Fabrication of ulvan based structures for cell culture in wound healing

Sylvia van Kogelenberg

Unless otherwise indicated, the views expressed in this thesis are those of the author and do not necessarily represent the views of the University of Wollongong.

Recommended Citation
Fabrication of ulvan based structures for cell culture in wound healing

Sylvia van Kogelenberg
Professor Gordon Wallace
Dr. Zhilian Yue
July 14, 2017

Abstract
Progress within the field of biofabrication, and specifically additive manufacturing, is dependent on the development of hydrogel formulations and printing strategies. Biofabrication is an important tool for the creation of engineered tissue constructs, resembling native tissue in the positioning of biologically relevant materials, biocompatibility, mechanical behaviour, and reproducibility. The objective of this study was to fabricate constructs for wound healing purposes mimicking the natural state of healthy skin with an ulvan based material. In this study several different printing strategies have been explored, implementing Poloxamer 407, a thermosensitive block copolymer with favourable mechanical properties, as a support for the printing of modified ulvan, a biologically favourable material. By implementing the different printing strategies, an attempt was made to develop a biofabrication approach to allow for all stages of the bioprinting process.
Layman’s summary

Fabrication of ulvan based structures for cell culture in wound healing

Sylvia van Kogelenberg

Skin, one of the largest organs of the body, functions as a protective barrier preventing invasions of pathogens and other harmful substances. When damaged, a natural regeneration process is instigated. However, if the wound encompasses all skin layers or a large area of the body, the healing process is hindered and clinical interference is necessary (Chandika et al., 2015; Chua et al., 2016; Ehrlich&Krummel, 1996; Wicket et al., 2006). A contemporary approach to wound healing is skin tissue engineering, and specifically biofabrication. Biofabrication, the manufacturing of complex biological products, may provide a reproducible and economical method for the creation of biological and mechanical appropriate constructs with a high spatial organization of cells (Mironov et al., 2009). A functional skin substitute it needs to provide a moist environment, adequate oxygen and nutrient exchange, prevent infiltration of pathogens, and enhance natural wound healing (Groebner et al., 2011; Pereira et al., 2013).

In this study an attempt has been made to develop a highly defined tissue construct resembling skin, by combining ulvan and Poloxamer 407 as a bioink for extrusion in additive manufacturing. Ulvan, a polymer derived from green seaweed, was used to provide biologically favourable attributes, such as antioxidant activity (Alves et al., 2012), while Poloxamer 407 demonstrates favourable mechanical properties (Melchels et al., 2016).

To create a printable construct four different methods have been established: 1. printing a grid structure with different amounts of Poloxamer 407, 2. combining a casted hydrogel with a defined structure on top to recreate the structure of the dermal layer of the skin, 3. using a support structure to create surface tension and increasing the height of the total construct, and 4. manufacture a support structure from Poloxamer 407 for an ink consisting of ulvan. All structures showed promise for further study, however, for skin regeneration with ulvan, further continuation of method 1, containing the high amount of Poloxamer 407, and method 4 are advised. Further study must be executed in regards to optimizing the constructs, dermal cell response to the materials and structures, and the incorporation of supplementary cells and layers of the skin.
3.4 Biological characteristics ........................................................................................................ - 36 -
  3.4.1 Cellular morphology .......................................................................................................... - 36 -
  3.4.2 Metabolic activity and Proliferation .................................................................................. - 39 -
4. Future directions ....................................................................................................................... - 42 -
5. Conclusion ................................................................................................................................. - 43 -
References .................................................................................................................................. - 36 -
Acknowledgements ....................................................................................................................... - 44 -
Abbreviations and Acronyms ...................................................................................................... - 49 -
Appendix ..................................................................................................................................... - 50 -
  A. Poster; ACES electromaterial symposium 2017 ................................................................... - 50 -
  B. Front page and abstract literary review; Submitted: 09-07-17 to Advances in Wound Care - 51 -
1. Introduction

Skin is a protective barrier with immunologic and sensorial functions, preventing invasions of pathogens and other substances harmful to the body (Chandika et al., 2015; Chua et al., 2016; Ehrlich & Krummel, 1996; Wicket et al., 2006). When damaged, a natural regeneration is instigated: first by forming a platelet plug, followed by inflammation, proliferation, and remodelling. However, when the wound encompasses all skin layers and regenerative elements, healing is delayed or inhibited. This results in poor regeneration, inadequate vascularization and scar formation (Ehrlich & Krummel, 1996; Pereira et al., 2013). To enhance the healing process, wound dressings are applied to the damaged area. However, current treatments are insufficient in serious burn victims, chronic wounds, and other wounds that encompass large areas of the body. Hence, there is an overwhelming need for the development of new skin replacement strategies. In this in vitro study an attempt is made to biofabricate an artificial skin replacement with ulvan, a green seaweed derivative, as base for the bioink. With this bioink and construct an attempt is made to addresses current complications in addressing large area wounds, such as immune rejection of allografts and donor shortage for autografts.

1.1 The skin

For the development of a well-functioning skin tissue replacement, a thorough understanding of healthy skin and the wound healing process is necessary. Healthy skin can be divided into three main layers: the epidermal, dermal, and hypodermal layer. The epidermal layer is the outer layer and in contact with the environment. It plays a significant role in the protection of the body against harmful agents and the prevention of moisture loss (Pereira et al., 2013a). 90-95% of the epidermis consists of densely packed keratinocytes; the remaining 5-10% consists of melanocytes, Langerhans cells, and merkel cells (Lamers et al, 2013). Under normal circumstances, the epidermis is renewed in 16-24 days. This starts with the division of a keratinocyte located in the stratum basale, after which one daughter cell remains and the other cell moves through the other layers. During that time, the cell body will flatten and grow, and before it reaches the stratum corneum, the outermost layer of the epidermis, the nucleus will start to break down. The stratum corneum consists only of dead keratinocytes, which are regularly detached and replaced in desquamation (Alberts et al., 2002). At the interface of the epidermis and the dermis, the basal lamina is located, responsible for attachment point for the epidermal cells and a separation of the layers. The dermis contains inter alia fibroblasts, collagen and elastin. Collagen and elastin are responsible for the unique properties of elasticity and mechanical integrity found in the skin. However, mechanical properties are also dependent on ageing, orientation of mechanical force, hydration, and region of skin (Lamers et al., 2012). Oxygen and nutrients are delivered to both the epidermal and dermal layer via the vessels present in the dermal layer (Michael et al., 2013; Pereira et al., 2013a). The thickness of the dermis varies between 0.05 mm at the eyelids, to 1.5 mm at the soles of the foot. The hypodermal layer, also known as the subcutaneous layer, consists of adipose tissue, where adipocytes are embedded in connective tissue (Lamers et al., 2013).
If skin is damaged, a natural wound healing process is instigated, which consists of three distinct phases: the inflammatory phase, new tissue formation, and tissue remodelling (Shäfer&Werner, 2008; Chandika et al., 2015). During the inflammatory phase, one to three days after initial injury, the wound is closed with a clot consisting of fibrinocytes, and recruitment of inflammatory cells, namely mast cells, neutrophils, lymphocytes and macrophages, via activation of the complement system, degranulation of platelets and the degradation of bacteria (Figure 1A).

The tissue formation phase occurs three to ten days after initial injury and consists of copious amounts of macrophages present in the wound tissue and angiogenesis in the newly formed tissue. In this stage, migration of keratinocytes and hair follicles occurs from the edge of the wound along the dermis (Figure 1B). Tissue remodelling occurs two weeks after injury; after the completion of the vascularization. The density of granulation tissue decreases and the extracellular matrix (ECM) is reformed (Figure 1C). Scar formation, which occurs in larger wounds, leads to a reduced tensile strength, thereby compromising one of the main functions of the skin (Chandika et al., 2015; Shäfer&Werner, 2008).

1.2 Considerations for skin tissue engineering

Traditional wound dressings, such as bandages, wool, or cotton, are still used regularly and provide a protection against pathogens and other environmental hazards to proper wound healing. However, as they absorb fluid extrusions and need to be removed while sometimes adhering to the wound, application of these dressing may lead to impairment of proper healing (Pereira et al., 2013).

Currently, autologous split-thickness grafts are a preferred method of skin regeneration, as it shows good adhesion, provides pain relief, and rejection of the graft is reduced compared to allografts. However, the amount of material is limited to the patient and available donor sites, thereby being greatly limited in patients with large area wounds, such as serious burn victims (>60% total body surface affected), elderly patients and children, due to small healthy skin area, low regeneration rate, and low total area respectively (Sood et al., 2010). Allografts, on the other hand, may lead to disease
transmission and immune rejection (Loss et al., 2000). Therefore, a pressing need for appropriate wound healing methods still remains.

To restore the skin to its natural healthy conditions, the tissue replacement should closely resemble the skin layering and composition. Because of the complexity of the skin, the development of a proper functioning replacement tissue faces many challenges still. Generally speaking, to engineer skin substitutes, four key requirements must be adhered to: 1. Engineered skin must be able to provide a moist environment; to promote healing. 2. Engineered skin must be able to enhance natural wound healing responses. 3. Replacement tissue must also be able to provide an adequate oxygen exchange with the surrounding environment. 4. It must limit the infiltration of potential harmful bacteria and pathogens (Groeber et al., 2011; Pereira et al., 2013a).

Important to consider in engineering of skin substitutes are also patient safety, degradation time, duration of cover, (neo)dermis formation, shelf life, cost, mechanical stability, scaffold vascularization, and amount of stages for completion of treatment (Yildirimer et al., 2012). Preferably, a replacement has the structural composition and mechanical properties similar to the ECM (Kirchmajer & Gorkin, 2015). Tissue engineered skin substitutes currently applied are epidermal substitutes, dermal substitutes and dermo-epidermal substitutes. However, currently these scaffolds are often difficult to handle, with poor adhesion to the wound bed, poor vascularization, no promotion of regeneration of full-thickness wounds, and high manufacturing costs (Pereira et al., 2013a). These complications are the result of the relative simplicity of the constructs (Hrabchak et al., 2006).

Improvements in wound healing can be made in regards to the high manufacturing costs, regeneration of the skin, immune rejection, ease of handle, and complexity of the material. High manufacturing costs may be addressed by the use of bioprinting, by bringing standardization to the fabrication process. This would reduce manual labour, reduce cost, creates reproducible scaffolds, and maintains the possibility of creating a patient specific process. Immune rejection and regeneration of the skin may be addressed by the adaptation of the scaffold in which the cells are seeded.

1.3 Biofabrication

To overcome current limitations in skin tissue engineering, higher complexity of the construct must be achieved (Hrabchak et al., 2006; Pereira et al., 2013a). The utilization of biofabrication strategies, foremost three-dimensional (3D) printing, enables controlled construction of tissue-like structures in three dimensions (Malda et al., 2013). This leads to increased spatial control of cells, structural integrity, closer imitation of natural tissues, and the possibility of the addition of different elements in one construct. Currently, three (bio)fabrication strategies have been explored to fabricate artificial skin tissues, namely: (drop on demand) inkjet printing, laser cell printing, and electrospinning (Lee et al., 2009; Lee et al., 2014; Koch et al., 2012; Pereira et al., 2013a; Planz et al., 2016).
1.3.1 Bioprinting

Lee et al. (2009) applied a stage controlled inkjet printer to create a layer-by-layer construct containing both primary adult human dermal fibroblasts and primary adult human epidermal keratinocytes, printed in a polydimethylsiloxane (PDMS) mould. The layers were constructed by printing collagen, onto which a layer of cells was printed. After each layer, the cell-containing gel was crosslinked via a surface coating of nebulized aqueous sodium bicarbonate solution. This design consisted of 10 layers of collagen, the second layer seeded with fibroblasts, and the eighth containing keratinocytes (Figure 2A). A viability of 95.0±2.3% fibroblasts and 85.5±5.7% keratinocytes was shown after printing, compared to 96.6±3.9% and 83.9±7.1% respectively for the control group, which indicated that inkjet printing had a minimal effect on the cell viability. However, the printed cells were still inhomogeneously organized.

To improve upon this method, Lee et al. (2014) altered the spatial organization of the collagen layers. In the attempt to approach the structure and spatial cell organization of native skin, more layers were

A.

![Diagram A](image)

B.

