# The Use of Human Blood Derived Protein Components and Crosslinked Hyaluronic Acid in Tissue Engineering and Regenerative Medicine

PhD thesis

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# List of Abbreviations

ANOVA	analysis of variance	
BDDE	butanediol-diglycidyl ether	
<b>CD44</b>	cluster of differentiation 44	
DVS	divinyl sulfone	
ECM	extracellular matrix	
EDTA	ethylenediaminetetraacetic acid dipotassium salt	
FBS	fetal bovine serum	
FCS	fetal calf serum	
FTIR	Fourier transform infrared spectroscopy	
НА	hyaluronic acid	
H-E	hematoxylin-eosin	
HSA	human serum albumin	
IR	infra-red	
MSC	mesenchymal stem cell	
NAG	N-acetyl glucosamine	
PRF	platelet-rich fibrin	
PRP	platelet-rich plasma	
RBC	red blood cell	
SEM	scanning electron microscope	
SPRF	serum from platelet-rich fibrin	
ХТТ	Cell Proliferation Kit II	

# **1. Introduction**

#### 1.1. Regenerative medicine and tissue repair

The aging population and increasing presence of chronic diseases require new solutions for promoting the regeneration of various tissues, thus the field of regenerative medicine and tissue engineering is growing constantly and rapidly since the 1990s (1, 2). The aim of regenerative medicine is to repair or replace damaged, missing, old, or diseased cells, tissues, and organs to restore their functions. In human body the potential of regeneration is extremely wide between different organs, while there are tissues with apparently lacking regenerative capacity (3), thus external help is often necessary. Generally, the three main areas of tissue engineering are cells, scaffolds, and growth factors (4). Stem cells are in widespread use in this field because of their capacity to form *de novo* tissue and promote innate repair (5), while synthetic or natural scaffolds are applied to arrange them in a three-dimensional architecture. Scaffolds, like hyaluronic acid (HA) also act as a carrier of growth factors and promotes their controlled release (6). Growth factors complete the role of scaffold materials by contributing to the regulation of stem cell fate (6). Human blood derivatives are prevalent sources of growth factors (7) hence they are often applied in tissue engineering.

Musculoskeletal disorders are an important section of regenerative medicine as they affect more and more people in the world, and they are one of the leading causes of loss in quality of life (8). Osteoarthritis is one of these diseases, and it is estimated, that one tenth of the population over 50 years will be affected (9). End stage hip and knee osteoarthritis is originally treated radically, by total hip or knee replacement surgery, while there are attempts to delay this painful and costly intervention by alternative solutions in earlier stages of the disease. Intraarticular injection of crosslinked or noncrosslinked high molecular weight hyaluronic acid is an option to improve the joint function through its lubricating effect (10) or by utilizing it as a cell delivery system (11). In addition, hyaluronic acid can be combined with growth factor containing agents, but growth factor concentrates may be used alone too. For example PRP (platelet rich plasma) is in widespread use with the intended use of slowing down the progression of joint diseases, promoting tissue repair and easing pain (12), and SPRF (serum from platelet-rich fibrin, also referred to as hyperacute serum, PRF releasate, or PRF exudate) was found to be suitable for the same indications (13).

#### **1.2. Blood derivatives**

Human blood products are defined fractions of human blood isolated for allogenic and autologous therapeutic uses. The concept of blood derivatives includes whole blood, cellular elements, as red blood cell (RBC) and thrombocyte concentrates; serum fractions, human serum albumin (HSA); coagulation factors, immunoglobulin concentrates, and plasma products isolated both from whole blood, and by apheresis. Serum products undergo the process of blood clotting; thus, they contain activated platelets and the growth factors released from them. By contrast, plasma products are produced using anticoagulants, most commonly citrate, potassium salt of ethylenediaminetetraacetic acid (EDTA) or heparin and additional platelet activation may be needed, generally using CaCl<sub>2</sub> and thrombin.

Human blood products are widely used in regenerative medicine; however, they have an important role as medium supplements as well. Many cell cultures need medium supplementation as a source of growth factors, transport proteins, vitamins, trace elements and hormones for their proliferation and growth in laboratory experiments and cell therapies. The most often used medium supplementation is fetal bovine serum (FBS), also called as fetal calf serum (FCS) (14), however, there is a strong need for an alternative to eliminate the drawbacks of this non-human serum. Its clinical applicability is questionable as well as its reproducibility, and animal welfare concerns. Human blood products, like HSA (15, 16), serum (17), PRP (18), and SPRF (19-21) could all be promising choices to replace FBS.

#### **1.2.1.** Plasma products

In regenerative medicine, one of the most commonly used plasma products is platelet-rich plasma. PRP is isolated from whole blood with anticoagulants, and the aim is to enrich the plasma fraction in platelets, which release a high amount of growth factors and cytokines (22), preferably inducing regeneration in the surrounding tissues. PRP is often used in tissue regeneration and reparation, also in soft tissue engineering, like scar management (23), but especially in orthopedics, like joint diseases, osteoarthritis (12, 24), and osteonecrosis (25). The most common application is treating the patients with intraarticular injection into osteoarthritic knee and hip to ease pain and improve the joint function and motility.

