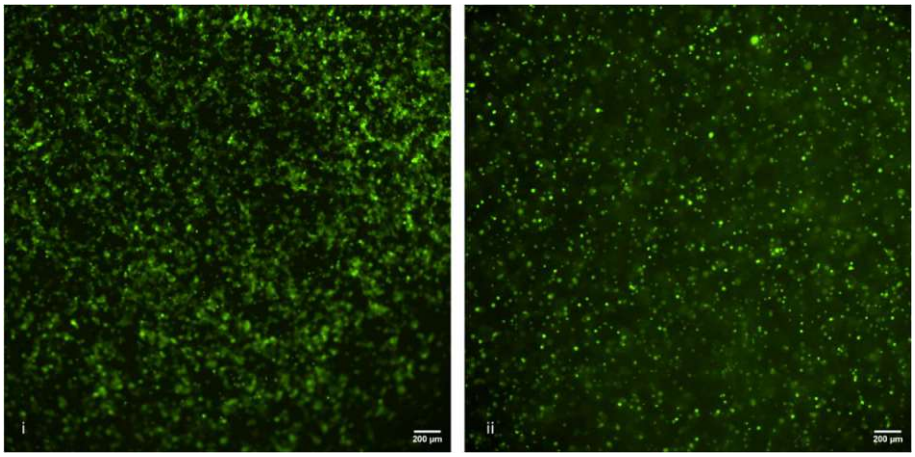

Figure 22: Distribution of GFP-HUVEC in fibrin hydrogel right after gelation. (i) fibrinogen 2.5 mg, 50 % hpS, gelation process finished after about 2 hours (ii) fibrinogen 2.5 mg, 25 % hpS, 25 % thrombin, gelation process finished within seconds

Right after the gelation process was completed, images of the samples were taken (Figure 22). Hydrogels without thrombin remain soluble right after the production of the formulation, incubating them for about 2 hours at 37 °C results in a full gelation of the hydrogel. In comparison, hydrogels with included thrombin formed within seconds a stable hydrogel. In both hydrogels cells were imaged right after gelation. In hydrogels without thrombin cells settled down, while in hydrogels with thrombin a homogenous cell distribution was observed. This suggests that gelling time takes too long. As displayed in the coagulation cascade, thrombin is a key factor for the stable formation of a clot.[138] Under certain conditions, such as the addition of specific magnesium salts or by decrease of pH and subsequent incubation at 37 °C, fibrinogen can also form stable hydrogels.[147,148]. As already observed in previous 2D experiments, hpS remains liquid at 37°C. Since human placental substrate is derived from native tissue components, it can be assumed that hpS also contains a small amount of thrombin, which would explain the formation of a gel, when mixed with fibrinogen.

After an incubation period of 5 days, the samples were examined again under the microscope. Within the group without thrombin, no re-migration into the hydrogels was observed and cells remained in one plane. Nevertheless, GFP-HUVEC arranged them into networks, which proves biological activity of hpS. In contrast, fibrin hydrogels, produced with thrombin, showed a homogeneous distribution of cells within the gels and an incipient formation of networks can be observed, which is not fully developed compared to gels without thrombin (Figure 23). This can be attributed to the reduced amount of hpS used but as well to reduced cell density in terms of the area. In the control group (co-culture of GFP-HUVEC with ASC), fully developed networks were established after 5 days. In all samples, no significant difference in cell behavior was observed regarding different fibrinogen concentrations. As described in literature, fibrinogen concentration can be varied, depending on the purpose of use.[149,150] Since

no significant differences were detected in previous experiments, a fibrinogen concentration of 2.5 mg/clot was used for further experiments and thrombin is added to achieve faster polymerization leading to homogenous distribution of the cells.

In another experiment, the hpS concentration in fibrin hydrogels was increased to 50 % (v/v) with embedded HUVEC. Since thrombin was used for polymerization a homogenous cell distribution within the hydrogel was achieved and network formation was observed (Figure 24, iii and v). In Control (HUVEC in co-culture with ASC) HUVEC established as well networks (Figure 24, i and iv), and in Fibrinogen-hpS gels produced without thrombin, as shown in the previous experiment, network formation remained stable after even 7 days of culturing (Figure 24, ii). A comparison of the fibrin hydrogels shows a clear morphological difference in HUVEC between the control and fibrin-hpS gels. In Control, HUVEC built capillary structures, which have been already observed as well in other materials like collagen or *Matrigel.*[151,152]

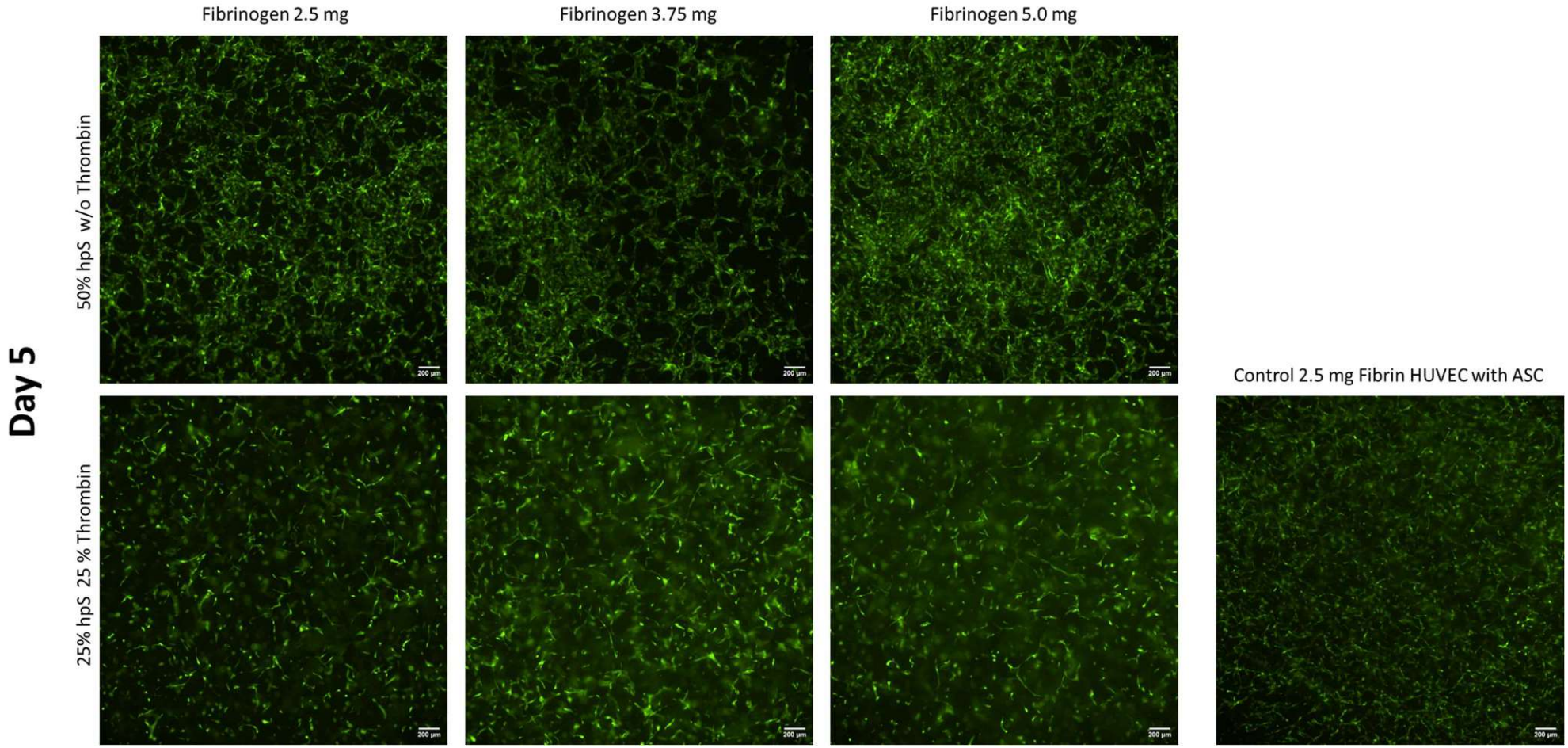

Figure 23: Different fibrinogen-based hydrogels in 3D.
Influence of fibrin hydrogel composition on GFP-HUVEC cell behavior 5 days post seeding. Fibrinogen was examined in concentrations of 2.5 mg, 3.75 mg and 5.0 mg, as well as the presence of thrombin and the hpS concentration (25 % and 50 %). Control: Co-Culture of GFP-HUVEC with ASC.

In contrast, in fibrin-hpS hydrogels HUVEC networks are formed but failed to achieve capillary-like structures. Instead, the cells aggregated into smaller colonies that were interconnected by networks, which has been already observed in 2D experiments with hpS.