![Diagram B](image)

**Figure 2**: Construction of two designs of layer-by-layer printing of collagen, human dermal fibroblasts, and keratinocytes. A. illustrates the design by Lee et al. (2008) containing 10 layers, with layer 2 consisting of collagen and embedded fibroblasts and layer 8 consisting of embedded keratinocytes, while B. shows the design of the follow up study by Lee et al. (2014) containing 13 layers, with layer three, six, and nine consisting of embedded fibroblasts, and layer twelve and thirteen consisting of embedded keratinocytes.
added to the design, whereby the keratinocytes were printed in a close range to each other, and the fibroblasts were further apart. The advanced design consisted of 13 layers, with the third, sixth, and ninth containing fibroblasts and the twelfth and thirteenth layer containing keratinocytes (Figure 2B). This design increased morphological and spatial cell distribution resemblance of skin. In this study, the cells were cultured for 7-14 days. The 3D printed constructs were better able to retain their shape after 7 days of culture, compared to manual deposition.

Koch et al. (2012) applied laser-assisted bioprinting (LaBP) to develop a 3D spatial controlled construct resembling human skin (Figure 3). A bi-layered construct was created, containing murine fibroblasts and human immortalized keratinocytes in Matriderm™. 10 days of culture demonstrated vitality and proliferation in all cell types. Also, a layer of laminin, the main component of the basal lamina, had formed in between the keratinocyte and fibroblast layer (Figure 3C). The formation of this layer shows the increasing complexity can be formed by the 3D spatial arrangement of the cells and the influence of multiple cell types in one structure. The intra-cellular communication was assessed by analysis of adherence- and gap-junctions. Adherence junctions were found in abundance between the keratinocytes, but to a lesser extend between the fibroblasts. A yellow dye penetrated from a destroyed keratinocyte into surrounding cells and further, demonstrated the presence of functional gap junctions. Similarly, Michael et al. (2013) applied LaBP to fabricate a bilayer cellularized skin substitute, assembling a construct containing 20 layers of keratinocytes on 20 layers of fibroblasts in Matriderm™. These constructs were tested in vivo in full thickness wound in mice. All animals survived the surgery and surrounding tissue connected with the implanted skin substitute; no inflammatory or necrotic processes were detected. Proliferation in the epidermal and dermal layer was found both in healthy mouse skin and the skin constructs, but not in the negative control. In the skin substitute, a blood vessel was formed after 11 days, but no complete vascularization could be achieved. Both studies show LaBP as a promising approach in skin tissue engineering. It is encouraging in regards to spatial cell arrangement, angiogenesis stimulation, and connecting with native tissue and should be further explored.
1.3.2 Melt electrospinning

Another (bio)fabrication method utilized in skin tissue engineering is electrospinning, mainly used to mimic biomechanical and structural characteristics of skin. Electrospinning is a technique whereby a polymer is extruded through a needle via forces applied through a high potential difference between needle and collector plate. In skin ECM, elastin and collagen fibres are present. Elastin fibres are the energy absorbers, while collagen fibres are important for load transfer (Planz et al., 2016). This technique also has relative low costs and high capacity for binding multiple molecules.

In 2014, Bonvallet et al. developed three scaffold types from polycaprolactone (PCL), 50:50 collagen I|PCL, and 70:30 collagen I|PCL. Immortalised J2 mouse fibroblasts were seeded onto the scaffolds. 160 µm pores between 70:30 collagen I|PCL fibres were filled with ECM after 14 days, which did not occur in 250 µm pores or for 50:50 collagen I|PCL fibres. Fibroblast viability after 21 days for 70:30 collagen I|PCL were over 90%. Mechanically, the scaffold constructed of PCL alone performed best in tensile testing conditions, no significant difference was found between the 50:50 and 70:30 collagen I|PCL scaffolds. After culturing fibroblasts on 70:30 collagen I|PCL for 10 days, keratinocytes were seeded onto the construct. Scaffolds of 70:30 collagen I|PCL with 160 µm pores, no pores, or a traditional gauze were implanted into 15 mm diameter full-thickness wounds in the skin of Sprague-Dawley rats. At every time point, up to 21 days, it was established that the 70:30 collagen I|PCL scaffolds with 160 µm pores the area of abnormal tissue was significantly smaller compared to the other scaffold type, or traditional bandage (Bonvallet et al., 2014). Bonvallet et al. (2015) repeated this experiment, comparing aforementioned scaffolds with an acellular microporous scaffold, demonstrating minimum abnormal tissue was found in 70:30 collagen I|PCL scaffolds with 160 µm pores.

In 2016, Planz et al. also attempted to mimic the mechanical integrity of skin ECM. Electrospun PCL fibre, PCL|gelatin blends, and PCL|gelatin with pure gelatin constructs were developed to imitate the biomechanical characteristics of human skin, and provide both the required stiffness and the flexibility of human skin. It was established that a high proportion of gelatin has a higher cell attachment. Blending PCL and gelatin lead to the highest mechanical resistance of the fibres, but a lower strain was required to break the material. Highest cell attachment was found in the PCL|gelatin hybrids, and culturing fibroblasts and keratinocytes together on a single structure retained the hierarchical organization. These studies show the promising possibilities of electrospinning in skin tissue engineering, as it shows good cell viability and infiltration into the scaffolds. Also, in regards to mechanical properties of the material, promising results have been found.

Further optimization of biofabrication techniques may lead to more complex tissues, including the organization of the skin and the different ECM components in each layer, allowing for the structural proteins collagen and elastin (Pereira et al., 2013). Application of these techniques can be applied in many areas of tissue engineering and provide the opportunity to study cells in a 3D environment. These techniques show potential in expanding current knowledge and in developing tissues closer resembling native tissues. In this study, the focus lies on extrusion printing of encapsulated cells. However, advantages can be gained at later stages by the incorporation of several techniques, such as
electrospinning, extrusion printing, and the assimilation of different types of hydrogels and other materials.

1.4 Bioinks

1.4.1 Material considerations

Essential in the development of tissue engineered constructs via 3D extrusion printing is the development of appropriate bioinks. Several requirements must be met in regards to mechanical and biological properties. Mechanical considerations include the shape vitality after extrusion, high viscosity, shear thinning behaviour, and adaptability of mechanical properties before and after printing (Mouser et al., 2016). Biological bioink design considerations include biocompatibility, support of cell vitality, differentiation, possible migration, and cell-cell communication. Furthermore, material must be appropriate for its specific end function in regards to cellular behaviour in response to material (Lee&Yeong, 2016).

Hydrogels have been applied in many variations as bioinks, because they mimic ECM behaviour and retain high water content, thereby supporting cell vitality. Furthermore, hydrogels can be tailored in biological and mechanical behaviour attuned for different cells and tissues. To provide an overview of mechanical behaviour in response to shear stress, rheological testing should be performed. The materials response to shear strain, recovery, and temperature should be determined. The importance of shear thinning, which is the decrease of viscosity under shear strain increase, in extrusion printing lies in the decrease of pressure necessary during extrusion while maintaining mechanical integrity in absence of pressure. (Ferris et al., 2015; Li et al., 2016; Melchels et al., 2014; Mouser et al., 2016). The consistency in shape in the absence of applied pressure is of particular importance to maximize definition and height of construct (Melchels et al., 2014). Additional crosslinking is required to permanently secure the desired design and prevent dissolution within cell culture medium at later stages. Furthermore, changing the mechanical properties after the printing process can also further direct the biological behaviour in the constructs (Chan et al., 2008). Before the start of the extrusion process, material response to temperature must be determined, with the focus on the sol-gel transition, as printing temperature needs to be set for gel formation. Lastly, it is argued that yield behaviour of the material may be of particular importance, as it provides control over the pressure needed to initiate the printing process (Mouser et al., 2016).

Besides the physical behaviour of material on biological behaviour, the chemical construct of a material influences the cellular environment. Popular hydrogels previously applied in biofabrication are collagen, gelatin, hyaluronic acid, chitosan, and alginate. Most of these need to be modified for crosslinking. These macromolecular structures have proven to improve cell proliferation, DNA synthesis, and production of ECM. These hydrogels can also be supplemented with growth factors, nutritional molecules, and other molecules influencing physiological behaviour of cells.
1.4.2 Marine derived biopolymers and ulvan

Traditionally, naturally derived materials have been used in wound healing to stimulate the natural healing process and prevent infections. Currently, increasing interest is shown in marine derived products in tissue engineering. Marine derived materials for wound healing, currently used in skin tissue engineering include collagen, alginate, chitin, chitosan, hyaluronic acids, and fatty acids. These materials can provide several advantages within the wound healing process by providing bioactive, biodegradable structures engineered to have an optimal porosity and mechanical properties. At the same time, marine derived materials negate the necessity of animal derived materials and accompanying disadvantages, such as high cost and autoimmunological responses. Furthermore, via the delivery of small molecule modulators, marine polymers may be able to direct signal transduction, thereby stimulating the wound healing process (Chandika et al., 2015).

An upcoming marine derived material in tissue regeneration is ulvan, a sulphated polysaccharide collected from the cell walls of green seaweed belonging to the ulvale family. It shows structural fibre similarities to glycosaminoglycan; specifically hyaluronic acid (Kaeffer et al., 1999). Ulvan has been proposed to have potential in wound healing, wound dressing, and skin tissue engineering due to its biocompatibility and economical advantage. Furthermore, ulvan is known to have antiviral, anticoagulant, and antioxidant properties (Alves et al., 2012). Thereby, it can be modified for photocrosslinking, similar to other hydrogels in biofabrication such as gelatin (Figure 4).

Ulvan is constructed from two major disaccharide repeating units; the ulvanobiuronic acid 3-sulfate type A and type B (Robic et al., 2009). It contains several biological interesting functional groups, such as sulphate, thamnose, xylose, glucuronic acid, and iodome (Lahaye&Robic, 2007). All of these groups may have an important effect on mediation of the wound repair process against degradation of the scaffold and in the general wound healing process (Andrés et al., 2005). The sulphate containing rhamnose group (L-rhamnose-3-sulfate) displays a chemical composition similar to that of other sulphated glycosaminoglycans, for example chondroitin sulphate. Chondroitin sulphate-based materials have been used before as biomaterials for tissue engineering due to their positive effect on cartilage specific gene expression, osteogenic differentiation, recovery of spinal cord injury, and promotion of the wound healing process. In addition, polysaccharides rich with rhamnose groups have been shown to stimulate cell proliferation, collagen biosynthesis, and decrease elastase activity. Also, hyaluronic acid regulates cell behaviour in wound healing and inflammation.

![Figure 4: Functionalization of Ulvan with methacrylic anhydride (Figure adapted from Morelli et al., 2010).](image-url)
(Xue et al., 2015). The close resemblance of the ulvan molecule may be of particular influence on wound healing behaviour. Considering the composition of the human skin, the molecular construct and rhamnose groups may prove to be of particular importance (Andrès et al., 2005).

Even though ulvan meets the biological requirements for a bioink, further modification is required in order to improve its printability and structural stability of 3D printed structures, due to its low viscosity (Lahaye & Robic, 2007). Ulvan forms a weak hydrogel through a cation-mediated physical crosslinking mechanism, which results in poor structural integrity. This may be improved chemically by producing stabilized polymer networks via chemical crosslinking or introducing another (temporary) material to the bioink. Attaching a methacrylate group to the ulvan chains has been reported to produce photocrosslinkable hydrogels to improve mechanical integrity of the end constructs (Figure 4) (Morelli et al., 2010). In this study, an attempt has been made to improve the mechanical characteristics of the bioink during and after the printing process. It was attempted to increase the viscosity of the methacrylated ulvan, via the addition of calcium chloride and boric acid, Gellan gum, or Poloxamer 407. After printing, the material was stabilized by photo crosslinking the methacrylate groups.

1.4.3 Gellan Gum

Gellan gum, an anionic linear polysaccharide derived from the Sphingomonas paucimobilis bacteria, is a promising material for the fabrication of bioinks, due to high yield, FDA approval, and low cost due to bacterial origin (Ferris et al., 2015; Giavasis et al., 2008; Oliveira et al., 2009). Gellan gum consists of three main structural components, namely glucose, glucuronic acid, and rhamnose (60%:20%:20% respectively) (Giavasis et al., 2008). The functional properties of this gel are the adaptable gel modulus, brittleness and elasticity, the clarity of the material, the flexibility of the temperature. Thereby, the material is cytocompatible, as it does not participate in specific cell binding interactions, and biodegradable (Morris et al., 2012; Stevens et al., 2016). Biodegradation was reported to be 10-15% over a period of 168 days (Stevens et al., 2016).