Another plasma product is cryoprecipitate, which was first used to treat antihemophilic factor deficiency in the 1950s (26). The precipitate is composed of fibrinogen, coagulation factor VIII, factor XIII, von Willebrand factor, fibronectin, and platelet microparticles that do not resolve at low temperature, thus they can be collected and resolved in a small volume of plasma (27). Later its indication was altered to treat massive hemorrhage, which caused critical fibrinogen level decrease in trauma, cardiac surgery, liver transplantation, or obstetric hemorrhage. However, its use as transfusion has been withdrawn in many countries due to safety concerns, namely transmission of pathogens or transfusion-related acute lung injury (26).

As a safer solution for promoting blood clotting, one of the most important ingredients of cryoprecipitate, fibrinogen may be purified from human blood and used for various indications. Fibrinogen is normally present in 1.5-4 g/L concentration in human plasma, but its level can be multiplied in cryoprecipitate. It is essential for hemostasis, wound healing, angiogenesis and inflammation, among other biological functions (28). Water-insoluble fibrin is the result of fibrinogen cleavage during hemostasis via a cascade of enzymatic reactions (28). Fibrin is often used for drug and growth factor delivery applications (29), as adhesives in surgical procedures, sealants in tissue engineering and wound healing (30), as fibrin glue (31, 32), for bone repair (33), or for tissue regeneration (34).

### 1.2.2. Serum products

Serum products are isolated from human blood without anticoagulants. Liquid and solid phase serum products are also used in regenerative medicine. One of the most frequently used serum product is platelet rich fibrin (PRF) membrane (35) which is commonly applied in hard tissue engineering, like dentistry (36, 37), in maxillofacial (38) and periodontal plastic surgeries (39, 40), besides in soft tissue engineering (41, 42) such as wound healing (43). PRF is isolated from whole blood usually contacting a negatively charged surface as titanium or glass to promote blood clotting. PRF is an advantageous biodegradable scaffold, because it does not require any anticoagulants for its isolation,

and therefore no additional activation is needed in contrast with plasma products. It contains the platelet-derived growth factors and cytokines, responsible for the biological effects, namely, that it has remarkable cell adhesive and proliferative effects, and promotes revascularization (44, 45). However, since PRF does not contain an extremely large number of platelets, its growth factor and cytokine milieu are close to natural (46), as distinct from platelet concentrates. When isolating PRF membranes, the serum has to be squeezed out from the fibrin clot to get a flat membrane. This serum is called serum from platelet rich fibrin, and it has the advantageous effects of PRF; however, it is a liquid, thus it has further possible applications in regenerative medicine. In recent studies, the positive effects of SPRF *in vitro*, cell proliferative and regenerative effects were proven on human mesenchymal stem cells (19, 21, 47), and on the cells of bone, cartilage and synovial membrane (13, 20). In the literature, possible utilizations of SPRF were reported in bone grafting (48), implant surface functionalization (49), in dental applications (50), or as another indication, treating osteoarthritis (13).

#### 1.3. The production of SPRF and a new device

There are different ways to prepare SPRF, however, these techniques have certain inconveniences, if the aim is to use SPRF in the field of regenerative medicine, thus a new device was needed to overcome these issues. For the production of SPRF, most of the existing methods isolate PRF in glass vacuum blood collection tubes as the first step and obtain the serum by pressing it out on a sterile grid (49), using a centrifuge, or shaker and a vortex (51). Besides, there is an existing medical device, the PRF box, which contains compression wells to press out the liquid from the PRF in a sterile environment, however the indication of this device is PRF membrane production for dental void filling, while serum is a secondary product (52).

The abovementioned method's disadvantage is that open handling of the PRF clot cannot be avoided, thus a laminar flow hood is required to achieve the production of sterile SPRF. Practically, the serum is injected intraarticularly to the patient's knee to treat osteoarthritis, thus sterility is essential. However, orthopedic clinics do not generally possess laminar flow hoods, hence there was a need for a new approach. To overcome this inconvenience, a syringe-shaped medical device was developed for autologous SPRF production in a closed system for point of care therapy by our research group (Figure 1). The device called HypACT Inject Auto was designed to draw venous blood from the patient and help blood coagulation by a glass part during centrifugation, thus PRF is formed inside the syringe. The serum can be squeezed out from the PRF clot using the plunger and directly injected to the patient's knee without open handing, maintaining sterility. With the depicted device, the aseptic processing of the whole blood to produce PRF and serum can be achieved (47).

Item number	Description		
1	Glass Cylinder		
2	Casing		
3	Distal Connector		
4	O-Ring		
5	Spacer		
6	Waste Container		
7	Syringe Piston		
8	Syringe Plunger		
9	Container Cap		$\sqrt{\circ}$
10	Waste Piston		$\odot$
11	O-Ring		
	4 (4) (10) (9) (10) (10) (10) (10) (10) (10) (10) (10		
		_5 cm	1

Figure 1: Visualization of the HypACT Inject Auto medical device and its parts (47).

