ArtiVasc-3D-Generation of a 3D vascularized skin substitute

Large scale skin defects following burn accidents, trauma or tumor resection need to be reconstructed frequently in clinical practice. Because donor sites often are limited, new approaches need to be established to overcome this shortage. ArtiVasc 3D is a multinational, interdisciplinary project to develop a bioartificial vascularized skin substitute.

M. Keck, Wien

ArtiVasc 3D will, for the first time, allow tissue replacement with optimum properties. Scientists, engineers and clinicians will research and develop an innovative combination of hi-tech engineering such as micro-scale printing, nano-scale multiphoton polymerisation and electro-spinning with biological research on biochemical surface modification and complex cell culture. In a multidisciplinary approach, experts in biomaterial development, cell-matrix interaction, angiogenesis, tissue engineering, simulation, design and fabrication methods work together to generate bioartificial vascularised skin in a fully automated and standardised manufacturing approach. The bioartificial vascularised skin engineered in ArtiVasc 3D will be of great value in a vast array of clinical treatments, e.g. as a transplant in trauma treatment.

Generation of a vascularized 3D skin substitute and soft tissue

A high number of patients is suffering from a loss of skin and soft tissue due to trauma, tumor removal or burns. Limitations in reconstructing these defects ask for new solutions such as tissue engineering approaches.

Nearly 6 million people in Europe need reconstructive surgery every year.[1] Additionally, 4.5 million people in Germany, and 30 million people in the five largest European countries suffer from chronic wounds like diabetic ulcers, which have to be treated with wound dressings.[2] And every year there are more than 20 000 cases of major burns that require skin transplantation in Europe.

There are significant limitations for current non-vascular skin equivalents when treating wounds caused by burns, trauma, surgical excisions and chronical ulcers. There are several limiting factors that prevent full integration such as insufficient nutrition supply, and a lack of fat tissue for rebuilding the connective tissue. These limitations can be overcome by utilising a partly or fully vascularised skin equivalent, which consists of an artificial vascularised fat cell layer, with patient specific autologous cells, a micro-vascularised dermal, and an epidermal cell layer. In addition to its potential clinical use the new bioartificial vascularised skin substitute can be used as an in vitro skin equivalent for pharmaceutical or chemical substance testing.

In vitro models of complex tissues represent a promising alternative to animal experimentation, which has the potential of reducing expensive, ethically disputed animal testing. Current in vivo experiments can only partially represent the situation in humans and are also raising ethical concerns. For the first time, the use of vascularised human tissue models will allow the complete simulation of the resorption path including the bilateral transport of substances between blood vessels and surrounding tissue, and the monitoring of the dynamic distribution in artificial circulation. In this way, not only can the cellular interaction of pharmaceutical, cosmetics and chemical substances be investigated but also the influence on the whole metabolism within complex tissue structures. As a result, using the skin model allows an accurate risk assessment for any tested substance.

Challenges in regenerative medicine

The use of bioartificial tissue for regenerative medicine offers great therapeutic potential, but also has to meet high demands with respect to the interaction of the bioartificial devices and natural tissues. Key issues for the successful use of bioartifical tissues as natural tissue replacements are their long term functional stability and biocompatible integration. Various approaches for the generation of bioartificial tissues have failed due to insufficient nutrition and oxygen supply. This is the reason why so far only tissue engineered products for non vascularised tissues such as cartilage or bone have been realized.

In the past, many approaches to soft tissue engineering have failed, due to insufficient metabolism. To solve this problem, it is necessary to introduce vascularised networks; otherwise sufficient metabolism can only be guaranteed to depths up to 200 µm thickness (the diffusion limit of oxygen).

Current research in vascularisation focuses on three areas:
- Scaffold design: Combination of suitable materials and scaffold geometries to stimulate cells to form vascularised tissue.
- Prevascularisation: seeding relevant vascular cells on tissue engineered constructs, to obtain vascular structures prior implantation.
- Angiogenic factor delivery: providing appropriate stimulus for the autonomous generation of vessels (angiogenesis).