To create a functional gel, gellan gum must be added to distilled water, with the addition of salts of calcium, sodium, potassium, or magnesium, as the divalent or multivalent cations are responsible for the gelation by crosslinking, creating aggregation of gellan gum to form a random coil state followed by multiple gellan helices aggregating into junction zones (Silva-Correia et al., 2013; Stevens et al., 2016). This is an ionotropic interaction, in which the (poly)cations and (poly)anions form electrostatic interactions (Kirchmajer & Gorkin, 2015). Maximum strength will be achieved with ± 0.004% w/v calcium, 0.005% (w/v) magnesium, ± 0.16% (w/v) sodium, or 0.12% (w/v) potassium. Also at 1% (w/v) gellan, the addition of MgCl2 or CaCl2 at 0.1% (w/v) showed high gel strength (800g/cm2). Concentration of gellan gum is on average 0.04-0.2% (w/v) (Giavasis et al., 2008).

1.4.4 Poloxamer 407

Poloxamer 407, also known as Pluronic F-127 or Lutrol, is a synthetic polymer consisting of a central poly(propylene oxide) (PPO) with poly(ethylene oxide) (PEO) on either side. It has been applied in wound dressing and artificial skin tissue engineering, as a shear protectant and sacrificial material in 3D tissue engineering (Müller et al., 2015).
Due to the hydrogen bond formation, the Poloxamer 407 is better soluble at low temperatures. The material forms a soft gel at 20% w/v. Strength increases in proportion with the increasing w/v (Gioffredi et al., 2015). Gelation of the Poloxamer 407 made is thermoreversible, which makes it an ideal material for mechanical support in 3D extrusion printing, as it can be removed at a later stage. The material displays inadequate mechanical properties to function solely as a tissue engineering gel. However, due to fast dissolution in a liquid environment and shear thinning behaviour it is an ideal material for 3D extrusion printing (Klouda&Mikos, 2007). For all additive manufacturing techniques, a control over the mechanical properties of the bioink is a must. It must be possible to handle the material at a liquid state before the printing process, as it should be possible to add cells, growth factors, or other biological components. However, for the actual printing process the material should exhibit a higher viscosity, often induced via chemical or physical crosslinking. After extrusion of the material, crosslinking is often induced via a photoionization process, to ensure durability and stability of the end construct. Poloxamer 407 allows liquid handling below the lower critical solution temperature (LCST). Additionally, the material shows shear thinning behaviour, which has been repeatedly reported to be desirable for extrusion printing purposes (Müller et al., 2015; Mouser et al., 2016).

1.5 Aims

The purpose of this project was to develop a fabrication approach to ulvan-based hydrogels. This would allow for controlled distribution of cells, while maintaining mechanical stability in order to optimize printability, which would promote a patient specific, reproducible approach in wound healing. The use of ulvan and fibroblastic cells would enable enhanced regeneration of the epithelial tissue, thereby decreasing the risk of infection and morbidity. Focus of this project is the adjustment of parameters affecting the printability of the ulvan, the polymers effect on cellular behaviour in regards to wound healing, and increasing the possible complexity of the total construct via biofabrication.

2 Materials and Methods

2.1 Ulvan methacrylate synthesis

Ulvan (PhycoTrix™; provided by Venus Shell Systems) was functionalized with methacrylate anhydride (MA) to enable photo cross linking to stabilize 3D printed structures and ensure the proper mechanical properties (Figure 4).

Before methacrylation, the raw material was purified by addition of 10% w/v ulvan into distilled water (DI) (60°C) and stirred until dissolution. Thereafter, solution was dialyzed for 24h against DI (1:10) (cellulose membrane with 12-14 kDa cut off, Sigma Aldrich). The remaining solution was freeze-dried. To functionalize the material, the lyophilized ulvan was dissolved 10% w/v in Milli-Q (resistivity 18.2 MΩ cm) at 60°C. Dimethylformamide (DMF) with equal volume to water was added at room temperature (RT) and vigorously stirred overnight to ensure homogeneity. Methacrylic anhydride (5 molar equivalents of disaccharide repeating units of ulvan) was then added
dropwise, after which the pH of the reaction system was retained at ~8.5 for the first 2h by adding 5N NaOH solution using a syringe pump. The reaction was left overnight under stirring at RT. The raw product was isolated by precipitation in ethanol, and then purified by dialysis in DI water. The lyophilized final product, ulvan methacrylate (UMA), was characterized with hydrogen-1 nuclear magnetic resonance (H NMR) spectrometry, showing a degree of modification of 1.1. All assessments of functionalization of UMA material were performed in collaboration with Xifang Chen (PhD).

2.2 Bioink formulation

Purification of a commercial low-acyl Gellan Gum (GG) (Gelzan™ CM; received as a gift from CP Kelco), was performed to remove divalent cations, based on an established method described by Kesti et al. (2015). Briefly; 1% w/v GG in Milli-Q was stirred for 1-2h (60°C). This mixture was dialysed against ethylenediaminetetraacetic acid (EDTA) at 0.001% w/v in Milli-Q for 24h and against Milli-Q for another 12h to form purified GG.

All GG inks were initially synthesized without the addition of UMA to assess the suitability of the material as an ink. GG was formulated according to the protocol described by Ferris et al. (2015), with variations in salt type and concentrations. In short; 0.2-1% w/v (in steps of 0.2% w/v) low acyl GG was dissolved at 80°C in Milli-Q. GG was then mixed with 10% w/v phosphate buffered saline (PBS), 20% w/v PBS, 0.07 mM CaCl₂, 0.085mM CaCl₂, 0.1 mM CaCl₂, or 1% w/v Dulbecco’s modified of Eagle’s Medium (DMEM) to initiate a cation-based physical crosslinking process (Table 1) (Ferris et al., 2015; Mouser et al., 2016; Oliveira et al., 2008).

Based on the previous results a selection of inks was made; 9% w/v|0.9mM/0.45mM|0.28mM UMA|H₃BO₃|CaCl₂, 9% w/v|0.45mM|1% w/v UMA|H₃BO₃|DMEM, 9%|1%|1% w/v UMA|GG|DMEM, and 20%|10% w/v Poloxamer 407|UMA (5°C) (Tab. 1). These inks were visually assessed for viscosity, homogeneity, and shape retaining properties after extrusion. Then a rheological study was performed on the most promising ink; 20%|10% w/v Poloxamer 407|UMA to which 41µl 3% w/v lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was added. After casting/printing of the ink UMA would be crosslinked and Poloxamer 407 removed via diffusion (Figure 5).

Table 1: Bioink formulations

<table>
<thead>
<tr>
<th>Initial tests</th>
<th>Subsequent tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial tests</td>
<td></td>
</tr>
<tr>
<td>0.2-0.8% w/v GG</td>
<td></td>
</tr>
<tr>
<td>1% w/v GG</td>
<td>1% w/v DMEM</td>
</tr>
<tr>
<td></td>
<td>0.28mM CaCl₂</td>
</tr>
<tr>
<td></td>
<td>0.9mM H₃BO₃</td>
</tr>
<tr>
<td></td>
<td>0.45mM H₃BO₃</td>
</tr>
<tr>
<td></td>
<td>1% w/v GG</td>
</tr>
<tr>
<td></td>
<td>1% w/v DMEM</td>
</tr>
<tr>
<td></td>
<td>0.45 mM H₃BO₃</td>
</tr>
<tr>
<td></td>
<td>1% w/v GG</td>
</tr>
<tr>
<td>1% w/v PBS</td>
<td>20% w/v Latrol</td>
</tr>
<tr>
<td>20% w/v PBS</td>
<td></td>
</tr>
<tr>
<td>0.07 mM CaCl₂</td>
<td></td>
</tr>
<tr>
<td>0.085 mM CaCl₂</td>
<td></td>
</tr>
<tr>
<td>0.1 mM CaCl₂</td>
<td></td>
</tr>
<tr>
<td>1% w/v DMEM</td>
<td></td>
</tr>
<tr>
<td>10% w/v UMA</td>
<td></td>
</tr>
</tbody>
</table>
Although rheological results showed promise, further study indicated no sufficient height in printing could be reached. Therefore, a total of 3 inks were developed and extruded during the course of this study, to which LAP was added to alter degree of crosslinking; Ink 1: 20%|10% w/v Poloxamer 407|UMA in PBS, Ink 2: 25%|10% w/v Poloxamer 407|UMA in PBS, and Ink 3: 10% w/v UMA in PBS. The last of these was reinforced a structured formed by 30% w/v Poloxamer 407 in PBS. In this study LAP was employed as a photoinitiater to control the stability of 3D printed structures via light-mediated chemical crosslinking mechanism.

2.3 Bioink characterization

Based on initial visual results, only 20%|10% w/v Poloxamer 407|UMA inks were characterized for printability. Although the formulation of this ink was adapted at a later stage, rheological characterization for the newly developed inks was not executed, due to time restraints and similarity in printing characteristics to the previous ink. All rheological characterization was performed with a stress controlled rheometer (AR-G2, TA instruments, New Castle, United states). Samples were loaded at RT, equilibrated at the experimental temperature for 5 minutes to forgo any mechanical memory of the ink. After geometry was lowered to gap settings, excess material was removed. Each sample was used for a single test.

2.3.1 Temperature sweep

A plate-plate temperature sweep (oscillatory) (⌀ 15mm; gap 500µm) was performed at 7-40°C, frequency 1.0Hz and strain 1% (n=3), to assess at which temperature the storage modulus would surpass the elastic modulus to determine the sol-gel transition and therefore the printability range. A strain and frequency sweep to assess the range of testing parameters at the correct temperature was performed. Considering the initial test was within the proper range, these values have been retained.

Figure 5: A. The in PBS dissolved Poloxamer 407 and UMA are printed/cast B. UMA is photo crosslinked to create a stable structure. C. The construct is placed in cell culture medium to reduce the amount of Poloxamer within the construct via diffusion to increase biocompatibility.
To determine if the addition of 41µl of a 3% w/v LAP solution (final concentration: 0.12% w/v) would not have influence rheological behaviour of the ink, a comparison was made between 20%|10% w/v Poloxamer 407[UMA, 19.2%|9.6% w/v Poloxamer 407[UMA, and 19.2%|9.6% w/v Poloxamer 407[UMA superimposed with 0.12% w/v LAP. These data were also used to assess the temperature dependence of the materials storage modulus (G’) and elastic modulus (G”). For convenience, any referencing to the ink will state the standard ink concentrations separately from added LAP concentration.

2.3.2 Strain & Frequency

A plate-plate (oscillatory) (⌀ 15mm; gap 500µm) strain sweep was performed at 37°C (based on previous results), this point determined in combination of the gelation point determined with a quick temperature sweep, the printability of the material, and the preferred temperature for optimal cell behaviour. The strain sweep was performed from 0.1 to 100% strain at a frequency of 1.0Hz. Subsequently, a frequency sweep was performed from 0.1-100Hz at 1% strain (protocol based on Zuidema et al., 2014).

2.3.3 Recovery

A plate-plate step strain (oscillatory) (⌀ 15 mm; gap 500µm) was performed at 37°C, frequency 1.0Hz and strain 1% (n=3). Seven increments, each of 5 minutes were performed, with strains alternating between 1% and 1000%. This experiment was performed to assess the recovery of the material after deformation, of particular importance within the printing process where a material is regularly exposed to varying pressure.

2.3.4 Viscometry

Rotational cone-plate (angle 2°; ⌀ 15mm; gap 55µm) measurements (37°C) were performed with strain rate: 0.1-100 s⁻¹ or stress ranging from 0.1-600 Pa to determine viscosity, the printability and shear behaviour (n=3). Flow behaviour of a material can be described by:

$$\tau = a + k\dot{\gamma}^{(n-1)}$$

Eq. 1

\(\tau\) describes the shear stress as a function of shear rate (\(\dot{\gamma}\) (s⁻¹)). K describes the consistency index, \(a\) in the index of the power law model. When \(n=1\), the material is a Newtonian fluid. Is \(n>1\) shear thickening behaviour is found, while if \(n<1\) the material shows shear thinning behaviours. The Herschel-Burkley yield stress is indicated by \(a\), when \(a>0\) the material will show a Bingham plastic behaviour (Kesti et al., 2015).