The syringe was developed and patented by our research group, and it was authorized as a Class IIa medical device (47). For medical device authorization the safety and effectiveness of the device has to be proven by clinical evaluation. One possible way for this is clinical investigation (53), the other approach is the demonstration of clinical equivalence (54) to an existing method or medical device in terms of clinical, technical, and biological properties. In the case of HypACT Inject we chose the second possibility, we compared device derived and manually isolated SPRF to each other in terms of mesenchymal stem cell viability promoting effects *in vitro*.

### 1.4. The role of hyaluronic acid in regenerative medicine

Tissue engineering aims to replace defective tissues and recreate their structure and function. The growing demand for tissue implants promoted investigations towards cell regeneration and appropriate scaffolds to arrange the cells into three dimensional structures, which established the discipline of tissue engineering (55). The remodeling cells can be seeded into the matrices before implantation, or scaffolds can be supported with cell attracting materials to induce the attachment of the surrounding cells. The ideal scaffolds mimic human tissue structure based on their physical and chemical similarities (56), thus hyaluronic acid may be an ideal base material for scaffold development.

Hyaluronic acid is a natural polysaccharide, a non-sulphated, linear glycosaminoglycan built of repeating units of D-glucuronic acid and N-acetyl-D glucosamine (57). The molecular weight highly affects its physical and biological properties, under 10<sup>3</sup> kDa it is classified as low molecular weight HA and above 10<sup>3</sup> kDa it is considered high molecular weight HA (58). Hyaluronic acid is a water-soluble, biocompatible, non-immunogenic, and biodegradable material. It has important biological roles in many parts of the mammalian body in the extracellular matrix, umbilical cord, loose connective tissues, synovial fluid, cartilage, tissues of the eye and the skin, lung, muscle tissues and brain (58-60). It is capable of communicating with cells via membrane receptor CD44 (cluster of differentiation 44) (61-63) and it can induce angiogenesis, cell proliferation, aggregation or migration (64). Additionally, HA affects tissue regeneration by regulating the process of inflammation, as high molecular weight HA was reported to have anti-angiogenic and anti-inflammatory effects, while low molecular weight HA fragments were found to have an adverse action, they induce inflammation and angiogenesis and they have immuno-stimulatory effect (65).

#### 1.5. Crosslinked hyaluronic acid

Hyaluronic acid is a biodegradable polymer thus its natural enzymatic and nonenzymatic degradation *in vivo* is rapid, which may be a drawback in the case of tissue engineering applications. Its presence in the body can be prolonged by crosslinking the HA chains covalently (65) so increasing its resistance against hydrolysis (66). The mechanical properties are improved, and a water-insoluble hydrogel can be produced,



В



*Figure 2*: *The structure of hyaluronic acid, and the crosslinking reactions with* **A**: *BDDE and* **B**: *DVS (67).* 

which is a hydrophilic three-dimensional polymeric network absorbing large amounts of water or biological fluids (68). Crosslinked HA hydrogels are outstanding base materials of biocompatible scaffolds for tissue engineering (69) with reduced degradation and

water-insolubility. Different crosslinking agents form various chemical bonds, one type of the crosslinkers react with the hydroxyl group; glutaraldehyde, divinyl sulfone (DVS) (70) and butanediol-diglycidyl ether (BDDE) (71) are parts of this type, while carbodiimides act on the carboxyl group (65) (Figure 2). Increasing crosslinker concentration results in higher crosslinking density with increasing degradation time and improved mechanical properties. The crosslinking density also affects the water uptake capacity of the hydrogels. It can be determined by calculating the swelling ratio: the quotient of the swollen and dry gels' weight (72). A strongly crosslinked, highly interconnected gel can absorb less water, while weakly linked hydrogels have a higher swelling ratio (73). It is crucial to find the ideal crosslinker concentration to get a strongly crosslinked, but elastic matrix: excess reagent can result in a rigid and fragile gel, while low crosslinker concentration results in disintegrating gels with an extremely high swelling ratio. Nevertheless, most of the crosslinkers are toxic for cells in their unreacted forms in larger amounts (74, 75).

### 1.6. Hyaluronic acid derivatives in tissue engineering and wound healing

Crosslinked hyaluronic acid based matrices can be ideal choices as scaffolds for soft tissue engineering (76-78) due to their biocompatibility and biodegradability, nonimmunogenicity (79), possible stimulatory effects on cell proliferation, migration, extracellular matrix secretion and differentiation (62). Soft tissue replacement may be needed in the cases of soft tissue defects like congenital malformation, extirpation, or trauma (80). The structure of hydrogels mimics natural tissues, thus surrounding cells adhere to this network, helped by the biological regulating ability of hyaluronic acid. In the case of soft tissue deficiency, the hydrogel can serve as a synthetic extracellular matrix filling up the space of the missing tissue due to its high water-content and soft structure. This synthetic ECM (extracellular matrix) helps organizing the attaching cells into a three-dimensional structure (81), which preferably induces the remodeling and vascularization of the scaffold (82).