Currently, the above mentioned objectives cannot be realised sufficiently. In the area of scaffold design, there is a lack of geometric and dimensional freedom, especially regarding the vessel diameters and mechanical properties needed. Also there is no way to manufacture artificial branched tube structures with a diameter range from 1 µm to 1 mm. Major problems in in vitro prevascularisation arise in adherence and function of vascular cells. Additionally the perfusion of the micro-scale network is challenging and the connection between in vitro micro-vascular scaffolds and the in vivo vascular systems could not be achieved. The crucial point with angiogenic factor delivery is the partially uncontrollable stability and release profile of biomolecules. Additionally the ingrowth of vessels from host is still needed to perform vascularisation.

The success of research within 3D focuses on the combination of all three aspects, overcoming present inadequacies as mentioned above. The innovative combination of process technologies allows the production of optimum scaffolds. Novel materials with improved mechanical properties provide important stimuli to maintain the functionality of vascular cells and tailor-made biofunctionalisation and detailed characterisation of matrix-tissue interactions will enable the optimised endothelialisation of the artificial vessel structures. A co-culture of relevant cell types, able to induce angiogenesis, ist being used in order to mimic the complex interdependencies in nature.

The development of an artificial vascularised tissue scaffold for the generation of in vitro tissue and skin systems for transplantation and use as a test system requires a multidisciplinary approach uniting biology, engineering and clinical practice. The complete research and development chain has to be covered to deliver a vascularised tissue scaffold that can enter the regulatory process at the end of the project. To achieve this ambitious aim the work in ArtiVasc 3D has been organized into 10 scientific and technical work packages that address the current limitations in their domain.

Premanufactured vascular system

To be able to increase the application field of tissue engineered products, the establishment of a complex vascular structure that fully resembles mature blood vessels in vivo must be addressed, as well as providing adequate structural support. The generation of adequate tissue substitutes often requires a functional vascular network, in order to supply nutrients and dispose metabolites. Furthermore, a scaffold matrix for improved and stable cell proliferation is needed. Therefore, the generation of vascularized artificial tissue constitutes a challenging combination of three individual tasks:
- 3D-micromanufacturing of blood vessels,
- the provision of a topographic scaffold for the mechanical framework of artificial tissue, and
- a cell adapted biofunctionalisation for both components, which allows optimal cell co-culturing and proliferation leading to a stable formation of artificial vascularised tissues.

The challenge is to build artificial scaffolds by technical means, which adequately mimic the natural 3D environment of growing cells.

Preliminary results

Generation of adipose tissue

Mature adipocytes are the predominant cell type in the native fatty tissue. They have an univacuolar cell morphology with the nucleus compressed to the plasma membrane.[4]

Adipose-derived stem cells (ASCs) are immature precursor cells located between mature adipocytes in adipose tissue. These cells can serve as an ideal autologous cell source for adipose tissue engineering approaches. These precursor cells are much more resistant to mechanical damage and ischemia than mature adipocytes.[5] Adipose-derived stem cells can be harvested during liposuction or resection of adipose tissue. It has been shown that they are able to proliferate rapidly and differentiate into a variety

of tissues such as bone and adipose tissue both in vitro and in vivo.

The in vitro fatty tissue will be generated by using electro-spun scaffolds (fleeces) combined with hydrogel components, seeded with ASCs and adipocytes (Fig. 1).

Figure 1: Schematic sketch of built-up artificial in vitro skin

Electrospinning has gained much interest as processing technique for scaffold structures in soft tissue engineering. This technique not only offers a high flexibility in material selection including synthetic and also natural polymers, but also provides nano- or microstructured three-dimensional scaffolds that resemble the extracellular matrix and support the mechanical stability of tissue. The electrospun scaffolds allow cells to detach and communicate with each other. They support extracellular matrix generation, cell differentiation and vascularization. An adequate porous structure of the scaffold is a crucial factor for cell differentiation and integration. Fabrication parameters of the electrospinning process can determine the fiber diameter, porestructure, as well as mesh density and thickness.