2.3.5 Swelling behaviour

Casted gels of 0.09 mL in disk shape (20%&25%|10% Poloxamer 407[UMA) (n=3) were used for swelling measurements. Gels were incubated for 0.5, 1, 2, 4, and 24 hours at 37 °C in cell culture medium. Cell culture medium was removed and scaffolds were weighted to obtain wet weight, after which the gels were lyophilized and dry weight was obtained. Swelling ratio was determined by:

$$R = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}}$$

Eq. 2
Additive manufacturing

Ink printing was facilitated via a 3D bio extrusion printer (3D Bioplotter®, EnvisionTEC, Gladbeck, Germany). Extrusion was driven by pneumatic pressure and positioned with a xyz-controlled robot head. Disposable syringes were used with a 200 µm diameter nozzle-tip. Velocity of the inkjet was set to 10 mm s\(^{-1}\). Pressure was determined for the optimal printing of 20%|10% w/v Poloxamer 407|UMA containing 0.12% w/v LAP at 37°C by printing single strands with changing parameters and by printing 10×10 mm grids with a theoretical fibre spacing of 100µm to assess the maximum amount of height that could be reached with different parameters. Printed constructs were crosslinked by 30-60 seconds exposure to visible light at 400nm wavelength (\(\lambda\)) at 5cm distance. At a later stage a subsequent layer would be deposited, containing keratinocytes, to further mimic the morphology of skin tissue (Figure 6).

2.4.1 Photocrosslinking

To determine irradiation of the visible light source (Omnicure® LX 400+, 400nm \(\lambda\), Lumen Dynamics, Mississauga, Canada), a LED light meter system (Omnicure, model # LM-2011/LS100, Lumen Dynamics, Mississauga, Canada; 50mW/cm²-25000mW/cm²) was positioned in concurrence with standard conditions; 5 cm from the light sensor. Measurements were taken within the first 10 seconds of exposure to the light source to ensure consistency. Due to the limited range of the LED light meter system, a slope of the irradiation curve was determined by measurements from 100 downwards, with intervals of 5, until the measurement system was unable to detect irradiation. Correlation between the experimental values and a linear trendline were assessed with the correlation coefficient (\(R^2\)). Degree of exposure and amount of LAP was adjusted during the course of this study; originally starting with 0.12% w/v LAP, later decreased to 0.06% w/v. For 25%|10% w/v Poloxamer 407|UMA this was further decreased to 0.03% w/v. Light intensity was decreased from the initial 30%, to the machine minimum of 15%. Material was exposed for 30sec.

Figure 6: Overall concept idea/goal: illustrating the printing process of the A. dermal layer (applied in this study) and the B. epidermal layer (to be addressed in a follow-up study)
2.4.2 Manufacturing methods

Several printing methods were implemented in order to create functional artificial skin tissue, namely: standard grid printing, combining traditional casts with high definition formation via bioprinting, increasing height via surface tension, and a Poloxamer 407 reinforcement structure. Initial rheological characterization was performed on Ink 1; 20%|10% w/v Poloxamer 407|UMA, followed by parameter tuning on the 3D bioprinter. Methods were disqualified if structures could not retain shape and/or definition, or if material showed biological incompatibility. Sequence of experiments has been illustrated in Figure 7.

For the determination of printing parameters for the printing of a standard lattice, the material was extruded to define the required pressure. Depending on material behaviour, the speed of extrusion could be directly determined (in case of strand formation at needle tip), or a manual parameter tuning would be performed (droplet formation at needle tip). The last of which was omitted at a later stage due to superseding effect of grid pattern over the effect pressure and speed. If strand formation occurred, parameters were determined according to standard manufacture manual protocol (EnvisionTEC, 2010). Briefly; speed was derived from by 2 second extrusion duration at a safe height, to determine resulting strand length over time. Initial parameters for ink 1 were examined via the manual parameter tuning. Parameters were determined via the extrusion of strands at 0.1mm height and 100mm in length, varied in pressure and speed. Based on this, further fine-tuning of parameters required was determined via the construction of the end scaffold; a 10mm² grid (Method 1). Parameters were varied until a stable, highly defined structure could be formed. Quality of a print was dependent on homogeneity, stability, and continuity of strands, maximum height of construct, and accumulation at crossing points. Parameter tuning based on the material response to extrusion in the end construct design was applied to 20%|10% w/v Poloxamer 407|UMA, 25%|10% w/v Poloxamer 407|UMA, and 30% w/v Poloxamer 407.
Following the initial complications in the sustainability of the 20%|10% w/v Poloxamer 407 grid construct, two other methods were developed and executed with this ink, in order to increase control over height, structure definition, and reproducibility. By combining 1mm high, 10mm ø disks with a printed structure, an attempt has been made to include the structure of the papillary dermis to the construct (Method 2). The inclusion of the papillary dermis should lead to an increase of surface area between the epidermal and dermal layer, thereby increasing nutrition exchange between skin layers and increasing mechanical connection in shear direction between layers, preventing slippage. To create the papillary layer with a high definition and defined strand spacing, a layer was printed on top of a casted construct. For this purpose, a mould (10mm ø) would be placed in a 6-well plate, which would to be filled with 20%|10% w/v Poloxamer 407|UMA with 0.06% w/v LAP by extrusion (Figure 8). Material was photo crosslinked at 15% light intensity for 30sec in standard crosslinking conditions. Afterwards, the standard grid structure was printed on top of the cast with a 0.2mm ø needle with pressure, speed, and distance to the top of the cast in accordance to previously determined layer height. Thereafter, the total construct was photo-crosslinked again for 30sec. The total construct was removed by filling the well with PBS and gently extricating the construct from the mould. Afterwards, constructs were either placed in PBS or cell culture medium.

**Figure 8:** Concept sketch of method 2 combining both the casted material and the printed material in one design is shown above. Top left shows the measurements of a 6-wells plate, for determination printing coordinates. Top right shows the construct design. Bottom shows the step sequence for construction of concept.
Simultaneously, an attempt was made to increase height of the grid construct by applying the thin grid construct as a support structure (Figure 9). The standard grid with 20%|10% w/v Poloxamer 407|UMA (0.06% w/v LAP) was produced and either dried or photo-crosslinked at 400nm λ intensity: 30% (standard conditions) (Step 1&2; Figure 9). Program specifications were 0.8 strand spacing, speed 5mm/s, pressure 1bar, 100µm layer height. Thereafter, the same ink was deposited in between the previously printed strands with a 1.3mm ø needle to form a thicker layer, intended to contain human dermal fibroblasts (HDFs). Thereafter, the whole construct was exposed to 400nm λ at 15% intensity (standard conditions)(Step 3&4; Figure 9). Due to insufficient shape retention, see result section 3.3.2, both concepts were disregarded before the additions of HDFs. For a grid construct similarly to the first method, but an increased construct height, layer integrity would have to be increased to form a self and subsequent layer supporting gel. There are several strategies to encourage the desired behaviour, from increasing the yield point to prevent flow below a certain amount of pressure, temperature adaptation between collector plate and cartridge, and/or photo/chemical curing of the individual layers (Kirchmajer &Gorkin, 2015; Mouser et al. 2016). With an increasing amount of Poloxamer 407 the viscosity increases. Therefore, w/v of Poloxamer 407 was increased from 20% to 25%. Exceeding
25% w/v material was not manageable for the encapsulation of cells as the high viscosity and adhesive properties prevented proper transfer between vials and homogeneous distribution of cells.

All previously described method incorporated Poloxamer 407 as a support material during printing, increasing overall viscosity of the material. Poloxamer 407 was to be removed in order to create a nanostructured hydrogel similarly to Müller et al. (2015). In method 4, this approach was abandoned in favour of reinforcing the overall construct instead of increasing viscosity of the material. Reinforcement was created by the extrusion of a 30% w/v Poloxamer 407 grid structure, alternating extrusion of Poloxamer 407 and 10% w/v UMA in PBS (0.06% w/v LAP). Pressure and speed necessary were determined for the extrusion of Poloxamer 407 by manufactures protocol (EnvisionTEC, 2010). Briefly; Material was extruded for 2sec at a safe height and strand length was measured to determine speed of printing. Subsequently a grid was printed at 80% height of material diameter (160 µm), speed 10mm/s, pressure 3-4bar. After each layer of UMA ink, material was photo-crosslinked at 15% light intensity in standard conditions (Figure 10). Crosslinking time (20sec) was determined by the minimum amount of exposure necessary to prevent subsequent layers sagging through extruded UMA strands. Before continuing the development of this method, a biological characterization on the combination construct was executed.

2.5 Biological characterization

2.5.1 General cell culture

Human dermal fibroblasts were cultured in high glucose DMEM supplemented with 10% foetalbovine serum (FBS) at 37 °C in a 5% CO2 incubator in standard 75cm² culture flasks. Cell culture medium was replaced twice weekly. At approximately 80-90% confluence, passaging was performed with 0.25% w/v Trypsin-EDTA. Once cells assumed the typical round appearance associated with detached cells, 7 mL cell culture medium was added to interrupt the reaction. Solution was then centrifuged at 1250 rpm (Thermo Scientific™ Heraeus™ Primo R, Waltham, USA), after which supernatant was removed and the remaining pallet of cells suspended in fresh media. Cells were then transferred into a new culture flask with a seeding density of approximately 0.7*10^6 cells per 75cm².

Figure 10: Method 4; Creating a Poloxamer 407 reinforcement for the creation of complex 3D bioprinted UMA constructs

(Figure adapted from Melchels et al., 2016).
2.5.2 Cell encapsulation

For harvesting of the cells, a similar procedure was conducted. However, after removal from the flasks, 1*10^6 HDF cells (unless otherwise stated) were suspended per mL in premade 20%|10% Poloxamer 407|UMA, 25%|10% w/v Poloxamer 407|UMA, or 10% w/v UMA with various amounts of LAP and re-suspended multiple times to ensure homogeneity of the cellular distribution. The ink was then transferred to either an inkjet for printing purposes or used directly in a mould. For cell culture within the constructs, media contained high glucose DMEM supplemented with 10% v/v foetalbovine serum (FBS) and 1% v/v Penicillin/Streptomycin. Constructs were stored at 37 °C in a 5% CO2 incubator in 24-well plates. Photo crosslinking was executed with previously described protocol; time ranging between 30-90 seconds and light irradiation of 15%/30% light intensity depending on ink and protocol applied.

2.5.3 Vitality, proliferation & morphology

Initially, the morphology of the fibroblasts was determined at day 1, 3, 7, and 14 (n=1) in casted disks to determine cellular response to the material. Disks were kept at 37°C. To remove the Poloxamer 407 samples were subjected to 2h of 4°C, before and after which images were taken. The samples were stained with 5 µM calcein-AM for 25 min, after which propidium iodide (PI) was added, end concentration 10μg mL^-1, and left for 5 min. Calcein, an esterase based cell permeable staining, passes through the cell membrane, while PI is cell-impermeable, and can therefore not pass through healthy cell membrane. When the cell membrane is compromised the PI will bind to the DNA to fluorescently stain the dead cells. After this, the samples were washed 2x 5min with PBS, after which samples were immediately imaged using a Zeiss Axiovert 40 CFL inverted fluorescence microscope (software: AxioVision, 4.9.1 SP1, 2013, München, Germany), with excitation and emission set at 488/530 nm and 530/580 nm to detect calcein and PI stained cells respectively. Three pictures were taken of each sample. Due to the high amount of background staining, the protocol of 25%|10% Poloxamer 407|UMA (0.06% & 0.03% w/v LAP) was adapted to increase the contrast between the cells and material. Exposure of material to calcein was tested for 5, 10, 25, and 90 minutes. Based on these results, constructs were exposed to 90 minutes of calcein. PI exposure time was only slightly increased (7 minutes) due to the negative effect on cell viability, thereby influencing the viability results.

A PrestoBlue® viability assay was performed according to manufacturer’s protocol (Invitrogen, 2012). In short, a stock solution PrestoBlue® was diluted 10x in cell culture medium. Fluorescence was measured in a Fluostar omega microplate reader (BMG Labtech, Germany) at 544 nm emission and 590-10 nm excitation. Fluorescence reading of empty scaffolds was subtracted from cell containing scaffolds.