Another investigated utilization of the HA hydrogels is to prepare films to cover wounds, as they were reported to facilitate wound healing (60). The natural steps of wound healing process are well-known: hemostasis, inflammation, proliferation, and remodeling (83). This procedure may be inhibited in various conditions, like diabetic or pressure ulcers (84, 85), thus the wound cannot be recovered without external help. Severe burn injuries covering a wide area of the skin also need to be treated to avoid infections and dehydration (86). To help the healing process, a wound dressing may be required, which prevents contamination, maintains adequate moisture, and removes excess exudates (84). Crosslinked hyaluronic acid may be used as wound dressing, as it is able to create an advantageous environment based on its rheological, hygroscopic, and viscoelastic properties (85). Moreover, high molecular weight HA was found to facilitate cell migration, re-epithelialization, and new soft tissue formation (60), in addition, a cytoprotective effect was also attributed to it. Low molecular weight HA was reported to induce angiogenesis (85), thus it also contributes to the healing process, although it was not found to have the aforesaid protective effects (87).

### 1.7. Hyaluronic acid supplemented with blood derivatives

Although crosslinked hyaluronic acid gels are promising base materials for soft tissue implantations, and wound dressings, high molecular weight HA was found to be bioinert and weakly promoting cellular adhesion and proliferation when it was applied alone (72). This inconvenience can be evaded by preparing hybrid scaffolds: in existing researches HA is mixed with silk-fibroin (69), chitosan (88, 89), gelatin (90), or collagen (91, 92), among others. Another option to increase the cell attachment capacity of the gel is by coating it with various materials, like extracellular matrix gel, collagen, fibronectin, or laminin (93). Moreover, in a study peptides were incorporated into the hydrogel, which was reported to enhance cell growth, proliferation, adherence, migration, and organization (82).

Similarly, blood derived proteins can be incorporated, polymerized, or crosslinked into the structure of the HA hydrogels. Elaheh Jooybar and her coworkers developed a cell-laden injectable hyaluronic acid-tyramine scaffold with incorporated platelet lysate to be used as a cell delivery system for cartilage regeneration. In this case the tyramine conjugated HA was crosslinked using horseradish peroxidase and H<sub>2</sub>O<sub>2</sub> in a platelet lysate solution (62). Şükran Şeker *et al.* created an autologous HA-NH<sub>2</sub> tissue engineering scaffold crosslinked with genipin, containing platelet lysate isolated from PRP (94), and Connie Y. Chang *et al.* prepared HA-blood hydrogels from HA functionalized with N-hydroxysuccinimide, and lysed whole blood for improving stem

cell delivery to the myocardium (95). In another study fibrin from leukocyte- and plateletrich plasma was used to form a hydrogel exploiting the natural polymerization of fibrin, and HA coils were entangled through physical crosslinking (96). Sang-Hyug Park *et al.* prepared a fibrin-HA composite gel with pelleted chondrocytes also utilizing the polymerization process of fibrinogen, but with covalent crosslinking (97).

In our study we subsequently supplemented covalently crosslinked HA hydrogels. SPRF was chosen as it has noted effects on cell proliferation and regeneration (13, 19-21, 47) and it can be immobilized into the scaffold using chemical crosslinkers. Furthermore, we planned to exploit the natural fibrin polymerization process as well because fibrin also has advantageous effects on cell attachment and proliferation (41). In contrast to SPRF there is no need to further crosslinking, because when cryoprecipitate is isolated from human plasma, the concomitant fibrinogen can be activated, and through polymerization fibrin fibers are entangled in the covalently crosslinked HA matrix. Additionally, the growth factors and cytokines present in cryoprecipitate, entrapped by the fibrin matrix may have further benefits on regeneration.

# 2. Objectives

We intend to achieve our experiments in our well-equipped laboratories, where we have access to all devices and materials needed for cell culture, additionally, a spectrophotometer, an inverse fluorescent microscope, and a freeze drier. Besides, we plan to cooperate with other laboratories, who are able to conduct animal experiments, histological analysis, have a SEM (scanning electron microscope), and an FTIR (Fourier transform infrared) spectrometer. The Institutional Review Board approval number for phlebotomy is 33106-1/2016/EKU, 12.07.2016., and the animal experiments are approved by the National Scientific Ethical Committee on Animal Experimentation (PEI/001/2706-13/2014, approval date: 17 December 2014).

Firstly, we plan to prove the clinical equivalence of the device derived and manually isolated serum on human bone marrow derived mesenchymal stem cells (MSCs). SPRF is planned to be isolated manually in the presence of glass beads, besides, it will be isolated using the medical device. However, when isolating with the device, the serum usually contains a small number of red blood cells, which can be harmful for the cells, but RBCs can be separated if a second centrifugation step is introduced. Thus, we aim to compare the effects of these three kinds of SPRF groups on the viability of MSCs, to examine if the usage of the HypACT Inject Auto medical device is as safe as the original SPRF isolating method for the preparation of an intraarticular injection for knee osteoarthritis. Secondly, we plan to investigate the proliferating effects of SPRF used as medium supplementation, compared to fetal bovine serum and human serum albumin. We aim to use MSCs to inspect the effect of the supplementing materials on cell viability and density in the wells.