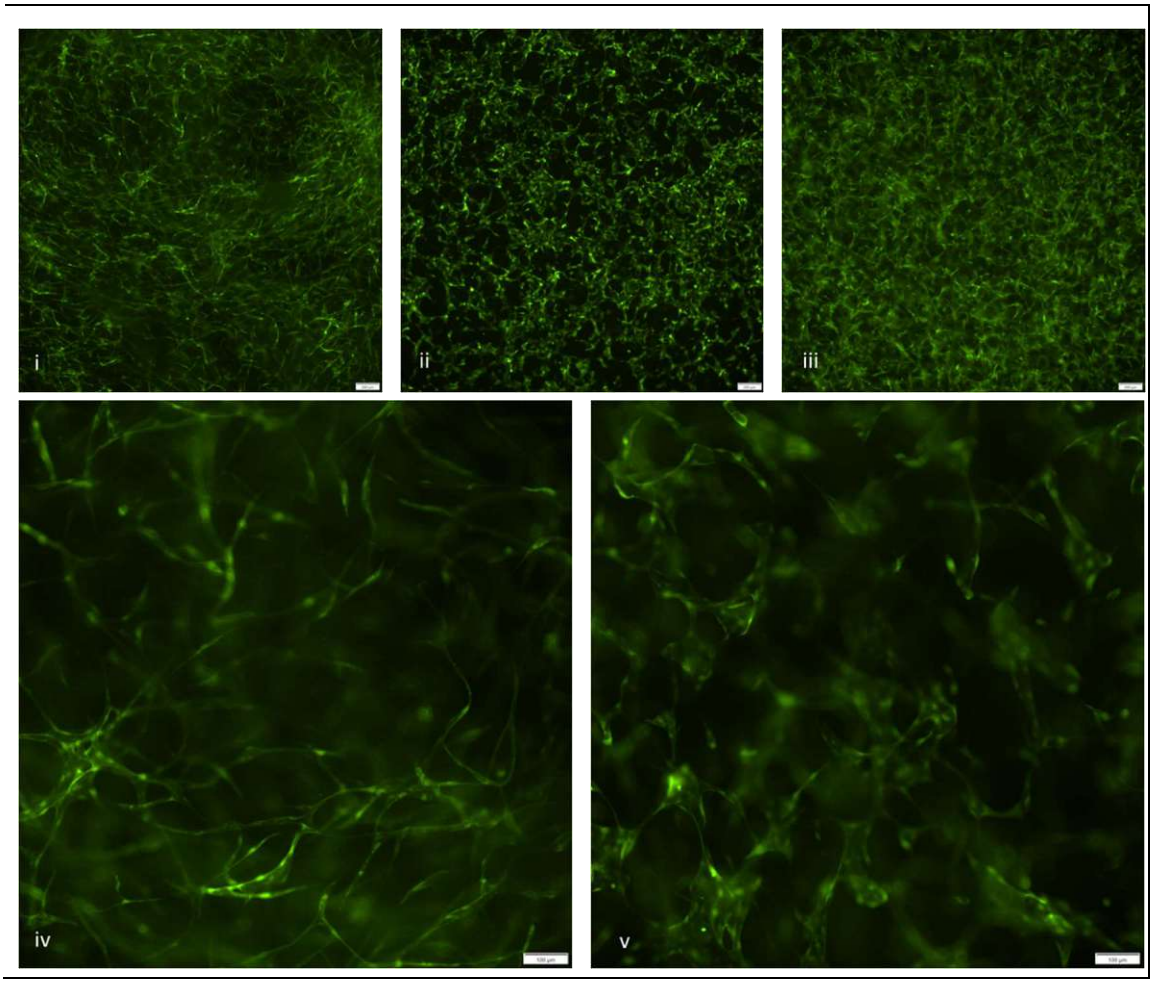

Figure 24: GFP-HUVEC 7 days after seeding in different fibrin hydrogel set-ups. (i) fibrin hydrogel (2.5 mg fibrinogen, 50 % thrombin) GFP-HUVEC co-cultured with ASC (ii) 2.5 mg fibrinogen, 50 % hpS, no thrombin (iii) GFP-HUVEC in fibrin-hpS hydrogel (2.5 mg fibrinogen, 50% hpS, 25 % thrombin) (iv) GFP-HUVEC (co-cultured with ASC) in fibrin hydrogel (2.5 mg fibrinogen, 50 % thrombin) (v) GFP-HUVEC in fibrin-hpS hydrogel (2.5 mg fibrinogen, 50% hpS, 25 % thrombin)

The results obtained show that hpS stimulates network formation, but their morphology differs from conventional capillary structures. In a study in which HUVEC co-cultures with ASC and BMSC (bone marrow derived stem cells) were examined, it was shown that vascular networks were formed in both co-cultures. However, only in BMSC co-cultures perfusable micro vessels were formed, whereas in ASC the networks were only semi-perfusable.[153] To make more precise determinations about the generated networks, cultivating them in chips that allow for perfusion tracking would be advantageous. This would enable more accurate assessments of the hydrogels used and the networks formed within them; however, this is beyond the scope of the current work.

Summary and Conclusion

One of the biggest challenges in RM is ensuring the supply of nutrients and oxygen as well as the removal of waste material. In a healthy person, this is carried out by the vasculature system. Damage or injuries of organs and tissues can lead to insufficient nutrient supply, which makes vasculature an important research field in regenerative medicine. There are already various materials available to examine vascularization processes. *Matrigel* is a commonly used material in bioscience investigating angiogenesis and vasculature processes. *Matrigel* is obtained from a tumor of mice and therefore not suitable for clinical applications.[110,154] A potential alternative is hpS (hpS), which is extracted from placental tissue.[82] The human placenta is a precious organ rich in proteins, but in most cases disposed as waste material after birth. During various experiments the potential of hpS in RM was investigated.

Initial experiments demonstrated that pure hpS remains liquid even when incubated at 37 °C, incorporating it into different biomaterials lead to polymerization and a potential bioactive material supporting vasculature. Comparing two different batches to each other demonstrated that great batch to batch variation occurs. This might be related to different donors or loss of biological activity over time. This suggests pooling of different batches prior use.

First incorporation of hpS into Gel-MA hydrogels (5wt%, 0.1% LAP, 50% hpS) lead to total detachment of cells from hydrogel surface after 8 hours. In order to investigate the exact reason for such behavior, the individual components of the formulation were examined more closely via live/dead staining and metabolic assays. Attention was paid to two different photoinitiators (irradiated and non-irradiated) - LAP and Irgacure 2959. Macromer concentration and solvent did not influence cell behavior. Both photoinitiators are classified as biocompatible, but results demonstrate that with increasing PI concentration viability and metabolic activity of cells decreased.
The LAP (photoinitiator) concentration was adjusted to 0.025 % and different types of gelatin-based hydrogels were prepared. All samples were tested as with/without incorporated or with hpS coated materials using HUVEC as endothelial cell model. HUVEC establish under certain conditions capillary structures and are known as “gold standard” in vascular research[155]. None of the tested hydrogels, except for Gel-MA, showed any network formation. Therefore, further focus was placed on Gel-MA hydrogels.

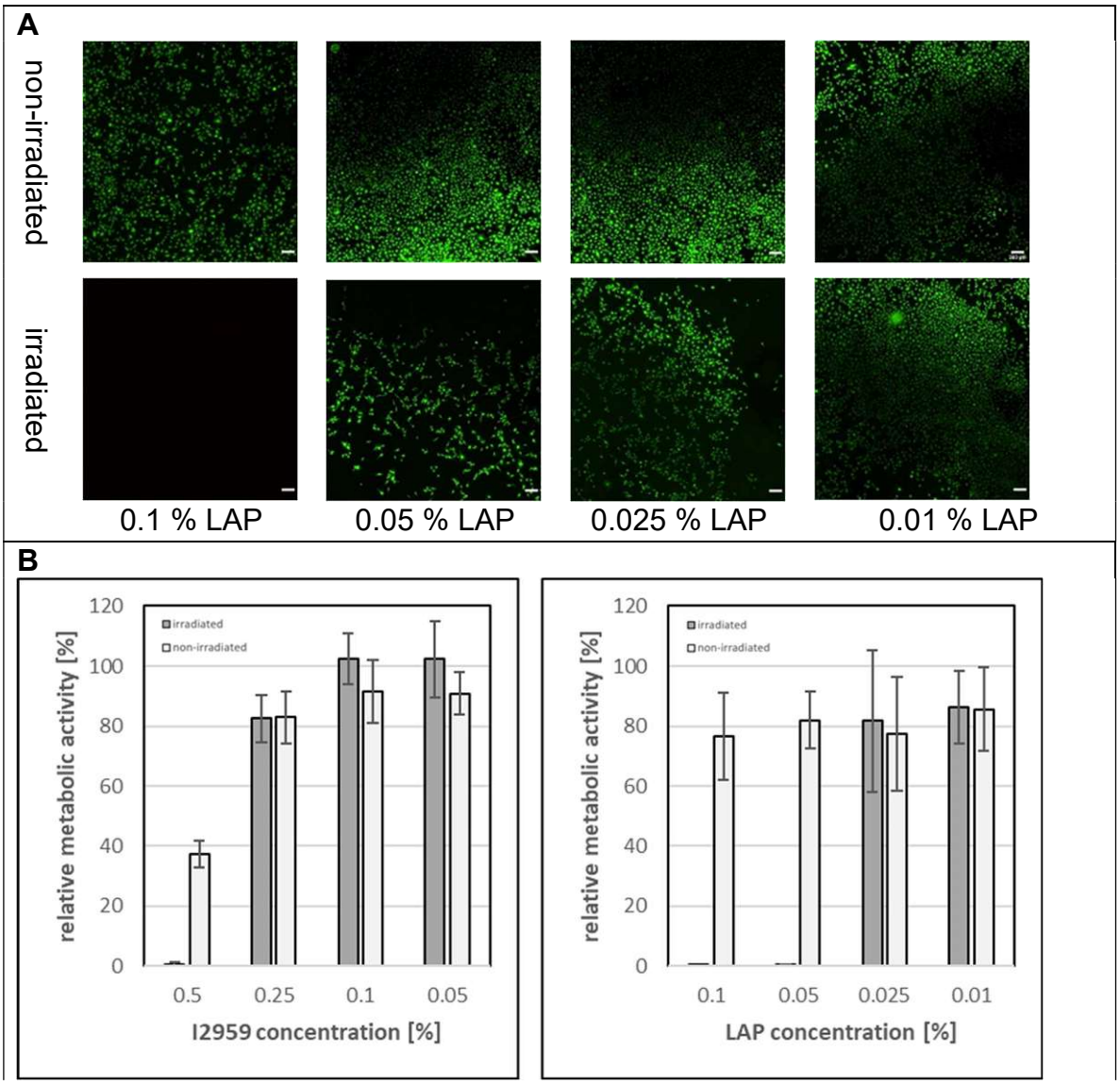

Figure 25: Investigation of individual component of Gel-MA hydrogel formulation. (A) Live/Dead staining 2 days post seeding with different LAP concentrations (irradiated/non-irradiated). Scale bar 200 µM. (B) Results of Tox-8 Assay, metabolic activity in different PIs (Irgacure 2959 and LAP)