The first assay was performed to determine the effect of extrusion through a 200µm needle at 1, 2, and 4 bar pressure at day 1, 3, and 7 according to manufacturing protocol (1h staining). Culture medium was changed 2 times a week, following standard culture conditions. For comparison, ink was extruded into the moulds (ϕ: 10mm, h: 1mm) and compared to the control: disks hand casted through standard issue 1 mL syringe. The encapsulation of the cells would have changed the rate of reduction of resazurin to resorufin compared to the same number of cells cultured on plate, as the material would affect the amount of PrestoBlue® reaching the cells. 500µL was added to each well of the 24-wells
plate to submerge the constructs and left in incubation for 1h. 100 µL of the reagent was then transferred to a 96-wells plate. Results were compensated against empty scaffolds to account for background fluorescence. After the fast decrease in mitochondrial activity, a quick experiment was executed on printed scaffolds with 25%|10% w/v Poloxamer 407|UMA (0.06% w/v LAP) with 1, 0.75, and 0.5 million cells per mL. All scaffolds were cultured according to standard cell culture. To assess effect of Poloxamer 407 removal via protocol described by Müller et al. (2015) with diffusion in standard cell culture conditions, mitochondrial activity of the cells was measured for both types in printed scaffolds containing 1.0*10⁶ cells per mL. Mitochondrial activity was assessed on day 0, 1, and 3 to assess proliferation.

As the Relative Fluorescence Units (RFU) after 1 hour incubation was very low, the incubation time was adapted from the initial standard operation procedure. To determine appropriate time of incubation, scaffolds were left for 1, 2, and 3 hours. At each time point fluorescence units were compared as described above and a visual comparison was made.

2.6 Statistics

To prevent inaccurate representation of data standard deviation in biological experiments is not shown between mechanical replicates. For proper representation standard deviation should only be shown for biologically independent samples (n>1) and not biological replicates. Although it is possible to calculate mean and/or standard deviation between the replicates, it would not provide information on the effect of cell encapsulation in the different materials/methods, but reflect on pipetting accuracy, thereby not providing information on the accuracy of the hypothesis (Blainey et al., 2014; Cumming et al., 2007).
3 Results & Discussion

3.1 Ink formulation

Purified GG was quickly discarded as a suitable material, for high concentrations would have been necessary to form stable, shape-retaining constructions. Presumably, the removal of cations in the purification process is responsible for the inability of the material to physically crosslink at the concentrations that are sufficient for the gelation of non-purified GG. Therefore, all further experiments requiring GG were performed with non-purified GG. Figure 11A illustrates six blends of various salt types and concentrations combined with GG and extruded with a 1mL syringe. Sample 1, 1% GG in DMEM, produced a brittle inhomogeneous gel unsuitable for printing. Samples 2 and 3, 1% GG in 20% PBS and 1% GG in 10% PBS, resulted in a gel unable to retain shape upon extrusion. Samples 4 and 5, 1% GG containing 0.085mM and 0.077mM CaCl\(_2\) respectively, formed homogeneous gels and could be extruded with relative ease (Tab. 2). The 0.085mM gel regained a parabolic shape (cross section), suggesting a visco-elastic behaviour with a relatively low yield point (Figure 11). Further increasing the CaCl\(_2\) concentrations resulted in an inhomogeneous gel that is inappropriate for printing purposes. Inhomogeneous gels were considered unsuitable for printing, due to the irregular pressure that would have to be administered for extrusion, as well as the unpredictable and non-uniform distribution of the material and cells within the gel. This would lead to poorly controlled and irregular structures. The standard concentrations of 1% w/v GG was based on literature and a pilot test for formulations containing 0.2-1% w/v GG with different salt types and concentrations (Ferris et al., 2015; Mouser et al., 2016; Oliveira et al., 2008).

Figure 11: Ink formulations extruded with a 1mL syringe. A: 1% GG with 1% DMEM, 2: 20% PBS, 3: 10% PBS, 4: 0.085 mM CaCl\(_2\), 5: 0.077 mM CaCl\(_2\) and 6: 0.1 mM CaCl\(_2\). B-D. show inks with 10% UMA, constructed B. with 0.9 mM H\(_3\)BO\(_3\) and 0.28 mM CaCl\(_2\), C. with 1% DMEM and 0.45 mM H\(_3\)BO\(_3\) and D. with 1% GG and 0.085 mM CaCl\(_2\).
Based on these results, a combination of 9% w/v 1% w/v 0.085mM UMA|GG|CaCl$_2$ was assessed for suitability and compared to an UMA ink made with previous protocol developed by Jeremy Di Noro (2016), and to an ink made with the same protocol, but with a slight adaptation to increase biocompatibility. This was done by substituting CaCl$_2$ for DMEM (containing 2 mM CaCl$_2$). Figure 11B-D shows formulations containing 10% w/v 0.9mM 0.28mM UMA|H$_3$BO$_3$|CaCl$_2$ (B), 10% w/v 0.45mM 1% w/v UMA|H$_3$BO$_3$|DMEM (C), 10% w/v 1% w/v 0.085mM UMA|GG|CaCl$_2$ (D), respectively. Each combination resulted in a brittle, in-homogeneously crosslinked hydrogel, which could not be extruded within the machine pressure range (0-6bar). This could not be circumvented by adjusting the order of the protocol or temperature of the materials. It is assumed that the increase of CaCl$_2$ in 10% w/v 0.45mM 1% w/v UMA|H$_3$BO$_3$|DMEM was directly responsible the increased viscosity shown compared to 10% w/v 0.9mM 0.28mM UMA|H$_3$BO$_3$|CaCl$_2$. Decreasing the amount of DMEM responsible for this increase would negate the biological advantage. An initial visual result showed that lowering the boric acid concentration lead to a decreasing viscosity, however, the ink remained inhomogeneous and is not suitable for printing. Formulations and visual results are noted in Tab. 2.

A new direction was taken by using Poloxamer 407 as a support material to enhance printability. Poloxamer 407 is primarily used as a thickening agent and shows thermal reversible behaviour. Interestingly, the material is more fluidic at lower temperatures (5°C) compared to higher temperatures (40°C) and it is a popular ink material for extrusion printing (Pereira et al., 2013b). In this study, a Poloxamer 407 solution (20% w/v in PBS) was used to formulate an UMA ink at 10% w/v that can be readily extruded via the bioprinter. Lowering the Poloxamer 407 concentration resulted in a rapidly decreasing viscosity, resulting in a material too fluidic for extrusion printing. Therefore, 20%|10% w/v Poloxamer 407|UMA remained the base of the bioink and its rheological behaviour was systematically characterized.

3.2 Bioink characterization

3.2.1 Temperature sweep

20%|10% w/v Poloxamer 407|UMA, 19.6%|9.6% w/v Poloxamer 407|UMA, and 19.6%|9.6%|0.12% w/v Poloxamer 407|UMA| LAP were evaluated for the temperature sensitivity and potential variations due to the photoinitiator addition with a temperature sweep (7-40°C). The ink formulations show a similar trend in both G’ and G” (Figure 12A&B), indicative of a negligible effect of the LAP addition on the rheological behaviour of ink. Therefore, only 20%|10% w/v Poloxamer 407|UMA was used for further tests. In this study, LAP is employed as a photoinitiator to control the stability of 3D printed structures via a light-mediated chemical crosslinking mechanism.
Table 2: Results regarding the initial and subsequent ink formulations in regard to viscosity, homogeneity, and shape retention (visually assessed).

<table>
<thead>
<tr>
<th>Initial tests</th>
<th>Viscosity</th>
<th>Homogeneity</th>
<th>Shape retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2-0.5% w/v GG</td>
<td>Low</td>
<td>Good</td>
<td>Low</td>
</tr>
<tr>
<td>1% w/v GG</td>
<td>Low</td>
<td>Good</td>
<td>Low</td>
</tr>
<tr>
<td>20% w/v PBS</td>
<td>Low</td>
<td>Good</td>
<td>Low</td>
</tr>
<tr>
<td>0.07mM CaCl₂</td>
<td>Good</td>
<td>Good</td>
<td>Low</td>
</tr>
<tr>
<td>0.085mM CaCl₂</td>
<td>Good</td>
<td>Good</td>
<td>Low</td>
</tr>
<tr>
<td>0.1 mM CaCl₂</td>
<td>Good</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>1% w/v DMEM</td>
<td>Medium-High</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subsequent tests</th>
<th>Viscosity</th>
<th>Homogeneity</th>
<th>Shape retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>9% w/v UMA</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>0.28mM CaCl₂</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>0.45mM H₂BO₂</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>1% w/v GG</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>1% w/v DMEM</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>0.45mM H₂BO₂</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>1% w/v GG</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

10% w/v UMA | 20% w/v Lutrol | Good | Good | Good |

Figure 12: Oscillatory temperature sweep (7 °C - 40 °C) of the storage modulus (G’) and the loss modulus (G”). In A, G’ and in B, G” of 19.6%|9.6% w/v Poloxamer 407|UMA, 19.6%|9.6%|0.12% w/v Poloxamer 407|UMA|LAP, 20%|10% w/v Poloxamer 407|UMA are shown (n=3). Graphs C-D show G’ and G” in relation to each other for 19.6%|9.6% w/v Poloxamer 407|UMA, 19.6%|9.6%|0.12% Poloxamer 407|UMA|LAP, 20%|10% Poloxamer 407|UMA respectively (n=3).
The storage modulus ($G'$) surpasses the loss modulus ($G''$) between 25°C and 29°C for all inks; illustrating the transition from a viscous fluid to viscoelastic gel (Fig 12C-E). A balance must be found with the sol-gel transition stage, to create a bioink that shows both fluidic properties at printing, in order to being extruded at a low pressure, and then solid properties below the yield point to retain and regain shape after extrusion. Temperature adjustments of both the ink and printing stage are necessary to find an optimal combination of mechanical properties, to meet both the printing and biological requirements (Kirchmajer&Gorkin, 2015).

3.2.2 Strain and Frequency sweep

Strain and frequency sweeps were performed to determine material response to the linear viscoelastic region (LVR) (37°C). Polymer network remains relatively stable between 0.3-4% strain for both $G'$ and $G''$, which is considered the strain plateau region, further increasing strain resulted in a decrease of $G'$, as a result of destabilization of the polymer network. The initial increase of $G'$ and $G''$ is the result of tightening of the polymer network (Figure 13A) (Zuidema et al., 2014). All frequencies above 0.1Hz reflect normal gel behaviour (Figure 13B). Interestingly, the frequency test (1% strain) showed higher modulus (6 KPa) at 1Hz compared to the strain experiment (1Hz) at 1% strain (1 KPa). Values for further testing were chosen within the plateau region; 1% strain and 1Hz frequency (Figure 13B).

3.2.3 Recovery

Time sweep and step strain measurements were performed at previously determined strain and frequency. The material showed a tightening of the polymer network during 10 minutes of constant applied strain (1%) and frequency (1Hz) (Figure 14A). Multiple applications of 1000% strain showed a decreasing trend in shear modulus when 1% strain was applied. However, decrease was not significant (p>0.05). Modulus at 1000% strain remained linear. This indicates that the mechanical behaviour of the material will not significantly change after multiple printing sessions (Figure 14B). Tests will be repeated if necessary, depending on the outcome of new frequency and strain sweep.

**Figure 13:** Illustrated are the A. Strain and B. Frequency sweep of 20%|10% w/v Poloxamer 407|UMA both displayed in logarithmic notation (n=3).
3.2.4 Viscometry

Rotational experiments executed with 20%|10% w/v Poloxamer 407|UMA demonstrate shear thinning behaviour, which can be described as decreasing viscosity in response to increasing shear strain, shown in Fig 15A, where the viscosity drop is 42.05±15.03 Pa.s over one decade. This conclusion is supported by applying the Herchel-Bulkey model on the relation between shear stress and shear rate in Figure 15B (n<1; Eq. 1) (Kesti et al., 2015).

The importance of yield stress for the printability of a material was supported by Mouser et al. (2016) due to the importance of an initial stress needed to induce flow. Yield stress indicates the amount of stress that must be applied to the material before it starts to flow. The material is deformed by shear forces above the yield stress and (slowly) reforms when the stress is removed. Where high viscosity only delays collapse of a deposited 3D structure, the presence of a yield stress can potentially prevent flow and collapse. For proper printing behaviour the material must be viscous after application of a shear stress below 2 bar, as to not damage the cells within the material, but remain its shape during printing (Billiet et al., 2014). A higher yield stress has the advantage of enabling a material to support a larger construct, without additional crosslinking, and prevents sedimentation of cells within the dispenser (Malda et al., 2013; Mouser et al., 2016). However, high pressure may damage the cells during the printing process. Similarly, to Mouser et al. (2016), who reported a yield stress of 48.2±3 Pa for the material most suitable for extrusion printing, we showed that our ulvan ink had a relatively high yield stress of 69.63±14.5 Pa. This implies a minimum pressure of 6.9×10^{-4} bar would have to be applied to extrude the material. Without the additional pressure the material will remain within the syringe.