The further objective is to develop a hyaluronic acid-based matrix, which can be used as a replacement in the case of soft tissue deficiency. To achieve this, we plan to crosslink hyaluronic acid to prolong its presence in the body after implantation and supplement it with human blood derived protein to increase the cell attachment capacity (62). Butanediol-diglycidyl ether (BDDE) and divinyl-sulfone (DVS) are planned to be used as crosslinkers in different concentrations to find the best crosslinker type and ratio to have a strongly crosslinked, non-cytotoxic hydrogel, which is resistant against enzymatic degradation. We want to achieve a product as drug or drug-device combination, thus well-documented (98, 99) crosslinkers were chosen. These materials have already been used in similar medical device formulations and their toxicity profile is well known (100, 101). We plan to examine the effects of the type and the concentration of the crosslinker by physical characterization. We measure the swelling ratio of the hydrogels to get information about the water uptake capacity and we aim to indirectly conclude the crosslinking density. We want to compare the resistance against degradation between the different hydrogel groups with the help of an *in vitro* enzymatic degradation test. SEM imaging shows the visual appearance and gives information about porosity, while FTIR reveals changes in chemical bonds of hyaluronic acid after crosslinking. We plan to supplement crosslinked HA with two blood derived proteins, one of them is SPRF and the other is fibrin. To immobilize SPRF, a second crosslinking step is needed to bind the protein to the HA matrix. In the case of fibrin, immobilization is simpler: cryoprecipitate is isolated from human plasma, and it is mixed with human thrombin and CaCl<sub>2</sub> and then poured onto the freeze-dried hydrogel. Subsequently, the liquid gets absorbed by the matrix and fibrinogen activation and fibrin chain formation takes place inside the scaffold. The advantage of this method is that it does not require additional toxic chemical reagents. We aim to characterize the modification using FTIR spectroscopy to investigate the protein content of the matrices, and we plan to use SEM to visualize the changes. MSCs are planned to be used to examine the cytotoxicity and cell attachment capacity of the scaffolds. Afterwards, we want to choose one gel type with the most promising physical and biological properties and test it for biocompatibility: we plan to homogenize and inject it subcutaneously into C57BL/6 mice. We aim to observe *in vivo* degradation and vascularization by microscopic imaging, then the implants are harvested, and histological analysis is planned to be conducted to detect if remodeling and new vessel formation occurred inside the scaffolds.

## 3. Results

#### **3.1.** The effect of SPRF as medium supplementation

To examine the effects of differently isolated serum products and to test the safeness of the HypACT Inject medical device, various supplementing materials were added to mesenchymal stem cell cultures: FBS as positive control, manually isolated SPRF, device derived SPRF, two times centrifuged device derived SPRF, and HSA, and serum free medium was used as negative control. Manually isolated SPRF was produced in a centrifuge tube using glass beads to promote blood clotting, while device derived SPRF was created with the HypACT Inject medical device. A third SPRF group was also examined, as the latter contains some red blood cells, which might be harmful for mesenchymal stem cells, thus a second centrifugation step was installed to get rid of them. The viability of the cells was measured using XTT (Cell Proliferation Kit II) reagent after six days culturing. Manually isolated SPRF was compared to device derived SPRF and twice centrifuged device derived SPRF. According to our results these three types of SPRF were equivalent to each other in terms of cell viability, no significant difference was observed. However, device derived SPRF without the second centrifugation step had slightly, but significantly lower proliferative effect than FBS, while manually isolated, and twice centrifuged device derived SPRF were as good as FBS in terms of MSC viability. As negative control the cells were cultured without serum supplementation. HSA supplementation did not have significantly better effect than serum free medium (Figure 3).

Finally, our results show that both manually isolated and twice centrifuged device derived SPRF have the same advantageous cell proliferating effects as FBS, however, residual RBCs in the medium may be harmful. As we expected, HSA does not possess the same supplementing effect as FBS and the serum products.



**Figure 3**: Viability of MSCs cultured in media supplemented with FBS, HSA, manually isolated SPRF, two times centrifuged device derived SPRF, device derived SPRF and in serum free medium. One-way analysis of variance (ANOVA, analysis of variance) with Tukey's post hoc test was performed to compare the means of groups using Prism 7 software. The significance level was p < 0.05, where \* means that p is between 0.01 and 0.001, and \*\*\* means that p is lower than 0.001, and ns means non-significant. The data are presented as mean  $\pm$  SEM, n = 4, (47).