Increasing macromer concentration contributes to increasing stiffness of hydrogels. Dual crosslinking by another level. With increasing stiffness, the establishment of a confluent monolayer was achieved, which is consistent with previous studies. On soft Gel-MA hydrogels with incorporated hpS typically network-like structures were built, indicating that lower macromer concentration and softer hydrogels promote vascularization, when biological active materials like hpS were incorporated into hydrogels. [113,125,128,156]

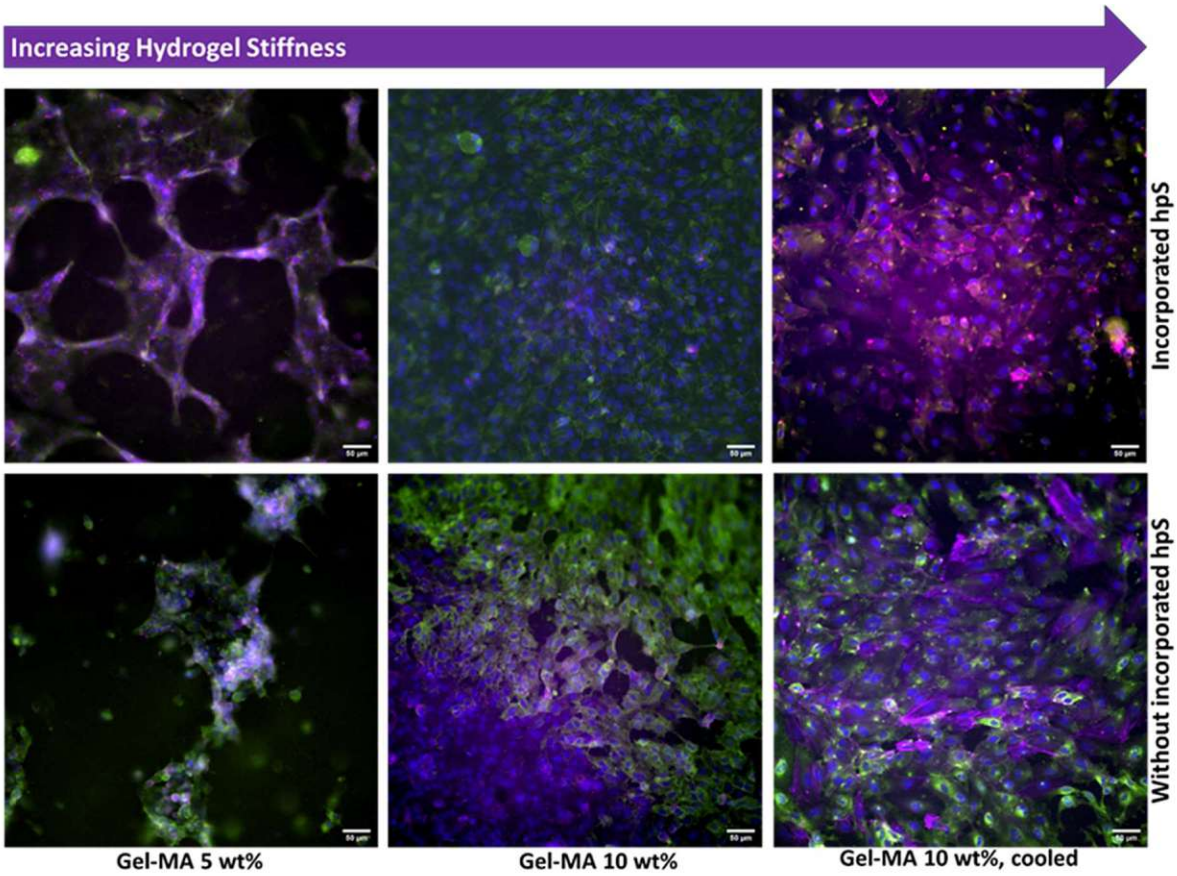

Figure 26: Different stiffness degrees of Gel-MA.

HUVEC seeded and two days cultivated on Gel-MA hydrogels (with/without incorporated hpS) with increasing stiffness. Immunostains: DAPI-blue, F-actin-red, VE-cadherin-green

In Gel-MA 3D experiments the impact of hpS was compared to co-culturing of ASC, which are known to support vascularization of endothelial cells.[132,157] In both hydrogels, cells within 3 days migrated out of the gel without establishing networks. Initial theory of lack of nutrient supply was disproved by reducing hydrogel thickness but obtained same results. This suggests that hydrogel composition is not biocompatible regardless of whether hpS was incorporated or not.

An alternative to gelatin-based hydrogels are fibrin hydrogels. Due to its versatile applications and high biocompatibility, experiments with incorporated hpS were performed to prove bioactive potential of hpS. As mentioned above, HUVEC can modulate vasculature structures when co-cultured with MSC like ASC. Since hpS substrate as well supports network formation, production of fibrinogen-hpS formulations display a promising alternative to already existing hydrogels.

Initially, fibrinogen-hpS hydrogels were produced without any separate addition of thrombin, resulting in hydrogels when incubated at 37°C for approximately 2 hours. Due to expanded gelation duration all cells accumulated on the basement surface of the gel and no homogenous cell distribution could be observed. Fibrin-hpS (25 % hpS

v/v) hydrogels (with thrombin) achieved homogenous cell distribution within the gels, and after five days of incubation initial network structures could be observed. When comparing these hydrogels with the control sample, it was observed that hydrogels containing 25 % hpS exhibited a slower formation of network structures. Increasing hpS concentration resulted in a dense HUVEC network, assuming hpS contains various bioactive molecules and proteins supporting vascularization. Since the control and the manufactured fibrin-hpS hydrogels significantly differ morphologically, additional investigations like presence of tubular structures would be beneficial.

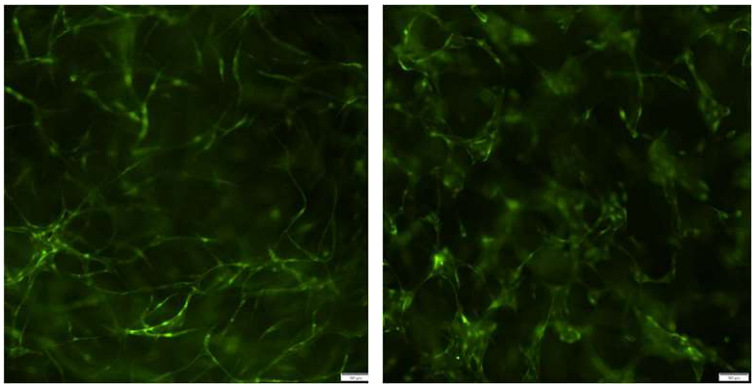

Figure 27: Network formation of HUVEC
(left) control sample - GFP-HUVEC (co-cultured with ASC) in fibrin hydrogel (2.5 mg fibrinogen, 50 % thrombin) (right) GFP-HUVEC in fibrin-hpS hydrogel (2.5 mg fibrinogen, 50% hpS, 25 % thrombin)

HpS has shown in previous trials that it consists of pro-vascularizing components that are beneficial for medical applications. However, at the beginning of the study, some difficulties had to be overcome. For example, a further increase in the concentration of hpS in various gels would probably achieve better results. But due to its physical and chemical properties, it is unfortunately not possible to produce Gel-MA hydrogels with an increased hpS content (more than 50% hpS). An attempt to dissolve lyophilized Gel-MA in pure hpS, for example, unfortunately did not work, as the protein content within the formulation was too high. Even in other hydrogels, such as Gel-NB with Trap6M or Gel-Norb, no formulation with hpS (50 vol%) could be produced. These limitations as well as the great batch-to-batch variance could pose challenges for clinical and research applications, as they affect reproducibility and consistency. Nevertheless, hpS has great potential for RM due to its properties, but further intensive research into its characterization and possible applications is still required before it can be used in clinical settings.