In our study electrospun poly(ester-urethane) and poly(ester-urethane-urea) fleeces were synthesized. ASCs were isolated out of human adipose tissue and cultivated on top of the electrospun fleeces. Viability was detected by fluorescence staining of cytoplasm of viable cells using fluorescein diacetate and the nuclei of dead cells were stained using ethidium-homodimer-1. ASCs on the scaffolds showed very high viability[6] and a physiological morphology. Actin fibers were well expressed and spread into cell processes. Cells aligned and spread in the direction of the polymer fibers.[6] For determination of the potential of ASC to differentiate into the adipogenic lineage, cells were grown on biomaterials for two days followed by induction of adipogenesis. On day 21 lipid droplet accumulation could be observed in the cells as a sign of differentiation into the adipogenic lineage.[6]

A variety of hydrogel materials were synthesized (acrylated hyaluronic acid, methacrylated hyaluronic acid, collagen and methacrylated gelatin) and tested regarding stability, cell viability, cell differentiation and cell adhesion. Hydrogels can serve as an appropriate ECM substitute and here, gelatin is a very promising material for in vitro 3D cell culture. It has a natural RGD content and is soluble at a physiologic pH. Further, it can build triple helices comparable to collagen type I.[7] To produce stable hydrogels at a physiologic temperature, gelatin has to be modified. In a preceding study we used methacrylated gelatin that was cross-linked by the use of UV radiation and a photoinitiator.[7]

Hydrogels (5%) were prepared with adipose derived stem cells. To analyze cell viability within the gel, life/dead staining and an MTT assay were performed 7 and 14 days after gel preparation. Results show viable cells within the hydrogels. ASCs proliferate and are also able to migrate out of the gel.

In addition ASCs within the gel were differentiated for 21 days. The accumulation of lipids was observed and results show that cells do differentiate into the adipogenic lineage and begin to build up lipid droplets.

Methacrylated gelatine seemed to be most promising regarding handling, stability and supporting cellular functions.