Figure 14: Graphs shown are; A. a time sweep at 1% strain and 1Hz frequency and B. a step-strain sweep, to illustrate the recovery behaviour after exposure to multiple sessions of 1000% strain (5min) intermitted with 1% strain sessions (5min). Both measurements were executed on for 20%|10% w/v Poloxamer 407|UMA
Rheological measurements were of 25%|10% w/v Poloxamer 407|UMA were forgone, as temperature response does not significantly change when more Poloxamer 407 is added besides an overall increase in viscosity. Thereby, the goal of this study was to create a biological compatible, printing compatible ink and not a material characterization study. Therefore, the printing parameters were considered to have priority over the characterization of rheological behaviour change of the material after an increase of Poloxamer. However, rheological information should be collected when determination of construct stability, strand definition, and biological characteristics have taken place, to support further studies in bioink manufacturing.

3.3 Additive manufacturing

3.3.1 Photocrosslinking

The 10% w/v UMA ink reinforced with Poloxamer 407 needed to be exposed for a minimum of 20sec per layer at 15% light intensity, to prevent the Poloxamer strands from sagging into layers below, thereby disconnecting the layers of UMA. As the 400nm λ LED light intensity needed to create an
ECM like hydrogel was below the LED light meter system range (50 mW/cm²–25000 mW/cm²), the irradiation could not be measured directly. Therefore, a trend line curve was derived with the measured intensity values (5% increments; Figure 16). Exposure of 15% and 30% of total light intensity translates to 33.32 mW cm⁻² and 44.48 mW cm⁻² respectively. In literature, time ranges from 2–15 min and irradiation <10 mW cm⁻². Irradiation necessary can vary for hydrogel amount and time of exposure (Lim et al, 2016; Mahadik et al., 2015; Shih & Lin, 2012; Lee et al. 2015). However, due to the extrapolation based on the linear trendline, actual irradiation may vary from calculated values. Increasing exposure time resulted in a decrease of swelling ratio for both 20% and 25% w/v Poloxamer 407 ink at 15% exposure, while swelling ratio remained stable over time.

3.3.2 Manufacturing

Pneumatic extrusion of 20%|10% w/v Poloxamer 407|UMA shows an overall increase in strand thickness when pneumatic pressure is increased for all inks. The ink at both 30°C and 37°C shows an increase in the strand thickness when deposited on a 23°C plate, as compared to the structure deposited on a 37°C plate (for all pressures applied). For the ink at 23°C, the strand width when deposited on a 23°C plate is smaller compared to a similarly heated strand deposited on a 37°C plate. (Figure 17A&B). Comparing the ink at three temperatures (23°C, 30°C, 37°C), when deposited onto a 37°C plate, the strand thickness printed using the 23°C ink is the largest, but then become the smallest when the pressure surpasses 2 bar (Figure 17A). Figure 17 illustrates the effect of pressure and temperature on the number of layers that can be formed while the filaments remain intact. Dotted lines indicate a non-homogeneous/interrupted strand. Improved shape retention at 37°C correlates to the rheological data found in the temperature sweep, where visco-elastic behaviour is shown at 30°C and above (Figure 17A&B).

Afterwards, material was extruded into the standard grid scaffold, adjusting parameters until a homogeneous, highly defined lattice could be formed. Layer height, programmed distance between strands, speed and distance to contour were all of influence on end result. Layer heights above 0.1 mm lead to a discontinuation of extrusion of material in subsequent layers (Figure 17C). Decreasing speed of cartridge movement, lead to an excess of material, thereby losing strand definition (Figure 17D). Conversely, this would occur with high pressure or a small strand distance as well. A large strand distance in combination with high speed would lead to discontinuation of the strands and accumulation.
of the material in certain areas of the print. Effect of low pressure is illustrated in Figure 17E. However, the combination of the various parameters is detrimental for the end result (Figure 17F). Similar strand characteristics could be created by, for example, increasing pressure and speed, or increasing stand distance while decreasing speed (Figure 17G&H). A sample of parameter combinations and the corresponding strand width and closed squares is shown in table 3. For the purpose of this study, end parameters were set to starting height: 0.1 mm, layer height: 100µm, speed: 6mm s⁻¹, maximum number of layers: 6, pressure: 2.3 bar, and distance between strands: 800 µm, with an ink and collector plate temperature of 37 °C. This resulted in constructs with height of 260±25µm, strand diameter: 365±16µm, distance between strands: 620±35µm (Figure 17I&J).

Upon introducing the end construct to cell culture media, construct was unable to retain shape. Height could not be further increased due to material properties. Subsequently, either the material or the method of extrusion would have to be adjusted to create a functional construct. In this study, several different strategies have been implemented simultaneously, to be retained or discarded dependent on construct stability, maximum precision possible in printing behaviour, and biological compatibility.

In the manufacturing of method 2, 20%|10% w/v Poloxamer 407|UMA (0.06% w/v LAP) was printed across the casted scaffold from the same material, to create a corrugated structure, similar to the papillary dermis (Figure 6, 19 A, C, and D). The casted scaffold needed to be crosslinked for 30 seconds at 15% light intensity to ensure the printed layer would not combine with the cast, which would result in a smooth surface layer. After extrusion of the uppermost layer, the construct was again exposed to 30 seconds of 400nm λ, 15% light intensity to secure the strands. Although a defined structure, strand width: 422±55µm and distance between strands: 289±32µm, corresponding with a programming distance of 800µm, could be printed on top of the casted material, only one layer could be deposited without losing strand definition. Furthermore, immersions of the combined construct into DMEM lead to the loss of the uppermost layer. Subsequently, this method of manufacturing was dismissed for this material.

| Table 3: Parameter tuning 20%|10% Poloxamer 407|UMA |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Initial height (mm) | Layer height (mm) | Pressure (bar) | Speed (mm/s) | Waiting time(s) | Closed squares (%) | Strand thickness (µm) |
| 0                | 0.1             | 1.7            | 6             | 30              | 60              | 743±41              |
| 0.1              | 0.08            | 1.5            | 6             | 30              | 80              | 845±147             |
|                  | 1.7             | 6              | 30            | 100             | 662±47          |
|                  | 2               | 8              | 0             | 0               | x               |
|                  | 8               | 30             | 13.333        | 668±79          |
|                  | 8               | 45             | 60            | 733±24          |
| 0.1              | 1.5             | 6              | 30            | 13.333          | 975±70          |
| 0.1              | 1.7             | 5              | 30            | 20              | 1516±346        |
|                  | 8               | 30             | 20            | 811±77          |
|                  | 10              | 30             | 26.66667      | 839±66          |
| 0.2              | 1.5             | 6              | 30            | 6.66667         | 1046±159        |
|                  | 10              | 30             | 1043±250      | 1043±250        |
Figure 17: Manual parameter tuning of 20%|10% w/v Poloxamer 407|UMA for A. Pressure and B. Speed of extrusion. Intermittent line indicates non-homogeneous extrusion of material. The diameter of a printed strand is shown in regards to the pneumatic pressure printed on room temperature plates or plates at 37 °C with the ink at 23°C, 30°C, and 37 °C showing the diameter response to pneumatic pressure at different ink temperatures. This is depicted as temperature ink/temperature collector plate. Error bars are the standard deviation derived from the mean. (n=3). Figures below illustrate the effect of programmed C. layer height, D. insufficient speed, and E. insufficient pressure. F. illustrates what is considered appropriate strand definition and scaffold formation in 2 layers. G&H Illustrate how the same effect can be created by adjusting the combination of parameters, while I. illustrates the end scaffold, with J. a depiction of the height of end construct.
Even though this method was not appropriate in combination with this material, it may have merit for further study. The inclusion of a papillary dermis in tissue constructs increases surface area between the dermal and epidermal layer of the skin, increasing possibility for nutrient exchange and creating friction between layers, thereby preventing sliding in between layers. Previously bioprinted do not include these constructs (Cubo et al., 2016; Koch et al., 2013; Lee et al., 2009; Lee et al., 2013). However, the unique properties of the different bioprinting techniques the opportunity to manufacture tissue constructs closely resembling skin tissue morphology, increasing precision in construct formation and providing opportunities the inclusion of microstructures and skin constructs. By creating two different layers the properties of both can be incorporated by adjusting the bioink to meet the mechanical support characteristics of the reticular dermis, while incorporating a second ink with higher porosity in the papillary dermis, to encourage vascularization necessary for nutrient and oxygen exchange with the epidermal layer, as the diffusion limit of oxygen is 100-200µm (Rouwkema et al., 2008). However, currently incorporating several inks manufactured specifically for each skin component is distant. For this study the further development of this method had to be discontinued, due to the incompatibility of the ink and method.

Figure 18: Image A. and D. illustrate the concept of method 2 and 3 respectively. Correspondingly B/C and E/F show the results of applying either method.
In the manufacture of method 3; a lattice was printed according to the first method of manufacturing, with ink 20%|10% w/v Poloxamer 407|UMA with 0.06% w/v LAP (Figure 9), on top of which the material was hand extruded through a 400µm needle between the previously printed construct (Figure 18E). However, extrusions in this manner lead to a quick height increase and could lead to relatively defined structures, as shown in Figure 18E, it generally lead to overflowing of the material from in between the supporting lattice. Also, to make this method viable, inkjet printing would be preferred over extrusion printing. In that manner less material would have to be deposited on areas where the extruded strands cross. Due to time constraints, this method was not further developed.

The adjusted material was initially extruded from a safe height to assess strand or droplet formation (Figure 19A&B). As droplet formation occurred, parameter tuning was executed via the extrusion of the square scaffold. Manual parameter tuning was omitted, as previous efforts had shown the superseding effect of scaffold formation on strand diameter and speed necessary. End parameters found most appropriate for the extrusion of a standard grid with a homogeneous, highly defined lattice were starting height: 100µm, layer height: 80µm, speed: 3mm s$^{-2}$, maximum number of layers: 8, pressure: 2.3bar, and distance between strands: 1400µm, the latter being of particular importance for the extrusion of continuous strands. This resulted in constructs with height of 876±167µm, strand diameter: 332±7µm (excluding contour), distance between strands: 148±2µm (Figure 19). Detriments in this method of constructions were the effluent at contours and the relative brittleness of...
the whole construct. Transportation of constructs could be problematic due to weak mechanical points in the inner grid structure, leading to breakage of the whole construct. LAP concentration was decreased to 0.03% w/v, however, constructs remained relatively brittle. This is behaviour is not shown in response to hydrogel swelling, as constructs remain stable over a period of two weeks, however, mechanical stimulation, as would be conducted by manual transport, could result in breakage.

Lastly, method 4 was executed. As 30% w/v Poloxamer 407, formed strands when extruded at a safe height; thus, standard material assessment as determined by the manufactures manual (Envisiontec, 2010). Programming parameters were layer height: 80% height of needle diameter (160µm), speed: 10mm/s. After each layer a layer of ink 3 with end concentration of 0.06% w/v LAP was added hand extruded through a 200µm needle and crosslinked for 20sec (15% light intensity). Lower amounts of exposure lead to an inability to support subsequent Poloxamer 407 layers and the formation of separate squares instead of connected strands. The end constructs were height 967±164µm, strand diameter: 32±8µm, and distance between strands: 47±6µm (Figure 20).

Both the first method with 25%|10% w/v Poloxamer 407|UMA and method 4 with 10% w/v UMA resulted in highly defined, sustainable, and reproducible constructs. These methods could therefore be assessed for biological compatibility and may be further developed. The constructs manufactured with 10% w/v UMA proved to be more pliable and tended to curl upon contact with PBS or DMEM, indicating a relatively soft material losing the brittleness typical for ulvan methacrylate, which was still present within the Poloxamer 407|UMA combination ink. UMA predisposes to the formation of aggregates, decreasing the amounts of methacrylate groups available for photocrosslinking, causing weak points within the construct (Morelli&Chiellini, 2010). Within the Poloxamer 407|UMA construct the combination of these weakly crosslinked areas, the relative thinness of the strands, and the rigidity of the material often caused the constructs to break during transfer between well plates.