Experimental Details

Equipment

Eppendorf Centrifuge 5430
Fluorescence microscope
Freezer (≤ - 20°C)
Fridge (+ 4 °C)
Incubator (37°C, 5% CO2)
Laminar Flow
Light microscope
LN2-Tank
PerkinElmer EnSpire 2300 Photometer
Pipets (0.5-10 µL, 10-100 µL, 100-1000 µL)
Ultra-deep freezer (≤ - 80°C)
Vortex mixer
Water bath

Materials

24-well plate
6-well plate
96-well plate (flat bottom)
Aluminium foil
Cellstar®Tubes (50 mL, 15 mL)
Eppendorf tubes (1.5 mL)
Glas slides
Omnifix Syringe
Parafilm®
Pipet tips (10 µL, 100 µL, 1000 µL)
Sterican needle ⌀ 0.80 x 50 mm
Sterile serological pipets (5 mL, 10 mL, 25 mL)
T25, T75 Cell culture flasks

Reagent	Manufacturer	Catalog number	Storage temperature	Additional information
Antibiotic Antimycotic Solution (100X)	Sigma	A5955-100ML	+ 4 °C	medium supplement
In Vitro Toxicology Assay Kit	Sigma	TOX8-1KT	+ 4 °C	Tox-8 Assay
Endothelial Cell Growth Medium 2	PromoCell	C-22011	+ 4 °C	medium
Hank' Balanced Salt Solution (1X)	Gibco	14025-092	RT	wash and buffer solution
Dulbecco's Phosphate Buffered Saline	Sigma	D8537-500ML	RT	diluent
TISSEEL Lyo	Baxter	n.a	+ 4 °C	aprotinin,fibrinogen, thrombin
Fetal Bovine Serum, Research Grade	Sigma	F0804-500ML	≤ -20 °C	medium supplement
$CaCl_2$-solution	Sigma	21115-100ML	+ 4 °C	thrombin solvent
Dimethyl sulfoxide	AppliChem	A3608,0500	RT	freezing supplement, solvent
Phalloidin-iFluor 647 Reagent	abcam	Ab176759	≤ -20 °C	staining of F-actin
Anti-VE Cadherin antibody - Intercellular Junction Marker	abcam	ab33168	≤ -80 °C	staining of VE Cadherin
DAPI (4′,6-diamidino-2-phenylindole)	Invitrogen	D1306	≤ -20 °C	staining of DNA (nucleus)
goat anti-rabbit secondary antibody	Thermo Fisher	A16118	≤ -20 °C	secondary antibody
Growth Medium 2 SupplementMix	PromoCell	C39216	≤ -20 °C	Medium supplement
HUVEC	Cell Chip Group	n.a.	LN_2	Endothelial Cells
ASC	Cell Chip Group	n.a.	LN_2	Co-Culturing with HUVEC
GFP-HUVEC	LBI	n.a.	LN_2	Endothelial Cells
Goat serum	Cell Chip Group	n.a.	≤ -20 °C	Blocking
Paraformaldehyde	Cell Chip Group	n.a.	+ 4 °C	Fixation of samples
BCA Protein Assay Kit II	Bio Vision	K813-2500	RT	Quantification of Protein content
Calecein AM	InvitrogenTM	C3099	≤ -20 °C	Live staining
Ethidium Homodimer-1	InvitrogenTM	E1169	≤ -20 °C	Dead staining
Matrigel™	Corning	n.a.	≤ -20 °C	ECM-equivalent
Human placenta substrate	THT Biomaterials	n.a.	≤ -80 °C	Supports network formation
Trypsin-EDTA	Cell Chip Group	n.a.	+ 4 °C	Cell cultivation
Tween	Cell Chip Group	n.a.	RT	Staining

Reagent Preparation

EGM-2 Medium (5 % FBS)	
Endothelial Cell Growth Medium 2	500 mL
Growth Medium 2 SupplementMix	10 mL
Fetal Bovine Serum	25 mL
Antibiotic Antimycotic Solution (100X)	5 mL
The medium was stored at 4 °C and warmed up in a water bath before use. The volumes were adjusted as required.	

Live / Dead Staining Solution	**Dilution**	**Example**
Endothelial Cell Growth Medium 2 (w/o FBS, w/o supplemental mix, w/o antibiotics)		1.0 mL
Calcein AM [4 mM]	1:2000	0.5 µL
Ethidium Homodimer-1 (EthD-1) [2 mM]	1:500	2.0 µL
The solution was freshly prepared and protected from light. The volumes were adjusted as required.		

Fibrinogen Stock (100 mg/mL)
Lyophilized fibrinogen is reconstituted in EGM-2 medium (without FCS) and placed in the incubator at 37 °C for 10 minutes. Afterwards, the solution is carefully swirled and filled into Eppendorf tubes at 100 µL each and stored at ≤ -20 °C until further use.

Methods

Cell Culture Procedure

In this study two different cell lines were used – HUVEC (human umbilical vein endothelial cells) and ASC (adipose-derived stem cells). For 3D-experiments in fibrin hydrogels GFP-labeled HUVEC were used (alone or as Co-culture with ASC), which were provided by the Cell Chip group at TU Vienna. ASC were used from passage 8 to 12 and HUVEC from 5 to 12. The passage number of the cells was not exceeded during the entire study to exclude missing or insufficient network formation due to the passage number or the age of the cells. HUVEC originate from different donors - pooled cell line – and a cell bank from one original vial was established to minimize batch to batch variance. All steps were performed under laminar flow unless otherwise specified.

Thawing

Frozen cryopreserved cells were obtained from a liquid nitrogen tank and the vial transferred into a pre-warmed water bath (37 °C). Once the cells are thawed, they were transferred immediately into an appropriate vessel (T75 or T25, depending on cell density) with medium. The cell suspension needs to be diluted at least 1:10. When cells attached (approximately after 3 hours) a medium exchange was performed. Alternatively, the thawed cell suspension can be diluted 1:50 and the medium exchange can be done next day. The transfer of the cells into medium is a critical step, since DMSO is toxic to cells.

Cell Seeding and Maintenance

Cells were passaged at a confluence of 80-90 %. For this the medium was discarded and the flask washed twice with DPBS to remove remaining medium. Trypsin-EDTA was added and the flask was placed into a humidified incubator until the cells detach. As soon as the cells were in suspension, they were counted. For this a counting chamber was used. The cells were seeded with a density of 10,000 – 20,000 cells / cm^2. The medium was replaced every 2-3 days.

Freezing

For long term storage in liquid nitrogen (LN_2), cells were passaged as described before and centrifuged for 5 minutes and 125 rcf. Then the supernatant was gently removed without destroying the pellet. The pellet was resuspended into a 10% DMSO-FBS-solution, aliquoted into cryovials, transferred into a cryo box and stored for 1 to 3 days at ≤ -80 °C. After that, the vials were placed into liquid nitrogen until further use. The freezing cell density should be between 500,000 and 1,000,000 cells / mL.

1 HUVEC on Matrigel

Matrigel was thawed at 4 °C overnight. All materials that had direct contact with the Matrigel (plate, tips) were also pre-cooled at 4°C, since *Matrigel* gels irreversibly at room temperature. The thawed *Matrigel* was stored on ice until further use. Matrigel™ was carefully aspirated with the pre-cooled tip and added to selected wells (avoided bubbles). The plate was then placed in an incubator (5 % CO_2, 37 °C) for 30 to 50 minutes. During this time, the cells were passaged and the required cell density was set. After the specified incubation time, the cells were seeded directly onto the gel without destroying it and the gels were covered with medium. First networks can already be observed after 4 hours and network formation was completed after 18 hours. The samples were imaged at different times.

2 HUVEC on human placenta substrate (hpS)

During this study, human placenta substrate (hpS) was used in different ways as pro-vascularizing substance – incorporating into gels, coating of wells and coating of gels. HpS was provided as raw material and had to be first UV-sterilized for 30 min before each use. As there was no empirical data on the product, the batch-to-batch variance was analyzed and the possible applications were tested.

300 µL hps / 24-well (volume could be adjusted for other well sizes) was added and incubated for 2 hours (the incubation time was varied in some experiments) at 37 °C and 5 % CO_2. HpS does not gel and remains liquid even after a 2-hour incubation period. The cells were carefully seeded onto the liquid and medium was added without mixing or forming air bubbles. The plate was incubated for further 24 to 48 hours and then analyzed microscopically.

3 Influence of hydrogel components on cell viability and metabolic activity

3.1 Live/Dead Staining

Medium was gently removed and hydrogels are washed twice with HBSS. Staining solution was prepared (described above) and 100 µL per well was added, so that entire hydrogel was covered with staining solution. Depending on 2D or 3D experiments, hydrogels are incubated for 30 minutes / 45 minutes at 37°C. Afterwards, hydrogels were washed twice with HBSS, EGM-2 Medium was added and hydrogels were imaged (FITC (live/green)) and TRITC (dead/read). In some experiments only live staining was performed.

3.2. Metabolic Activity - Tox-8 Assay

Metabolic activity was evaluated in various experiments to assess which materials or hydrogels support metabolic activity. The relative metabolic activity compared to cells under natural cultivation conditions was analyzed. After the cells were seeded onto the gels or separate components such as photoinitiators or macromers were added, a Tox-8 assay was performed.

For this purpose, an assay was conducted after two days of incubation at 37°C and 5% CO_2. The gels or cells were washed twice with DPBS, and 300 µL 10% Tox-8 solution in EGM-2 was added. The samples were then incubated for 2 hours at 37°C and 5% CO_2. Subsequently, the supernatant was collected, technical replicates (triplicates) generated and analyzed using a plate reader (Settings are listed in Table 3).