To support our previous experiment and to visualize the shape and density of the cultured cells affected by different supplementing media, live-dead staining was conducted on the MSCs. Using this method living cells can be distinguished from dead cells, as living cells are stained by Calcein-AM, and they appear in green, dead cells stained by ethidium homodimer in yellow, while all nuclei are stained in blue using Hoechst. The mesenchymal stem cells were cultured in media supplemented with the three above mentioned kinds of SPRF, FBS, HSA and also without serum, and on the sixth day they were visualized by live-dead staining (Figure 4). The microscopic pictures confirm the results of XTT measurement, which were shown in Figure 3, based on the



**Figure 4**: Live-dead staining of MSCs cultured in media supplemented with A: FBS; B: serum free; C: HSA; D: manually isolated SPRF, E: device derived SPRF; and F: two times centrifuged device derived SPRF. Living cells are green, dead cells are yellow and nuclei are blue (47).

density of living cells. MSC density is roughly equal in FBS and SPRF containing wells, and residual RBCs did not cause visible difference in cell density (Figure 4, E). The number of dead cells were negligible in each case. Additionally, cell morphology also seemed to be normal if any kind of SPRF was used as supplementation. (The positive control was FBS containing medium, which is often called stem cell medium.) In the cases of serum free medium and HSA supplementation the cells seemed to be smaller, and their density was also lower, however, very few dead cells could be observed in the wells.

### 3.2. The preparation of the hyaluronic acid scaffolds

A crosslinked hydrogel with suitable properties was developed by tuning the crosslinking conditions, and the methods are described to help better understanding of the results. The hydrogels used in our experiments were prepared according to the following protocol (Figure 5):

BDDE or DVS in 2, 5 and 10 V/V% was mixed with pH=13 NaOH solution and poured onto 1.34 MDa molecular weight freeze-dried sodium hyaluronate. The compound was immediately vortexed until we got a homogenous mixture, it was centrifuged to get a flat gel, and allowed to crosslink. The crosslinked gels were washed and swollen until equilibration in 3 steps. They were autoclaved, and the sterilized gels were freeze-dried.

Swelling ratio and *in vitro* enzymatic degradation measurements, the first part of FTIR analysis, and SEM imaging were conducted using these freeze-dried gels.

In the case of SPRF linking serum was mixed with 5 V/V% DVS and NaOH to reach pH=12. The DVS containing alkaline serum solution was poured onto the freezedried gel pieces. It was let to crosslink and washed three times.

Cryoprecipitate was isolated from human plasma by thawing it at 3°C and centrifuging. The supernatant was resolved in 40% of the original plasma volume, it was activated with CaCl<sub>2</sub>, and human thrombin and it was immediately poured onto the freezedried HA matrices. The gels absorbed the plasma and clotting began inside the scaffolds, fibrinogen converts into fibrin polymers, getting caught by the matrix. Swollen SPRF or fibrin containing gels were used for cell culture experiments, or they were freeze-dried for the second part of FTIR spectroscopy, and SEM imaging.

5% DVS containing gels with and without fibrin were homogenized and injected into each leg of the mice, control to one leg, and fibrin containing to the other.



*Figure 5*: *Steps of preparing the hyaluronic acid-based hydrogels used for the experiments.* 

### 3.3. Crosslinking hyaluronic acid with different percentages of DVS and BDDE

We aimed to produce solid but elastic hydrogels by crosslinking high molecular weight sodium hyaluronate using BDDE and DVS as crosslinkers in different concentrations. Figure 6, I. shows the physical appearance of the crosslinked hydrogels. Interestingly, crosslinking with BDDE resulted in clear and flexible hydrogels, while in the case of DVS the matrices were a bit rougher and more rigid, but still flexible. The gels containing 10% crosslinker were too rigid, fragile, and moldered during the washing procedure. Based on this experience we concluded that 10% of both crosslinkers is a too high ratio, while 2 and 5% crosslinker concentrations resulted in hydrogels with advantageous properties. Thus, we only continued conducting experiments with 2 and 5% BDDE and DVS containing gels.



*Figure 6*: The crosslinked hyaluronic acid gels. *I.* The appearance of the matrices: A: 2% BDDE gel; B: 5% BDDE gel; C: 2% DVS gel; D: 5% DVS gel. *II:* SEM image of the structure of A: 2% BDDE; B: 5% BDDE; C: 2% DVS; and D: 5% DVS gels (67).

To explore the inner structure and porosity of the crosslinked hydrogels, SEM analysis was conducted. The crosslinked gels were washed and freeze-dried, and cut with a scalpel, then their cross-section was observed by SEM. The structure of the hydrogels was porous and honeycomb-shaped both if DVS and BDDE were used (Figure 6, II.), and no significant difference was observed between the pore sizes examined using ImageJ software (data not shown). Porosity is an essential property of hydrogels, however, we expected, that the pore sizes would decrease with increasing crosslinker ratios. The explanation of our result may be that the hydrogels could not be cut from the same angle, hence the pore sizes could not be measured properly.

### 3.4. Swelling ratio

The swelling ratio is an important property of the hydrogels inversely referring to the crosslinking density of the matrix. It relates to the water uptake capacity, and it can be calculated from the weights of the freeze-dried and swollen gels using the following formula:

Swelling ratio =  $W_s / W_d$ 

Where  $W_s$  is the weight of the swollen gel (g) and  $W_d$  is the weight of the freezedried gel (g) (72).

The more connections are existing between the HA chains, the less water it can absorb into its structure. In our case, when the same percentage of crosslinking reagent was used, the DVS containing gels were found to have lower water uptake capacity and significantly lower swelling ratio. As it was presumed according to the literature (102), we also observed that the 5% crosslinker containing samples were significantly less swollen than the 2% crosslinker containing ones both in the case of BDDE and DVS (Figure 7), which suggests stronger crosslinking.