Figure 20: Illustrates the reinforced 10% w/v UMA scaffold in A. Top view and B. side view.
3.4 Biological characteristics

3.4.1 Cellular morphology

Figure 21 shows the initial cellular response to encapsulation in 20%|10% Poloxamer 407|UMA (0.06% w/v LAP) at day 1, 3, 7, 14, and 21 (Figure 21&22). Freezing the cell-containing material did not seem to affect the viability of the cells. However, cells had not attached to the material and were removed together with the temperature-sensitive Poloxamer 407. Regarding the morphology; cells showed first attachment for 15% and 30% light intensities after 7 and 3 days respectively. Due to the low number of cells present in the samples, no quantitative conclusions were drawn. This experiment was considered an initial indication on cell response to encapsulation and photocrosslinking time/intensity. As printed structures with 20%|10% w/v Poloxamer 407|UMA were not functional and disappeared, no further experiments were conducted with ink containing 20% w/v Poloxamer 407. Due to the large migration of cells, the protocol of Poloxamer removal was adapted to exclude the liquefaction of Poloxamer 407 by decreasing the temperature. Instead, material was removed via diffusion at 37 °C.

Initial staining of the printed 25%|10% w/v Poloxamer 407|UMA (0.06% w/v LAP) could not sufficiently show the contrast between cells and the constructs. The necessary protocol adaptation was determined by staining the samples with calcein for 5, 10, 25 (standard procedure), and 90 minutes (Figure 22). In all samples, material staining interfered with the quantification of live and dead cells. However, by adjusting image parameters within the software, cell morphology and distribution could be visualized. Highest contrast could be found in the 90 minute staining. Additional complications are the result from the toxicity of the PI staining; as increasing staining time of PI negatively influence cell viability. Therefore, for quantification of cell viability and proliferation a PrestoBlue® staining was applied. As material stiffness may interfere with the penetration of the staining and the probable effect of the mechanical behaviour of the bioink on the cell morphology, LAP amount was decreased to 0.03%w/v. However, contrast between cells and surrounding material remained low.

Cell retained their round morphology for both time points and both materials (day 1 and 3; Figure 24&25). The calcein staining time adjustment necessary for proper visibility of the live cells, indicates a similar need for the adjustment of staining time of PI. However, as PI is cytotoxic, changing staining time affects the viability of the cells. Thereby, the difference in exposure time of the outer cells compared to the cells encapsulated in the middle of the construct would lead to an under- or overestimation of cell viability at different areas of the gel. Therefore, viability was not determined with LIVE/DEAD assay.
Figure 21: In vitro culture of HDF in casted 20%|10% Poloxamer 407|UMA scaffolds at 30% light intensity at day A. 1 (before poloxamere 407 removal), B. 1 (after removal), C. 3, D. 7, E. 14. Light blue circles indicated stretched cells in the material. All scale bars indicate 100 µm.

Figure 22: In vitro culture of HDF in casted 20%|10% Poloxamer 407|UMA scaffolds crosslinked at 15% light intensity at day A. 1 (before poloxamere 407 removal), B. 1 (after removal), C. 3, D. 7, E. 14. Light blue circles indicated stretched cells in the material. All scale bars indicate 100 µm.

Figure 23: Adjusted calcein staining time protocol determination printed samples of 25%|10% Poloxamer 407|UMA from left to right showing calcein staining time of 5, 10, 25, and 90 minutes. Respectively the samples were stained with PI for 2, 5, and 5 minutes. No contrast between material and cells could be visualized.
Further evaluation is needed in regard to the effect of the photocrosslinking process on the overall construct and cell viability and morphology. Mainly the effect of the mechanical properties on the cell morphology needs to be assessed. Unpublished data by Chen (2016) showed cell material interaction after 1 day for cells seeded on top of 10% UMA in PBS, crosslinked at 15% light intensity for 60sec, after which the material was lyophilized and reintroduced to cell culture media. This indicates the interference in the cell-material interaction is a probable result of the encapsulation process. Therefore, it is currently hypothesized that the mechanical behaviour of the bioink after crosslinking is responsible for the round morphology of the cell. Many mature cells, such as dermal fibroblasts, are able to sense material stiffness and change their adhesion and morphology accordingly (Chan&Leong, 2008). As the material stiffness affects the adhesion and cytoskeleton formation within the cells, adjusting extend of crosslinking in the bioink may positively influence cell-material interaction. Thereby, the fibroblasts are able to exert traction stress on the surrounding material, with an ideal relation between traction and to gel strain of 3-4% (Discher et al., 2005).

A softer gel leads to a decrease in contraction force applied by the cells. Especially cells in the 25%|10% w/v Poloxamer 407|UMA bioink have been exposed to a relatively stiff, non-elastic material. Further testing needs to be performed, by correlating cell behaviour with mechanical behaviour of the construct and assess mechanobiological effect correlating LIVE/DEAD morphology assessments with mechanical behaviour of the material. Thereby, a comparison of encapsulated cells with cells seeded on top should be executed to assess the effect of encapsulation on fibroblast behaviour. Adjusting mechanical stiffness could be achieved by decreasing light intensity by increasing the distance between light and hydrogel or decreasing w/v of UMA. Assessment of material stiffness and elasticity should be performed with a compression clamp with a flat top and bottom plate to allow for mechanical variation in different areas of the intricate design. These analyses should be performed for both the 25%|10% w/v Poloxamer 407|UMA and 10% w/v UMA inks.

Other possible influences on cell behaviour is the exposure to 400nm λ light, as the cells seeded on top of the constructs were not exposed, or the freeze-drying process, which introduces porosity through the crystallization of water particles. In the 20%|10% w/v Poloxamer 407|UMA bioink for 15%

![Figure 24: LIVE/DEAD staining 25%|10% Poloxamer 407|UMA (0.03% w/v LAP) for A. Day 1 and B. Day 3 (n=1).](image)
and 30% light intensity first cell stretching was seen after 7 and 3 days respectively. However, as neither the 25%|10% w/v Poloxamer 407|UMA inks show indication of cell-material interaction after 3 days of culture, it is assumed the effect increased amount of Poloxamer 407 on the cell morphology is negotiable. For conclusive results in regards to cell stretching after an increased culture time, the encapsulation process would have to be repeated.

Within the field of biofabrication the LIVE/DEAD has its disadvantages due to the nature of the constructs, as a LIVE/DEAD staining in a 3D construct is quite sensitive to researcher bias. Whereas in 2D culture it is possible to count all cells within the frame, within 3D culture it is necessary to count only the cells in the focus plane, as it is not possible to determine how much cells are visible in other layers of the construct. However, this means that the researcher will never have the exact same plane between different samples. To circumvent this, it should be advised to determine cell viability over 9 pictures per sample, to compensate for density at different heights of the sample and avoid researcher bias. Still, cell count can be affected by material fluorescence and thickness of the sample. Calcein and PI have to diffuse into the sample thereby exposing the outer cells to more fluorescence than the cells situated in the middle of the sample, resulting in an uneven distributed number of cells.

3.4.2 Metabolic activity and Proliferation

Comparison between the Bioplotter extruded and hand cast scaffolds showed a decreased relative fluorescence units (Figure 26A). This indicates a decreased mitochondrial activity of the cells (Boncler et al., 2014; Lall et al., 2013). A large decrease of cell activity was seen over all samples during the subsequent days. These results are in concurrence with literatures findings on extrusion induced shear effect on overall cell viability. Billiet et al. (2014) investigated the correlation between cell viability (hepatocarcinoma cells) and extrusion pressure and found exceeding pressure above 2 bar had a negative effect on cell viability. Chang et al. (2008) reported a decreasing cell viability compared to control (~85%) for HepG2 liver cells for all printing pressures >0.3 bar with a nozzle diameter of 150µm (< 70%). Cell vitality was restored to > 80% after 24h when cells were dispensed.
at 2.8 bar in a 400µm and 250µm nozzle. Nair et al. (2009) found a similar result with endothelial cells. However, the endothelial cells were less affected when printed with a 250µm and 400µm nozzle, showing a cell viability >80% immediately after printing. To enable proper cell viability within the printing process it was aimed to develop a material that could be printed at a low pressure (maximum 2 bar).

To rapidly determine the source of the RFU decrease a short experiment was executed applying a different washing method, based on the Poloxamer 407 removal method described by Müller et al. (2015). Interestingly, a large increase was found in the 1.0*10^6 cells mL^(-1), where standard culture conditions were applied. Therefore, further testing excluded the washing step and cell density remained 1.0*10^6 cells mL^(-1). Also, because relative fluorescence units were higher in scaffolds left in standard culture conditions, it was determined was to forgo the Poloxamer 407 removal step described, as described by Müller et al. (2015), and depend on slow diffusion and refreshing of the culture media twice weekly (Figure 26B).

Response to PrestoBlue® showed no distinction after an increase of 33% in cell and material amount after 1 hour of staining. Therefore, time was increased to 3 hours (Figure 27A). Although PrestoBlue® is a reliable method in describing the relative viability and proliferation between days and samples, in 3D culture and especially in 3D printing it is impossible to determine the viability of the cells at day 1 in relation to the relative fluorescence unit. In either method (print or cast), it is impossible to compare the material with a control, as the rate of reduction will be affected by the amount of material reaching the cells within the determined time span. With casted disks this could be circumvented by comparing different densities to fluorescence intensity, by determining the cell viability before seeding. However, in 3D printing there a stress factor is added to construction: cell printing. As shown in Figure 26A and in previous literature, pressure induced shear stress can have a
negative effect on the cell viability (Hua et al., 1993; Chan et al., 2008). However, as the shape of the scaffold does affect the surface area of the construct, thereby affecting the rate of reduction in a different manner than the disk casted scaffolds, it is impossible to determine the number of live cells within the scaffold. Therefore, all data are represented in relative fluorescence units instead of percentage of live cells to assess metabolic activity and proliferation over time and between different types of samples.

Even though the viability cannot be accurately assessed in the LIVE/DEAD staining of the 25%|10% w/v Poloxamer 407|UMA experiment, due to the need to adjust microscope parameters per sample to allow for visible contrast between cell and material and due to the adjustments necessary to the staining time leading to an under or overestimation of dead cells in different areas of the construct, there seems to be an inordinate decrease of active metabolism in the PrestoBlue® assay compared to the live cells present in the samples at day 3 (Figure 27B&C). To assess the accuracy and difference of both the LIVE/DEAD assay in these hydrogel constructs, a quantitative DNA/GAG assay would be appropriate. Via Picogreen® assay, quantified against a standard curve, and normalized against wet weight, and a DMMB assay, the DNA and GAG can then be quantified respectively and proliferation and cellular activity over time can be assessed. Briefly, DNA content is then quantified via a Picogreen® staining, where a fluorescence dye binds to double stranded DNA, after which fluoresces is measured in a microplate reader (485nm/520nm excitation/emission). GAG content is measured by staining with 1,9-dimethylmethylene blue and reading at 525/595 nm excitation/emission. Results are then normalized against the DNA content. By dissolving the construct, the influence of the diffusion rate on the end results is eliminated, which should increase accuracy of the results and increase objectivity of the results compared to the microscope dependend LIVE/DEAD staining.

The decreasing amount of metabolic activity in relation to the live cells may indicate dying cells, however, it may also be the result of material changes over time trapping the reagent, thereby influencing material fluorescence. Although theoretically PrestoBlue® could be interfering with the
viability of the cells, no cytotoxicity of PrestoBlue® has been reported previously, making this explanation unlikely (Invitrogen, 2012; Berrington et al., 2013). As the samples for the LIVE/DEAD assay and PrestoBlue® were generated simultaneously, the cause being a biological difference between the samples can be rejected.

### 4 Future directions

Even though the method based on combining a printed structure on top of a casted disk has not been sufficiently developed to draw any established conclusions, there are some advantages that can be applied in further developments of skin constructs. First, the simple manner in which this method could have been adapted to an industrial approach and second, the incorporation of the papillary dermal structure that has not previously been seen in any bioprinting strategy. For further development of this bio-mimicking method, a different bioink formulation should be applied. Similarly, the method based on printing the material in between a supportive grid relying on surface tension to gain height, may provide advantages in future pursuit of bioprinting tissue constructs. If applied with an inkjet printer, a higher control over droplet placement could overcome the current problem of accumulation of material at the crossing points, thereby increasing the height of the construct significantly with any material, which would have been previously unsuitable for biofabrication due to mechanical insufficiency. Absence of different methods and designs within bioprinting may limit progress within the field of biofabrication significantly. Only by combining existing methods and applying new concepts in such an emerging field, speed of progress can be maintained. Biofabrication is a tool that may be applied alone or in combination with other cell culture methods in the quest to develop functioning, highly specified tissue constructs.