Table 3: Settings of Tox-8 measurement on plate reader. Each plate was measured twice (measurement A, measurement B)

Plate type	
Name of the plate type	96 General
Number of rows	8
Number of columns	12
Number of the wells in the plate	96
Height of the plate	14.35 mm
Diameter of the well	6.5 mm
Volume of the well	300 µl
Calculations	
No calculations.	
Operations (Shake)	
Duration	5 s
Speed	60
Diameter	3 mm
Shake mode	Linear
Plate location	Inside
Measurement	
Meas	A + B
Tech	Fluorescence
Shake Duration	5 s
Speed	60
Diameter	3 mm
Shake mode	Linear
Plate location	Inside
First assay/plate repeat affected	1
Last assay/plate repeat affected	1
Exc. filter	N/A
Using of excitation filter	Bottom
Excitation wavelength	562 nm
Emission wavelength	585 nm
Measurement height	3 mm
Number of flashes	100
Number of flashes integrated	100

4 Different gelatin-based hydrogels in 2D

Different types of hydrogels were provided by Raffael Reichsöllner at the Institute of Applied Synthetic Chemistry. The formulations were either sterile filtered or, if filtration was not possible, UV sterilized post polymerization. To investigate the influence of hpS in different hydrogels on network formation of HUVEC per hydrogel three different hydrogel-variants were produced:

1. Hydrogels without hpS
2. Hydrogels with incorporated hpS (different amounts of hpS)
3. Hydrogel without incorporated hpS, but coated with hpS

For polymerization, one drop of the formulation was pipetted onto a Teflon block and pressed flat with a glass plate to create a flat surface. In the next step, depending on the PI, LAP or Irgacure, the still liquid formulation was polymerized with a specific wavelength. The platelets with the gels adhering to them were then carefully lifted without destroying the gel and transferred to the wells. The gels were then washed twice with DPBS, the cells were seeded directly onto the gel and then incubated for two hours at 37 °C and 5 % CO_2. After the cells had adhered to the gels, additional EGM-2 medium was added so that the gels and cells were completely covered with medium.

5 3D Gel-MA hydrogels

Gel-MA (5wt%) formulations were provided by Raffael Reichsöllner at the Institute of Applied Synthetic Chemistry. HpS was prior incorporation 30 min UV-sterilized. Afterwards cells (ASC and HUVEC) were harvested, counted and centrifugated at 150 rcf for 5 minutes. Afterwards, supernatant was discarded, and cells were re-suspended into prepared Gel-MA with appropriate cell density. Under the laminar flow, 50 µL of formulation (with incorporated cells) were pipetted into each well and polymerized. After polymerization, EGM-2 medium was added so that the gels and cells were completely covered with medium. Plates were incubated at 37 °C and medium was exchanged every 2-3 days.

5.1 Immunostaining

At specific time points, hydrogels were fixed with *para*-formaldehyde (PFA). For this purpose, they were washed three times with HBSS and then fixed with PFA (PFA 4% in HBSS) at room temperature (10 minutes for 2D gels, up to 30 minutes for 3D gels). After fixation, the wells were washed three times with HBSS, and a permeabilization solution (0.1% Tween 20 in HBSS) was added for 30 minutes at room temperature. The hydrogels were then washed three more times with HBSS and incubated with

blocking buffer (2% goat serum in HBSS) for at least one additional hour at room temperature (incubation can be extended for several days at 4°C).

During the staining process, the following components were targeted: VE-Cadherin, F-actin, and cell nuclei. First, a VE-Cadherin antibody was diluted 1:1000 in blocking buffer, added to the wells, and incubated overnight at 4°C in the dark. The next day, the wells were washed three times with HBSS, and a staining solution containing DAPI (1 µg/mL) and Phalloidin i-Fluor 647 reagent (1:200) in blocking buffer was added and incubated for another hour at 4°C. After three more washes with blocking buffer, the buffer was added back to the wells, and the samples were subsequently imaged using microscopy.

6 3D Fibrin-hpS-Hydrogels

Fibrinogen (100 mg/mL) is thawed at room temperature, and thrombin (stock 500 U/mL) is diluted to 2 U/mL in $CaCl_2$. Before preparing the hydrogels, round glass slides are washed in 70% ethanol, air-dried, and then stored in sterile well plates. The cells are then harvested, counted, centrifuged, and resuspended at the desired cell density as previously described. Fibrinogen is added to the cell suspension (note the final concentration of fibrinogen per clot).

The following steps must be carried out quickly, as the gels polymerize within seconds after the addition of thrombin. Two pipettes are prepared for this purpose. In the experiments conducted, 50 µL clots were prepared, requiring two 100 µL pipettes. One pipette was set to 30 µL, and the other to 50 µL. Depending on whether and how much thrombin was to be included in the hydrogel, the procedure was as follows:

- **Fibrin hydrogel, 50 % Thrombin, without hpS (control)**

  Add 30 µL of the fibrinogen-cell mix, then add 30 µL thrombin (2 U/mL). Mix by repeatedly aspirating and dispensing 50 µL with the pre-set pipette and carefully pipette a drop onto the glass slide without creating air bubbles. Afterwards, the clots incubated at 37°C in an incubator for 20 minutes.

- **Fibrin hydrogels with hpS and thrombin**

  The desired amount of hpS and thrombin per hydrogel determines the procedure. For example, for a hydrogel with 50% hpS and 25% thrombin, the cell suspension-fibrinogen mix (25%) must be prepared with the appropriate concentration to achieve the desired final concentrations of cells and fibrinogen. Then, hpS is pipetted into the cell-fibrinogen mix and mixed. Finally, thrombin is added, mixed with the pre-set pipette as described, and pipetted onto the glass slide. The proportions of hpS and thrombin can be adjusted as needed, but it is

important to always add thrombin last. Afterwards, the clots incubated at 37°C in an incubator for 20 minutes.

- **Fibrinogen-hpS hydrogels without thrombin**:

  The cell suspension was mixed with fibrinogen, and the desired amount of hpS was added. The required amount of the formulation was then gently pipetted onto a glass slide and incubated at 37°C in an incubator for 2 hours. (Caution during transport – the formulation does not polymerize before incubation.)

After the incubation period, EGM-2 medium is added to the respective clots and further incubated at 37°C in the incubator. Medium was exchanged every 2-3 days. At desired time points, images were taken, and additional assays were performed for further analysis.

Research Tools

Literature search was conducted using Google Scholar and PubMed. Writing was done in Microsoft® Word® (Microsoft® 365 MSO) and arrangement of images in Microsoft® PowerPoint® (Microsoft® 365 MSO). Microscopic images were edited using ImageJ.[158] ChatGPT 3.5 and DeepL were used for paraphrasing and text editing. Citations were added using Mendeley Reference Manager software.

References

1. HARRISON, J. H., MERRILL, J. P. & MURRAY, J. E. Renal homotransplantation in identical twins. *Surg Forum* **6**, 432–436 (1956).

2. Tan, S. Y. & Merchant, J. Joseph Murray (1919–2012): First transplant surgeon. *Singapore Med J* **60**, 162 (2019).

3. Allgemeines über die Organtransplantation in Österreich. https://www.oesterreich.gv.at/themen/gesundheit_und_notfaelle/organtransplantation/Seite.2510001.html.

4. Mao, A. S., Mooney, D. J. & Paulson, J. A. Regenerative medicine: Current therapies and future directions. doi:10.1073/pnas.1508520112.

5. Poss, K. D. Advances in understanding tissue regenerative capacity and mechanisms in animals. *Nat Rev Genet* **11**, 710 (2010).

6. Vieira, W. A., Wells, K. M. & McCusker, C. D. Advancements to the axolotl model for regeneration and aging. *Gerontology* **66**, 212 (2020).

7. Rosenthal, N. Prometheus's vulture and the stem-cell promise. *N Engl J Med* **349**, 267–274 (2003).

8. Holt, D. R. *et al.* Effect of age on wound healing in healthy human beings. *Surgery* **112**, 293–298 (1992).

9. Poojari, A. S., Wairkar, S. & Kulkarni, Y. A. Stem cells as a regenerative medicine approach in treatment of microvascular diabetic complications. *Tissue Cell* **85**, 102225 (2023).

10. Okamoto, R., Mizutani, T. & Shimizu, H. Development and Application of Regenerative Medicine in Inflammatory Bowel Disease. *Digestion* **104**, 24–29 (2023).

11. Tavakol, D. N., Fleischer, S. & Vunjak-Novakovic, G. Harnessing Organs-on-a-Chip to Model Tissue Regeneration. *Cell Stem Cell* **28**, 993 (2021).

12. Petrosyan, A. *et al.* Regenerative medicine applications: An overview of clinical trials. *Front Bioeng Biotechnol* **10**, 942750 (2022).

13. Sayed, N. *et al.* Gene therapy: Comprehensive overview and therapeutic applications. *Life Sci* **294**, 120375 (2022).

14. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).

15. Li, G. *et al.* Gene editing and its applications in biomedicine. *Sci China Life Sci* **65**, 660–700 (2022).

16. Hoang, D. M. *et al.* Stem cell-based therapy for human diseases. *Signal Transduction and Targeted Therapy 2022 7:1* **7**, 1–41 (2022).