*Figure 7*: Swelling ratio of the crosslinked gels. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to compare the means of groups using Prism 7 software. The significance level was p < 0.05, where \* means that p is between 0.01 and 0.05, \*\* means that p is between 0.01 and 0.001, and \*\*\* means that p is lower than 0.001, and data are presented as mean  $\pm$  SEM, n = 8 (67).

#### 3.5. In vitro enzymatic degradation

Degradation is a cardinal aspect of hydrogels, as biodegradability is required, however gels, which are resorbed precociously cannot be used as scaffolds. To compare the speed of the degradation of the different matrices, the crosslinked gels were soaked in hyaluronidase solution (hyaluronidase from bovine testis, type I, these enzymes randomly cleave  $\beta$ -N-acetylhexosamine-[1 $\rightarrow$ 4] glycosidic bonds in hyaluronic acid). The mixtures were measured using Ehrlich's reagent, which detects the N-acetyl glucosamine (NAG) concentration (103-105), one product of hyaluronic acid degradation. Ehrlich's solution reacting with NAG gives a color reaction, and the absorbance is directly proportional to NAG concentration, it can be evaluated on a calibration curve. The measurement was conducted for four days (106), while degradation increased with time with different rate in distinct matrices. Significant difference was observed between 2% DVS and 5% BDDE and between 2% DVS and 5% DVS gels. However, 2% DVS gel seemed to degrade faster than 2% BDDE gel, no significant difference was measured. Thus, 5% DVS and BDDE containing gels seemed to degrade slower than 2% crosslinker containing matrices (Figure 8), which is in good accordance with our expectations, as crosslinker concentration affects the degradation time.



**Figure 8**: In vitro enzymatic degradation of the crosslinked HA gels. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to compare the means of groups using Prism 7 software. The significance level was p < 0.05, where \* means that p is between 0.01 and 0.05, \*\* means that p is between 0.01 and 0.001, and \*\*\* means that p is lower than 0.001, and data are presented as mean  $\pm$  SEM, n = 3 (67).

### 3.6. FTIR analysis of the crosslinked gels and protein containing scaffolds

Successful crosslinking causes changes in the structure of hyaluronic acid, in addition, protein addition results in further structural differences. Observing these alterations can give information about the outcome of the modifications. The chemical changes in the structure of the hydrogels were examined using FTIR spectroscopy. At first natural hyaluronic acid was compared to crosslinked derivatives, then the modification with proteins was analyzed. The characteristic absorbance changes could be followed in the IR (infra-red) spectra. In the images the spectra were shifted vertically to avoid overlapping, and in parts A and B the absorbances were normalized to the peak at 1038 cm<sup>-1</sup>, and in part C to the 1533 cm<sup>-1</sup> peak.



Figure 9: FTIR analysis of the crosslinked and protein containing The matrices. *A*: spectrum of native sodium hyaluronate can be seen at the bottom of the image (HA) and above it in a vertical sequence the crosslinked derivatives: 5% DVS, 2% DVS, 5% BDDE and 2% BDDE are shown. B: FTIR spectra of SPRF containing HA matrices. From bottom to top 5% DVS. 2% DVS, 5% BDDE, and 2% BDDE crosslinked derivatives are shown. C: Fibrin coated HA derivatives. From bottom to top: 5% DVS, 2% DVS, 5% BDDE, and 2% BDDE crosslinked derivatives with fibrin (67).

In Figure 9, A the differences between initial sodium hyaluronate and crosslinked HA are visible. If the spectrum of initial and crosslinked HA is similar, it indicates that no crosslinking happened, while decreasing, augmenting, and newly appearing peaks can reveal new bonds. In the case of the crosslinked derivatives the peak of the OH-group around 3300 cm<sup>-1</sup> decreased because of the newly formed bonds, besides, at 2931 and 2875 cm<sup>-1</sup> the absorbance peaks of CH stretching vibrations of the CH<sub>2</sub>OH groups augmented (106). When HA was crosslinked with DVS, at 1280 cm<sup>-1</sup> a new peak appeared (70). At 1038 cm<sup>-1</sup> the C-OH vibration related to alcohol sidebands are visible in the case of natural hyaluronic acid, which is shifted to lower frequencies when the HA was crosslinked. Amide related carbonyl peak can be observed at 1606 cm<sup>-1</sup> both in crosslinked and in unmodified materials.

The crosslinked HA matrices were supplemented with blood derived proteins. SPRF was crosslinked to the matrices using DVS and fibrin was immobilized with CaCl<sub>2</sub> and thrombin to promote fibrinogen conversion into fibrin chains inside the porous structure of HA matrices.

Figure 9, B shows the effects of SPRF crosslinking into the HA matrices. In the first step HA was crosslinked either with BDDE or with DVS, but SPRF was linked in each case to the hydrogel using DVS. If DVS was used in the first step, the spectra did not show much difference to DVS crosslinked HA without protein addition, however, when BDDE was used as first crosslinker, new peaks appeared at 1238 and 1307 cm<sup>-1</sup>. When DVS was added in the second step, the amide A band of the proteins, which reflects the N-N stretching can be observed at 3280 cm<sup>-1</sup> on top of the broad OH stretching.