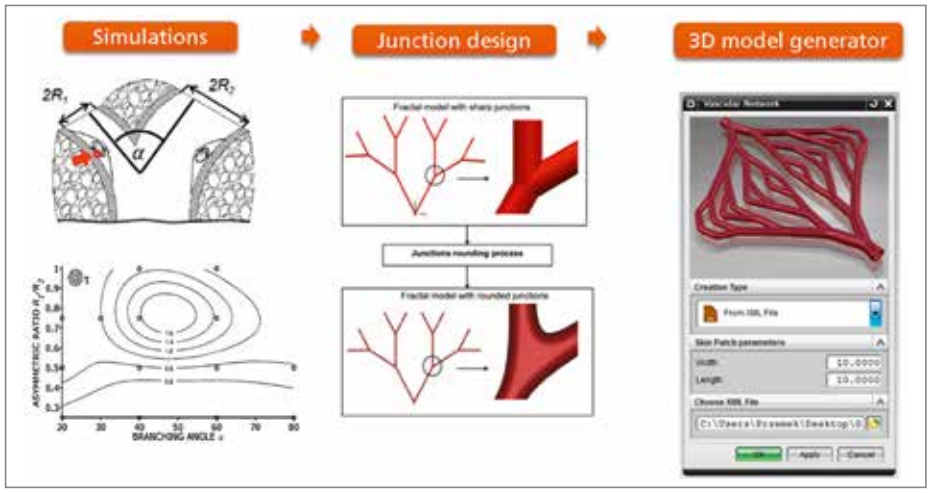

Figure 2: Simulation and design of vascular branched system

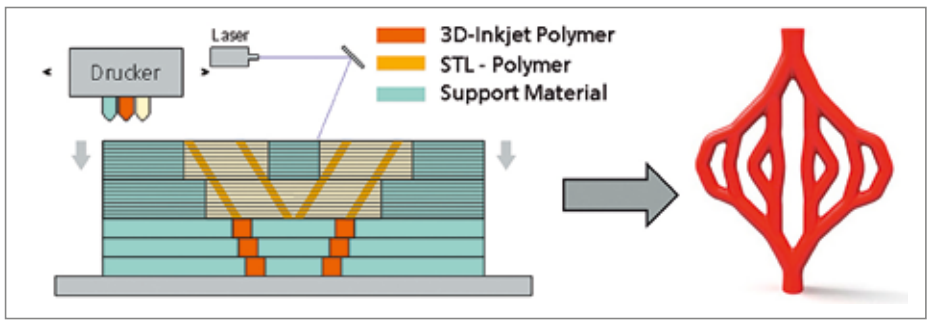

Figure 3: Process combination for layer-by-layer vessel structuring

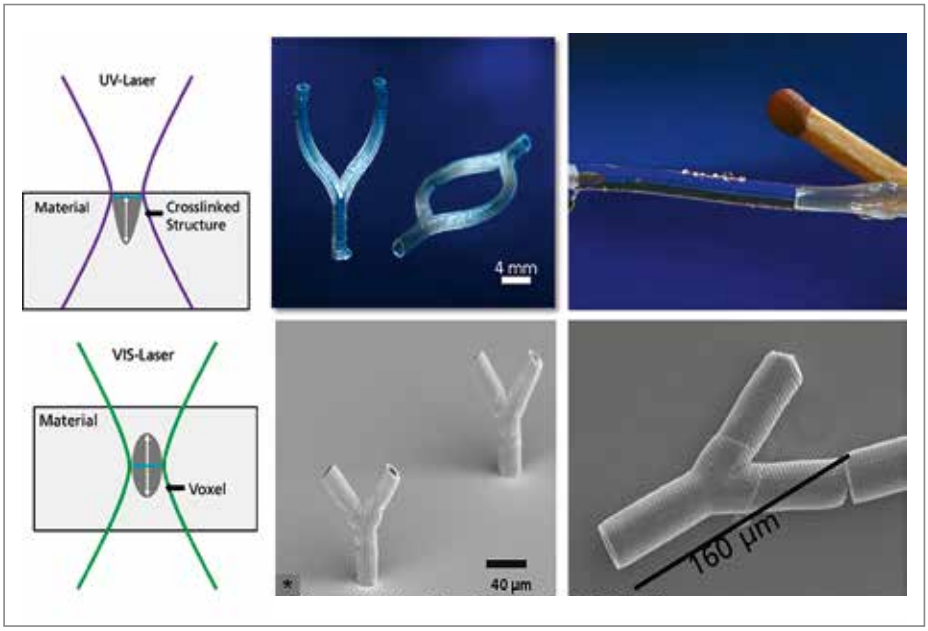

Figure 4: Stereolithography and two-photon polymerisation for vessel structuring; * Meyer W et al: J Funct Biomater 2012; 3: 257-268[8]

Production of vascular system

To allow nutrition supply a branched vascular system will be build up. The vascular system should be surrounded by the electrospun fleeces and the adipocyte containing hydrogels. Since the vascularized fatty tissue should have a size of 10cm x 10cm just a single tube would not be sufficient. A branched system which mimics the natural system with branches and vessels with decreasing diameter will be build up. The vessel geometry, the wall thickness and the wall elasticity, as well as wall porosity are under investigation. In mathematic simulation vessel structures are designed which allows an optimized nutrition supply within the fat. With these data an automated combined rapid manufacturing process can be fed (Fig. 2). The process is a combination of 3D-Inkjet printing, stereolithography and multiphoton polymerization (Fig. 3). Inkjet printing will allow the production of large vessels with wall thicknesses of more than 200 µm. For smaller vessels material will be positioned layer-by-layer by inkjet printing. Afterwards UV-polymerisation by stereolithography with a laser source will structure thin vessels with pores within these layers. In the area of very small vessels a two-photon-process allows the fine structuring of pores with diameters of few micro-meters (Fig. 4).

Linear porous tubes are produced by stereolithography and post process laser drilling. The long-term goal is to build the branched porous tubes by process combination immediately. The tubes will be seeded with endothelial cells and pericytes and will then be combined with the hydrogels and fatty tissue. Neoangiogenesis should start from vessel pores. These new vessels should provide the nutrition for the whole tissue construct. Artificial blood vessels and processes have special requirements which have to be fulfilled by a material which is suitable for processing. The material must be biocompatible and elastic for being a blood vessel and it needs to have the right viscosity for inkjet printing and needs to be UV-polymerizable for laser processing. Therefore a new designed material based on acrylates is under investigation.[8] First tests for surface functionalization and cytocompatibility as well as cultivation with endothelial cells showed promising results.[9]

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