17. Aly, R. M. Current state of stem cell-based therapies: an overview. *Stem Cell Investig* **7**, 8 (2020).

18. Leung, C. M. *et al.* A guide to the organ-on-a-chip. *Nature Reviews Methods Primers 2022 2:1* **2**, 1–29 (2022).

19. Approved Cellular and Gene Therapy Products | FDA. https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products.

20. Landfeldt, E. Gene Therapy for Neuromuscular Diseases: Health Economic Challenges and Future Perspectives. *J Neuromuscul Dis* **9**, 675 (2022).

21. Chan, B. P. & Leong, K. W. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *European Spine Journal* **17**, 467 (2008).

22. Almouemen, N., Kelly, H. M. & O'Leary, C. Tissue Engineering: Understanding the Role of Biomaterials and Biophysical Forces on Cell Functionality Through Computational and Structural Biotechnology Analytical Methods. *Comput Struct Biotechnol J* **17**, 591–598 (2019).

23. Cao, Y., Vacanti, J. P., Paige, K. T., Upton, J. & Vacanti, C. A. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. *Plast Reconstr Surg* **100**, 297–304 (1997).

24. Vacanti, J. P., Otte, J. B. & Wertheim, J. A. Introduction: Regenerative Medicine and Solid Organ Transplantation from a Historical Perspective. *Regenerative Medicine Applications in Organ Transplantation* 1–15 (2014) doi:10.1016/B978-0-12-398523-1.00001-X.

25. Cao, Y., Vacanti, J. P., Paige, K. T., Upton, J. & Vacanti, C. A. Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear. *Plast Reconstr Surg* **100**, 297–304 (1997).

26. Aird, W. C. Spatial and temporal dynamics of the endothelium. *J Thromb Haemost* **3**, 1392–1406 (2005).

27. Pugsley, M. K. & Tabrizchi, R. The vascular system: An overview of structure and function. *J Pharmacol Toxicol Methods* **44**, 333–340 (2000).

28. Udan, R. S., Culver, J. C. & Dickinson, M. E. Understanding vascular development: WIRE Developmental Biology (2012). *Wiley Interdiscip Rev Dev Biol* **2**, 327 (2013).

29. Tucker, W. D., Arora, Y. & Mahajan, K. Anatomy, Blood Vessels. *StatPearls* (2023).

30. Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature 2000 407:6801* **407**, 249–257 (2000).

31. Ng, H. Y., Lee, K. X. A., Kuo, C. N. & Shen, Y. F. Bioprinting of artificial blood vessels. *Int J Bioprint* **4**, (2018).

32. Naito, H., Iba, T. & Takakura, N. Mechanisms of new blood-vessel formation and proliferative heterogeneity of endothelial cells. *Int Immunol* **32**, 295–305 (2020).

33. Makanya, A. N., Hlushchuk, R. & Djonov, V. G. Intussusceptive angiogenesis and its role in vascular morphogenesis, patterning, and remodeling. *Angiogenesis* **12**, 113–123 (2009).

34. De Spiegelaere, W. *et al.* Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. *J Vasc Res* **49**, 390–404 (2012).

35. Phng, L. K. & Gerhardt, H. Angiogenesis: a team effort coordinated by notch. *Dev Cell* **16**, 196–208 (2009).

36. Gerhardt, H. *et al.* VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* **161**, 1163 (2003).

37. Uccelli, A. *et al.* Vascular endothelial growth factor biology for regenerative angiogenesis. *Swiss Med Wkly* **149**, w20011 (2019).

38. Semenza, G. L. & Wang, G. L. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* **12**, 5447–5454 (1992).

39. Krock, B. L., Skuli, N. & Simon, M. C. Hypoxia-induced angiogenesis: good and evil. *Genes Cancer* **2**, 1117–1133 (2011).

40. Ke, Q. & Costa, M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* **70**, 1469–1480 (2006).

41. Mahon, P. C., Hirota, K. & Semenza, G. L. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* **15**, 2675–2686 (2001).

42. Rey, S. & Semenza, G. L. Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. *Cardiovasc Res* **86**, 236 (2010).

43. Prabhakar, N. R. & Semenza, G. L. Oxygen Sensing and Homeostasis. *Physiology* **30**, 340 (2015).

44. Jawad, S. F. *et al.* The strict regulation of HIF-1α by non-coding RNAs: new insight towards proliferation, metastasis, and therapeutic resistance strategies. *Cancer and Metastasis Reviews* **43**, 5–27 (2024).

45. Salih, V. & Thomas, D. Fundamentals of cell and matrix biology for tissue engineering. *Standardisation in Cell and Tissue Engineering: Methods and Protocols* 3–17 (2013) doi:10.1533/9780857098726.1.3.

46. Alberts, B. *et al. Molecular Biology of the Cell*. (W. W. Norton & Company, New York, 2022).

47. Dzobo, K. *et al.* Fibroblast-derived extracellular matrix induces chondrogenic differentiation in human adipose-derived mesenchymal stromal/stem cells in vitro. *Int J Mol Sci* **17**, (2016).

48. Mienaltowski, M. J., Gonzales, N. L., Beall, J. M. & Pechanec, M. Y. Basic Structure, Physiology, and Biochemistry of Connective Tissues and Extracellular Matrix Collagens. *Adv Exp Med Biol* **1348**, 5–43 (2021).

49. Walker, C., Mojares, E. & Del Río Hernández, A. Role of extracellular matrix in development and cancer progression. *Int J Mol Sci* **19**, (2018).

50. Bukhari, S. N. A. *et al.* Hyaluronic acid, a promising skin rejuvenating biomedicine: A review of recent updates and pre-clinical and clinical investigations on cosmetic and nutricosmetic effects. *Int J Biol Macromol* **120**, 1682–1695 (2018).

51. Matarasso, S. L. Understanding and using hyaluronic acid. *Aesthet Surg J* **24**, 361–364 (2004).

52. Petrey, A. C. & de la Motte, C. A. Hyaluronan, a crucial regulator of inflammation. *Front Immunol* **5**, (2014).

53. Gupta, R. C., Lall, R., Srivastava, A. & Sinha, A. Hyaluronic acid: Molecular mechanisms and therapeutic trajectory. *Front Vet Sci* **6**, (2019).

54. Marinho, A., Nunes, C. & Reis, S. Hyaluronic Acid: A Key Ingredient in the Therapy of Inflammation. *Biomolecules* **11**, 1518 (2021).

55. Aamodt, J. M. & Grainger, D. W. Extracellular matrix-based biomaterial scaffolds and the host response. *Biomaterials* **86**, 68–82 (2016).

56. Petreaca, M. & Martins-Green, M. The dynamics of cell–extracellular matrix interactions, with implications for tissue engineering. *Principles of Tissue Engineering* 93–117 (2020) doi:10.1016/B978-0-12-818422-6.00007-1.

57. Xu, Y., Yu, Y. & Guo, Z. Hydrogels in cardiac tissue engineering: application and challenges. *Mol Cell Biochem* (2024) doi:10.1007/S11010-024-05145-3.

58. Sudhakar, C. K. *et al.* Hydrogels—Promising Candidates for Tissue Engineering. *Nanotechnology Applications for Tissue Engineering* 77–94 (2015) doi:10.1016/B978-0-323-32889-0.00005-4.

59. Huang, G. *et al.* Functional and Biomimetic Materials for Engineering of the Three-Dimensional Cell Microenvironment. *Chem Rev* **117**, 12764 (2017).

60. Caliari, S. R. & Burdick, J. A. A Practical Guide to Hydrogels for Cell Culture. *Nat Methods* **13**, 405 (2016).

61. Arjun Uppuluri, V. N. V., Sathanantham, S. T., Bhimavarapu, S. K. & Elumalai, L. Polymeric Hydrogel Scaffolds: Skin Tissue Engineering and Regeneration. *Adv Pharm Bull* **12**, 437 (2022).

62. Place, E. S., George, J. H., Williams, C. K. & Stevens, M. M. Synthetic polymer scaffolds for tissue engineering. *Chem Soc Rev* **38**, 1139–1151 (2009).

63. Gan, X., Wang, X., Huang, Y., Li, G. & Kang, H. Applications of Hydrogels in Osteoarthritis Treatment. *Biomedicines* **12**, (2024).

64. Place, E. S., George, J. H., Williams, C. K. & Stevens, M. M. Synthetic polymer scaffolds for tissue engineering. *Chem Soc Rev* **38**, 1139–1151 (2009).

65. Bashir, S. *et al.* Fundamental Concepts of Hydrogels: Synthesis, Properties, and Their Applications. *Polymers (Basel)* **12**, 2702 (2020).

66. Hu, W., Wang, Z., Xiao, Y., Zhang, S. & Wang, J. Advances in crosslinking strategies of biomedical hydrogels. *Biomater Sci* **7**, 843–855 (2019).

67. Husar, B. *et al.* Photopolymerization-based additive manufacturing for the development of 3D porous scaffolds. *Biomaterials for Bone Regeneration: Novel Techniques and Applications* 149–201 (2014) doi:10.1533/9780857098104.2.149.

68. Nguyen, A. K., Goering, P. L., Reipa, V. & Narayan, R. J. Toxicity and photosensitizing assessment of gelatin methacryloyl-based hydrogels photoinitiated with lithium phenyl-2,4,6-trimethylbenzoylphosphinate in human primary renal proximal tubule epithelial cells. *Biointerphases* **14**, 021007 (2019).