In this study, Poloxamer 407 has been utilized as a printability modifier, and is expected to elute from the 3D printed structures during the course of application and/or when exposed to cell culture medium (Figure 5). This process is facilitated by its sol-gel transition behaviour that depends critically on polymer concentration. To optimize the mechanical characteristic of the eventual construct, the effects of the photoinitiator concentration and photo energy level on the mechanical properties of the construct need to be examined. The first method executed with 25%|10% w/v Poloxamer 407|UMA could be further pursued by decreasing stiffness of the material. Further steps could be taken by increasing distance between material and LED light, decreasing crosslinking time, decreasing amount of LAP, and/or decreasing amount of UMA. However, as UMA demonstrations aggregative behaviour, due to its charged and hydrophilic nature, brittleness of the material may remain (Morelli & Chiellini, 2010). End construct should be assessed for the secreted Poloxamer 407 and development of nanostructures.

In 2005, Sun and colleagues showed that in 3D electrospun polystyrene scaffolds, the co-culture of fibroblasts and keratinocytes and the co-culture of fibroblast and epithelial cells had a positive effect on proliferation, and viability of all three cell types. This positive effect of co-culturing multiple cell types on skin tissue engineering was also reported by *inter alia* Koch et al. (2012) and Lee et al. (2012). Therefore, in the continuation of this study it should be behaviour of both keratinocytes and fibroblast should be assessed in regard to their interaction to the 3D printed ulvan structures, and their interaction with each other. Multiple studies have reported the spontaneous development of the dermal epidermal junction (Boehnke et al., 2006; Schoop et al., 1999; Smola et al., 1998). Koch et al. (2012) reported a
similar development of the basal lamina in 3D printed co-cultured cells. This junction is detrimental for molecule exchange and cohesion between the two main components of the skin (Marionnet et al., 2006). Components found in the dermal epidermal junction are laminin, collagen (type IV &VII), perlecan, and nidogen. This chemical exchange between the two skin layers is responsible for *inter alia* keratinocyte proliferation, growth regulation, cell migration, and adhesion (Goulet et al., 1996; Maas-Szabowski et al., 2000). Therefore, the logical continuation would be the assessment of the formation of the basal membrane. One of the main protein components of the basal membrane is laminin (Koch et al., 2012). Therefore, an anti-laminin fluorescence staining would be an appropriate indication of basement membrane development, and an improving 3D arrangement mimicking the morphology of natural skin. High control over fibre deposition and fibre size provides in the reinforcement method provides the ideal opportunity to create differently structured layers and incorporate a dense layer with strand diameters <100µm to mimic the papillary layer and a thicker layer containing larger strands <400µm, taking into account the maximum oxygen diffusion distance (Rouwkema et al., 2008). Therefore, further developments of this method of printing could be advantageous in improving the biomimicking process.

5 Conclusion

For further development of bioprinting within the field of skin tissue engineering, more materials need to be developed with biological and mechanical properties appropriate for both printing and replacing skin tissue. Current skin replacements still face problems of infection, immune rejection, scar formation and insufficient vascularization (Ehrlich & Krummel, 1996; Pereira et al., 2013). By incorporating ulvan in the development of a bioink a promising direction has been taken, however further optimization and developments are needed to utilize all possibilities.

Several methods have been presented to fabricate a bioink for 3D printing skin with ulvan as a base mater. Further optimization is necessary in regard to photocrosslinking and printing parameters for cell printing. Although the cast and print combination and surface tension based method could not be further developed, both have shown promise for application in biofabrication. Either method could be applied as a means to increase height of construct and precision of strand placement in materials not ideal for traditional grid printing. Mechanical behaviour and printing definition of the 25%|10% Poloxamer 407|UMA ink is promising. The reinforcement method, applying 10% UMA as ink, has shown high strand definition and easy handling after fabrication. Further research will have to be done in regard to biological characterization and mechanical characterization of the end constructs. The approaches described here can potentially be used for a bi- or multi-layered construct encompassing all layers of the skin. Research with these biofabrication approaches and inks can be continued in pursuit of manufacturing skin tissue constructs, but may also be applied in the engineering of other tissue constructs.
References


Acknowledgements

I want to acknowledge my daily supervisor Zhilian Yue, and first and second examiner Gordon Wallace, affiliated to the Intelligent Polymer Science Institute and the University of Wollongong, and Jos Malda, affiliated to the University Medical Centre in Utrecht, for their support. I also want to acknowledge funding from the Australian Research Council (ARC) Centre of Excellence Scheme (CE140100012). Thereby, I would like to acknowledge the use of facilities at the University of Wollongong Electron Microscopy Centre, support of the Australian National Fabrication Facility (ANFF). Lastly, my research in the Biofabrication master’s program has been made possible by the EU ICI ECP international joint program biofabrication mobility grant.

Abbreviations and Acronyms

3D Three-dimensional
PDMS polydimethylsiloxane
PPO poly(propylene oxide)
PEO poly(ethylene oxide)
LCST lower critical solution temperature
GG Gellan Gum
EDTA ethylenediaminetetraacetic acid
UMA Ulvan methacrylate
MA Methacrylate
RT Room temperature
DMEM Dulbecco’s modified of Eagle’s Medium
ECM extracellular matrix
LAP lithium phenyl-2,4,6-trimethylbenzoylphosphinate
PBS Phosphate buffered saline
HDF human dermal fibroblasts
LVR linear viscoelastic region
DMF Dimethylformamide
λ Wavelength
Appendix

A. Poster: ACES electro material symposium 2017

Fabrication of ulvan based structures for cell culture in wound healing

Sylvia van Kogelenberg

Supervisors: Professor Gordon Wallace, Dr. Zhilian Yue, Associate Professor Chris Baker

Background

Due to a consistent need for advancements in regenerative medicine, there is a need to develop fabrication of materials that can promote cell culture and differentiation, which are vital for tissue engineering. Ulvan, a natural polysaccharide, has shown potential in wound healing and tissue regeneration. This study aims to explore the use of ulvan in this context.

Objectives

- Develop a fabrication approach to mimic the dermal layer with an ulvan-based hydrogel
- Create an in situ mechanically stable during the printing process
- Develop a hydrogel with mechanical similar properties to the dermal layer
- Develop an adaptable, but repeatable printing process
- Control spatial arrangement of cells

Blomiromy

To create a skin substitute, it is important to study formation, mechanical properties, and unique composition of the skin. (Lee et al., 2005). The following skin consists of these main components:

- The epidermis, containing mainly keratinocytes, is primarily responsible for maintaining the skin’s barrier function and maintaining the water content in the skin.
- The dermis, consisting of smooth and hard connective tissues, helps maintain the strength, elasticity, and suppleness of the skin.
- The subcutaneous tissue is composed of fat cells that provide flexibility and cushioning for the skin.

Ulvan

- Anti-inflammatory
- Anti-oxidant activity
- Carbohydrate
- Nutrient replacement
- Cost-effective

Yield point

The yield point can be defined as the point at which the material is in the permanent strain. This point is important to determine the amount of force needed to deform the material. The yield point reflects the material's ability to withstand a certain amount of stress without permanent deformation.

Future

Following the initial mechanical characterization:
- Mechanical evaluation of total construct
- Further optimization printing parameters
- Porous scaffold within the material

Following the development of a dermal biode:.
- Porous scaffold within the material
- Optimization of seating keratinocytes
- Assessment keratinocytes response to printing
- Assessing influence of 3D spatial arrangement of multiple cell types

Acknowledgements

University of Wollongong
Professor Gordon Wallace
Dr. Zhilian Yue

University of Wollongong, School of Technology and Innovation (Computing, Engineering, and Mathematics), Wollongong, New South Wales, Australia

Appendix A: Poster at ACES Electro Material Symposium 2017

Fabrication of ulvan-based structures for cell culture in wound healing

Sylvia van Kogelenberg

Supervisors: Professor Gordon Wallace, Dr. Zhilian Yue, Associate Professor Chris Baker

Background

Due to a consistent need for advancements in regenerative medicine, there is a need to develop fabrication of materials that can promote cell culture and differentiation, which are vital for tissue engineering. Ulvan, a natural polysaccharide, has shown potential in wound healing and tissue regeneration. This study aims to explore the use of ulvan in this context.

Objectives

- Develop a fabrication approach to mimic the dermal layer with an ulvan-based hydrogel
- Create an in situ mechanically stable during the printing process
- Develop a hydrogel with mechanical similar properties to the dermal layer
- Develop an adaptable, but repeatable printing process
- Control spatial arrangement of cells

Blomiromy

To create a skin substitute, it is important to study formation, mechanical properties, and unique composition of the skin. (Lee et al., 2005). The following skin consists of these main components:

- The epidermis, containing mainly keratinocytes, is primarily responsible for maintaining the skin’s barrier function and maintaining the water content in the skin.
- The dermis, consisting of smooth and hard connective tissues, helps maintain the strength, elasticity, and suppleness of the skin.
- The subcutaneous tissue is composed of fat cells that provide flexibility and cushioning for the skin.

Ulvan

- Anti-inflammatory
- Anti-oxidant activity
- Carbohydrate
- Nutrient replacement
- Cost-effective

Yield point

The yield point can be defined as the point at which the material is in the permanent strain. This point is important to determine the amount of force needed to deform the material. The yield point reflects the material's ability to withstand a certain amount of stress without permanent deformation.

Future

Following the initial mechanical characterization:
- Mechanical evaluation of total construct
- Further optimization printing parameters
- Porous scaffold within the material

Following the development of a dermal biode:.
- Porous scaffold within the material
- Optimization of seating keratinocytes
- Assessment keratinocytes response to printing
- Assessing influence of 3D spatial arrangement of multiple cell types

Acknowledgements

University of Wollongong
Professor Gordon Wallace
Dr. Zhilian Yue

University of Wollongong, School of Technology and Innovation (Computing, Engineering, and Mathematics), Wollongong, New South Wales, Australia

Appendix A: Poster at ACES Electro Material Symposium 2017
CRITICAL REVIEWS

3D Printing and Cell Therapy for Wound Repair
Sylvia van Kogelenberg1,2, Zhilian Yue1, Jeremy N. Dimor0, Christopher S. Baker1,4, Gordon G. Wallace1
1ARC Centre of Excellence for Electromaterials Science, Intelligent Polymer Research Institute, Innovation Campus, University of Wollongong, NSW, Australia.
2University of Utrecht, the Netherlands.
3Department of Dermatology, St Vincent’s Hospital Melbourne, VIC, Australia.
4Department of Medicine (Dermatology), University of Melbourne, VIC, Australia.

*Correspondence Zhilian Yue. Intelligent Polymer Research Institute, ARC Centre of Excellence for Electromaterials Science, AIIM Facility, Innovation Campus, University of Wollongong, Wollongong, New South Wales 2522, Australia (Tel: +61 (2) 4221 3833; Fax: +61 (2) 4221 3114; E-mail: zyue@uow.edu.au). Gordon G. Wallace, Intelligent Polymer Research Institute, ARC Centre of Excellence for Electromaterials Science, AIIM Facility, Innovation Campus, University of Wollongong, Wollongong, New South Wales 2522, Australia (Tel: +61 (2) 4221 3137; Fax: +61 (2) 4221 3114; E-mail: gwallace@uow.edu.au).

Word Count: 3693
Keywords: 3D printing, wound repair, skin tissue engineering, biofabrication

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801
ABSTRACT

Significance: Skin tissue damage is a major challenge and a burden on healthcare systems, from burns and other trauma to diabetes and vascular disease. Although the biological complexities are relatively well understood, appropriate repair mechanisms are scarce. 3D bioprinting is a layer-based approach to regenerative medicine, whereby cells and cell-based materials can be dispensed in fine spatial arrangements to mimic native tissue.

Recent Advances: Various bioprinting techniques have been employed in wound repair based skin tissue engineering, from laser induced forward transfer (LIFT) to excursion-based methods, and with the investigation of the benefits and shortcomings of each, with emphasis on biological compatibility and cell proliferation, migration, and vitality.

Critical issues: Development of appropriate biological inks and the vascularization of newly developed tissues remain a challenge within the field of skin tissue engineering.

Future Direction: Progress within bioprinting requires close interactions between material scientists, tissue engineers, and clinicians. Microvascularization, integration of multiple cell types and skin appendages will be essential for creation of complex skin tissue constructs.