69. Tomal, W. & Ortyl, J. Water-Soluble Photoinitiators in Biomedical Applications. *Polymers (Basel)* **12**, 1073 (2020).

70. Groll, J. *et al.* A definition of bioinks and their distinction from biomaterial inks. *Biofabrication* **11**, (2018).

71. Ovsianikov, A., Khademhosseini, A. & Mironov, V. The Synergy of Scaffold-Based and Scaffold-Free Tissue Engineering Strategies. *Trends Biotechnol* **36**, 348–357 (2018).

72. Chichkov, B. N., El-Tamer, A., Hinze, U. & Chichkov Zusammenfassung, B. N. 3D Mikro- und Nano-Strukturierung mittels Zwei-Photonen-Polymerisation. *Additive Manufacturing Quantifiziert* 117–132 (2017) doi:10.1007/978-3-662-54113-5_8.

73. Harinarayana, V. & Shin, Y. C. Two-photon lithography for three-dimensional fabrication in micro/nanoscale regime: A comprehensive review. *Opt Laser Technol* **142**, 107180 (2021).

74. Wu, Y. *et al.* Additively manufactured materials and structures: A state-of-the-art review on their mechanical characteristics and energy absorption. *Int J Mech Sci* **246**, (2023).

75. Faraji Rad, Z., Prewett, P. D. & Davies, G. J. High-resolution two-photon polymerization: the most versatile technique for the fabrication of microneedle arrays. *Microsyst Nanoeng* **7**, (2021).

76. Jing, X., Fu, H., Yu, B., Sun, M. & Wang, L. Two-photon polymerization for 3D biomedical scaffolds: Overview and updates. *Front Bioeng Biotechnol* **10**, 994355 (2022).

77. Dobos, A. *et al.* Thiol–Gelatin–Norbornene Bioink for Laser-Based High-Definition Bioprinting. *Adv Healthc Mater* **9**, 1900752 (2020).

78. O'Halloran, S., Pandit, A., Heise, A. & Kellett, A. Two-Photon Polymerization: Fundamentals, Materials, and Chemical Modification Strategies. *Advanced Science* **10**, 2204072 (2022).

79. Yang, G., Mahadik, B., Choi, J. Y. & Fisher, J. P. Vascularization in tissue engineering: fundamentals and state-of-art. *Prog Biomed Eng (Bristol)* **2**, 012002 (2020).

80. Rouwkema, J., Rivron, N. C. & van Blitterswijk, C. A. Vascularization in tissue engineering. *Trends Biotechnol* **26**, 434–441 (2008).

81. Kleinman, H. K. & Martin, G. R. Matrigel: Basement membrane matrix with biological activity. *Semin Cancer Biol* **15**, 378–386 (2005).

82. Hackethal, J. *et al.* Novel Human Placenta-Based Extract for Vascularization Strategies in Tissue Engineering. *Tissue Eng Part C Methods* **27**, 616–632 (2021).

83. Zhao, K. yu *et al.* The biological macromolecules constructed Matrigel for cultured organoids in biomedical and tissue engineering. *Colloids Surf B Biointerfaces* **247**, 114435 (2025).

84. Nguyen, E. H. *et al.* Versatile synthetic alternatives to Matrigel for vascular toxicity screening and stem cell expansion. *Nat Biomed Eng* **1**, 0096 (2017).

85. Pogozhykh, O., Prokopyuk, V., Figueiredo, C. & Pogozhykh, D. Placenta and Placental Derivatives in Regenerative Therapies: Experimental Studies, History, and Prospects. *Stem Cells Int* **2018**, 4837930 (2018).

86. Dahlberg, A. & Milano, F. Cord blood transplantation: rewind to fast forward. *Bone Marrow Transplant* **52**, 799–802 (2017).

87. Broxmeyer, H. E. *et al.* Human umbilical cord blood: a clinically useful source of transplantable hematopoietic stem/progenitor cells. *Int J Cell Cloning* **8 Suppl 1**, 76–91 (1990).

88. Cursiefen, C., Rummelt, C., Beckmann, M. W. & Kruse, F. E. Amniotic membrane-covered bio-onlays for treatment of ocular surface disease. *Br J Ophthalmol* **91**, 841 (2007).

89. Lee, S. H. & Tseng, S. C. G. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol* **123**, 303–312 (1997).

90. Faulk, W. P. *et al.* Human amnion as an adjunct in wound healing. *Lancet* **1**, 1156–1158 (1980).

91. Silini, A. R., Cargnoni, A., Magatti, M., Pianta, S. & Parolini, O. The Long Path of Human Placenta, and Its Derivatives, in Regenerative Medicine. *Front Bioeng Biotechnol* **3**, 162 (2015).

92. de Laorden, E. H. *et al.* Human placenta-derived mesenchymal stem cells stimulate neuronal regeneration by promoting axon growth and restoring neuronal activity. *Front Cell Dev Biol* **11**, 1328261 (2023).

93. Pavlov, N. *et al.* Angiogenin distribution in human term placenta, and expression by cultured trophoblastic cells. *Angiogenesis* **6**, 317–330 (2003).

94. Moore, M. C., Pandolfi, V. & McFetridge, P. S. Novel human-derived extracellular matrix induces in vitro and in vivo vascularization and inhibits fibrosis. *Biomaterials* **49**, 37 (2015).

95. Gundacker, C. & Ellinger, I. The unique applicability of the human placenta to the Adverse Outcome Pathway (AOP) concept: the placenta provides fundamental insights into human organ functions at multiple levels of biological organization. *Reproductive Toxicology* **96**, 273–281 (2020).

96. Cindrova-Davies, T. & Sferruzzi-Perri, A. N. Human placental development and function. *Semin Cell Dev Biol* **131**, 66–77 (2022).

97. Roy, A., Mantay, M., Brannan, C. & Griffiths, S. Placental Tissues as Biomaterials in Regenerative Medicine. *Biomed Res Int* **2022**, (2022).

98. Burton, G. J. & Fowden, A. L. The placenta: a multifaceted, transient organ. *Philosophical Transactions of the Royal Society B: Biological Sciences* **370**, 20140066 (2015).

99. THT Biomaterials - Humanizing Tissue Engineering. https://tht-biomaterials.com/.

100. Hackethal, J. *et al.* Novel Human Placenta-Based Extract for Vascularization Strategies in Tissue Engineering. *Tissue Eng Part C Methods* **27**, 616–632 (2021).

101. Reichsöllner, R. *Functionalized, Biointeractive Hydrogels for Biomedical Applications*. (Wien, 2022).

102. Rashid, A. Bin, Showva, N. N. & Hoque, M. E. Gelatin-based scaffolds: An intuitive support structure for regenerative therapy. *Curr Opin Biomed Eng* **26**, 100452 (2023).

103. Su, K. & Wang, C. Recent advances in the use of gelatin in biomedical research. *Biotechnol Lett* **37**, 2139–2145 (2015).

104. Van Den Bulcke, A. I. *et al.* Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules* **1**, 31–38 (2000).

105. Chen, S., Wang, Y., Lai, J., Tan, S. & Wang, M. Structure and Properties of Gelatin Methacryloyl (GelMA) Synthesized in Different Reaction Systems. *Biomacromolecules* **24**, 2928–2941 (2023).

106. Mugnaini, G., Gelli, R., Mori, L. & Bonini, M. How to Cross-Link Gelatin: The Effect of Glutaraldehyde and Glyceraldehyde on the Hydrogel Properties. *ACS Appl Polym Mater* **5**, 9192–9202 (2023).

107. Qin, X. H. *et al.* Three-dimensional microfabrication of protein hydrogels via two-photon-excited thiol-vinyl ester photopolymerization. *J Polym Sci A Polym Chem* **51**, 4799–4810 (2013).

108. Baudin, B., Bruneel, A., Bosselut, N. & Vaubourdolle, M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc* **2**, 481–485 (2007).

109. Chandel, S., Kumaragurubaran, R., Giri, H. & Dixit, M. Isolation and Culture of Human Umbilical Vein Endothelial Cells (HUVECs). *Methods Mol Biol* **2711**, 147–162 (2024).

110. Kleinman, H. K. & Martin, G. R. Matrigel: basement membrane matrix with biological activity. *Semin Cancer Biol* **15**, 378–386 (2005).

111. Saunders, R. L. & Hammer, D. A. Assembly of Human Umbilical Vein Endothelial Cells on Compliant Hydrogels. *Cell Mol Bioeng* **3**, 60 (2010).

112. Zudaire, E., Gambardella, L., Kurcz, C. & Vermeren, S. A Computational Tool for Quantitative Analysis of Vascular Networks. *PLoS One* **6**, e27385 (2011).

113. Jalali, S., Tafazzoli-Shadpour, M., Haghighipour, N., Omidvar, R. & Safshekan, F. Regulation of Endothelial Cell Adherence and Elastic Modulus by Substrate Stiffness. *Cell Commun Adhes* **22**, 79–89 (2015).

114. Aisenbrey, E. A. & Murphy, W. L. Synthetic alternatives to Matrigel. *Nat Rev Mater* **5**, 539 (2020).

115. Background 1. What is Corning Matrigel matrix? Amounts of Growth Factors (GF) Present in Corning Matrigel Matrix vs. Growth Factor Reduced (GFR) Corning Matrigel Matrix Growth Factor Range of GF Concentration in Corning Matrigel Matrix Average GF Concentration in Corning Matrigel Matrix Typical GF Concentration in GFR Corning Matrigel Matrix Corning ® Matrigel ® Matrix Frequently Asked Questions.

116. Yue, K. *et al.* Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. *Biomaterials* **73**, 254–271 (2015).

117. Lin, R. Z., Chen, Y. C., Moreno-Luna, R., Khademhosseini, A. & Melero-Martin, J. M. Transdermal regulation of vascular network bioengineering using aphotopolymerizable methacrylated gelatin hydrogel. *Biomaterials* **34**, 6785–6796 (2013).

118. Im, G. B. & Lin, R. Z. Bioengineering for vascularization: Trends and directions of photocrosslinkable gelatin methacrylate hydrogels. *Front Bioeng Biotechnol* **10**, 1053491 (2022).

119. Xu, H., Casillas, J., Krishnamoorthy, S. & Xu, C. Effects of Irgacure 2959 and lithium phenyl-2,4,6-trimethylbenzoylphosphinate on cell viability, physical properties, and microstructure in 3D bioprinting of vascular-like constructs. *Biomedical Materials (Bristol)* **15**, (2020).

120. Soliman, B. G. *et al.* Development and Characterization of Gelatin-Norbornene Bioink to Understand the Interplay between Physical Architecture and Micro-Capillary Formation in Biofabricated Vascularized Constructs. *Adv Healthc Mater* **11**, (2022).

121. Genç, H. *et al.* Adjusting Degree of Modification and Composition of gelAGE-Based Hydrogels Improves Long-Term Survival and Function of Primary Human Fibroblasts and Endothelial Cells in 3D Cultures. *Biomacromolecules* **24**, 1497–1510 (2023).

122. Lau, L. F., Pumiglia, K., Cote, Y. P. & Feinstein, M. B. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of

platelet-derived secondary mediators. *Biochemical Journal* **303**, 391 (1994).

123. Lv, B. *et al.* Recent advances in GelMA hydrogel transplantation for musculoskeletal disorders and related disease treatment. *Theranostics* **13**, 2015 (2023).

124. Wu, Y. *et al.* The influence of the stiffness of GelMA substrate on the outgrowth of PC12 cells. *Biosci Rep* **39**, BSR20181748 (2019).

125. Lavrentieva, A. *et al.* Fabrication of Stiffness Gradients of GelMA Hydrogels Using a 3D Printed Micromixer. *Macromol Biosci* **20**, (2020).

126. Athirasala, A. *et al.* A Novel Strategy to Engineer Pre-Vascularized Full-Length Dental Pulp-like Tissue Constructs. *Sci Rep* **7**, (2017).

127. Reinhart-King, C. A., Dembo, M. & Hammer, D. A. The dynamics and mechanics of endothelial cell spreading. *Biophys J* **89**, 676–689 (2005).

128. Yeh, Y. T. *et al.* Matrix Stiffness Regulates Endothelial Cell Proliferation through Septin 9. *PLoS One* **7**, e46889 (2012).

129. Califano, J. P. & Reinhart-King, C. A. A Balance of Substrate Mechanics and Matrix Chemistry Regulates Endothelial Cell Network Assembly. *Cellular and Molecular Bioengineering 2008 1:2* **1**, 122–132 (2008).

130. Mutschall, H. *et al.* Bone tissue engineering using adipose-derived stem cells and endothelial cells: Effects of the cell ratio. *J Cell Mol Med* **24**, 7034 (2020).

131. Verseijden, F. *et al.* Prevascular structures promote vascularization in engineered human adipose tissue constructs upon implantation. *Cell Transplant* **19**, 1007–1020 (2010).

132. Pill, K., Hofmann, S., Redl, H. & Holnthoner, W. Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. *Cell Regeneration* **4**, 8 (2015).

133. Rohringer, S. *et al.* Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. *Angiogenesis* **17**, 921–933 (2014).

134. Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature 2000 407:6801* **407**, 249–257 (2000).

135. Cheng, M. H. *et al.* 3D-Bioprinted GelMA Scaffold with ASCs and HUVECs for Engineering Vascularized Adipose Tissue. *ACS Appl Bio Mater* **7**, 406–415 (2024).

136. Occhetta, P. *et al.* VA-086 methacrylate gelatine photopolymerizable hydrogels: A parametric study for highly biocompatible 3D cell embedding. *J Biomed Mater Res A* **103**, 2109–2117 (2015).

137. Lin, C. H., Lin, K. F., Mar, K., Lee, S. Y. & Lin, Y. M. Antioxidant N-Acetylcysteine and Glutathione Increase the Viability and Proliferation of MG63 Cells Encapsulated in the Gelatin Methacrylate/VA-086/Blue Light Hydrogel System. *Tissue Eng Part C Methods* **22**, 792–800 (2016).

138. Beudert, M., Gutmann, M., Lühmann, T. & Meinel, L. Fibrin Sealants: Challenges and Solutions. *ACS Biomater Sci Eng* **8**, 2220–2231 (2022).

139. Bolton-Maggs, P. H. B. & Pasi, K. J. Haemophilias A and B. *Lancet* **361**, 1801–1809 (2003).

140. Robertson, S. & Miller, M. R. Ambient air pollution and thrombosis. *Part Fibre Toxicol* **15**, 1 (2018).

141. Rojas-Murillo, J. A. *et al.* Physical, Mechanical, and Biological Properties of Fibrin Scaffolds for Cartilage Repair. *International Journal of Molecular Sciences 2022, Vol. 23, Page 9879* **23**, 9879 (2022).

142. Ceccarelli, J. & Putnam, A. J. Sculpting the blank slate: How fibrin's support of vascularization can inspire biomaterial design. *Acta Biomater* **10**, 1515–1523 (2014).

143. Orellano, I. *et al.* Engineering Vascular Self-Assembly by Controlled 3D-Printed Cell Placement. *Adv Funct Mater* **32**, 2208325 (2022).

144. Margiana, R. *et al.* Clinical application of mesenchymal stem cell in regenerative medicine: a narrative review. *Stem Cell Research & Therapy 2022 13:1* **13**, 1–22 (2022).

145. Ding, D. C., Shyu, W. C. & Lin, S. Z. Mesenchymal stem cells. *Cell Transplant* **20**, 5–14 (2011).

146. Wolberg, A. S. & Campbell, R. A. Thrombin Generation, Fibrin Clot Formation and Hemostasis. *Transfus Apher Sci* **38**, 15 (2008).

147. Hense, D. & Strube, O. I. Thrombin-Free Fibrillogenesis and Gelation of Fibrinogen Triggered by Magnesium Sulfate. *Gels* **9**, (2023).

148. Rieu, C. *et al.* Thrombin-free polymerization leads to pure fibrin(ogen) materials with extended processing capacity. *bioRxiv* 2020.05.12.091793 (2020) doi:10.1101/2020.05.12.091793.

149. Uehara, K. *et al.* The effect of fibrin formulation on cell migration in an in vitro tendon repair model. *J Orthop Sci* **26**, 902 (2020).

150. Montero, A. *et al.* Effect of fibrin concentration on the in vitro production of dermo-epidermal equivalents. *Int J Mol Sci* **22**, 6746 (2021).

151. Andrée, B. *et al.* Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels. *Scientific Reports 2019 9:1* **9**, 1–11 (2019).

152. Yavvari, P. *et al.* 3D-Cultured Vascular-Like Networks Enable Validation of Vascular Disruption Properties of Drugs In Vitro. *Front Bioeng Biotechnol* **10**, 888492 (2022).

153. Mykuliak, A. *et al.* Vasculogenic Potency of Bone Marrow- and Adipose Tissue-Derived Mesenchymal Stem/Stromal Cells Results in Differing Vascular Network Phenotypes in a Microfluidic Chip. *Front Bioeng Biotechnol* **10**, (2022).

154. Hughes, C. S., Postovit, L. M. & Lajoie, G. A. Matrigel: a complex protein mixture required for optimal growth of cell culture. *Proteomics* **10**, 1886–1890 (2010).

155. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756–760 (2006).

156. Wu, Y. *et al.* The influence of the stiffness of GelMA substrate on the outgrowth of PC12 cells. *Biosci Rep* **39**, BSR20181748 (2019).

157. Hade, M. D., Suire, C. N. & Suo, Z. Mesenchymal Stem Cell-Derived Exosomes: Applications in Regenerative Medicine. *Cells* **10**, 1959 (2021).

158. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods 2012 9:7* **9**, 671–675 (2012).

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Abbreviations

2D	two-dimensional
3D	three-dimensional
ASC	adipose derived stem cells
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EC	Endothelial Cells
EDTA	Ethylenediaminetetraacetic acid
EGM-2	Endothelial Cell Growth Medium 2
EthD-1	Ethidium Homodimer-1
FCS	fetal calf serum
FDA	food and drug agency
GFP	Green fluorescent protein
HBSS	Hank’s Balanced Salt Solution
hps	Human placenta substrate
HUVEC	Human Umbilical Vein Endothelial Cells
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
LBI	Ludwig Boltzmann Institute
LN_2	Liquid nitrogen
n.a.	not applicable
PFA	Paraformaldehyde
rcf	relative centrifugal force
RM	Regenerative Medicine
RT	Room temperature
TU	Technical University
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
DTT	Dithiothreitol
PEG	Polyethylene glycol
PA	Polyacrylamide