Fibrin containing scaffolds can be observed in Figure 9, C. The amide I, II and III bands of proteins caused three intense new peaks at 1643 cm<sup>-1</sup>, at 1533 cm<sup>-1</sup> (107), and at 1390 cm<sup>-1</sup>. The peak of the C-OH vibration of the alcohol sidebands at 1038 cm<sup>-1</sup> decreased, probably because of the fibrin coating. Besides, the two new peaks, which appeared in the case of SPRF crosslinking were also visible at 1238 and 1307 cm<sup>-1</sup> in the case of fibrin addition.

In summary, it can be observed that crosslinking caused major changes compared to native sodium hyaluronate, additionally, fibrin supplementation resulted in more visible differences in the spectra than SPRF crosslinking.

### 3.7. Scanning electron microscopy (SEM) of the protein containing scaffolds

After protein crosslinking the cross-section of the freeze-dried gels were examined with SEM to observe the changes caused by protein incorporation. According to our expectations, even after protein addition the matrices remained porous, this modification step did not alter the advantageous basic structure of the HA scaffolds. In the first part of the image the SPRF containing gels can be seen (Figure 10, I.). SPRF was linked covalently with DVS in a second crosslinking step. The protein can be seen in the pictures as a cancellous layer on the inner surfaces of the HA, which seems to be connecting weakly, only physically. In the second part (Figure 10, II.) fibrin containing gels can be observed. Fibrin was covalently polymerized inside the freeze-dried gel, which may be immobilized more strongly to the HA matrix. The fibrin chains can be noticed as thin fibers intertwisted with each other attaching to the edges or the surface of the HA lamellas forming a film on the inner surfaces.

### 3.8. In vitro cytotoxicity measurement

Chemical crosslinking reagents are toxic materials to living cells; thus, it is essential to get rid of the non-reacted rest after the crosslinking step. The hydrogels were tested after the crosslinking reaction, and SPRF containing gels were also examined, as SPRF linking required a second reaction using DVS. However, fibrin containing gels were not tested for cytotoxicity after protein addition, because we did not use any toxic reagents during the procedure of fibrin polymerization. The gels were washed with distilled water in several steps, and after that cytotoxicity measurement was conducted on MSCs. The cells were cultured in the presence of the gels, while there were control wells without HA gels. Viability was measured after 7 days using XTT, and it was calculated using the following formula: the absorbance values of the cells cultured in the gel containing wells was divided with the absorbance values of the control wells. Statistical analysis was performed, and successfully, none of the gels was cytotoxic (Figure 11).



**Figure 10:** SEM imaging of the structure of protein containing crosslinked HA matrices in 100x (I. and II. A-D), in 1000x (I. and II. E-H) and in 2000x (I. and II. I-L) magnifications. I: SPRF containing gels: the protein addition resulted in a spongious layer on the HA matrix, indicated with arrows; II: fibrin containing gels: fibrin fibers can be seen as interconnected filaments, or as a more compact coating, which attach to the HA surfaces, indicated with arrows. First column: 2% BDDE containing gels; second column: 5% BDDE containing gels; third column: 2% DVS containing gels; fourth column: 5% DVS containing gels (67).



**Figure 11:** In vitro cytotoxicity measurement of crosslinked HA and SPRF containing matrices. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed to compare the means of groups using Prism 7 software. The significance level was p < 0.05, where \* means that p is between 0.01 and 0.05, \*\* means that p is between 0.01 and 0.001, and \*\*\* means that p is lower than 0.001, and ns means non-significant. The data are presented as mean  $\pm$  SEM, n = 4 (67).

#### 3.9. In vitro cell attachment test

We aimed to test cellular adhesion to our fabricated hydrogels, as an ideal soft tissue implant promotes cell attachment and then the scaffold is digested and remodeled by the adhering cells. Crosslinked hyaluronic acid alone does not promote cellular adhesion (108), hence it cannot be used as a scaffold. However, when it is supplemented with blood derived protein, this drawback can be evaded. SPRF and fibrin containing crosslinked HA gels were cultured with MSCs in ultra-low attachment plates to examine their MSC adherence and proliferation promoting effect, and they were visualized by livedead staining similarly as in chapter 3.1. On the gels, which were crosslinked with BDDE and contained SPRF, no cells could be seen (data not shown). In contrast, if the first crosslinking reagent was DVS, some cells were visible on the SPRF containing gels. On fibrin interlaced scaffolds cell attachment seemed to be more effective, even if the crosslinker was BDDE, there were adhered MSCs on the matrices. The best result was achieved if HA was crosslinked with DVS and contained fibrin. In this case confluent regions could be observed on the hydrogels after two weeks culturing. However, cell adherence was not homogenous on neither of the gels, there were preferred regions, where the surface may have been more beneficial for the MSCs. Live-dead staining visualizes

living cells in green, dead cells in yellow, and all nuclei are bule. However, no dead cells were visible on the scaffolds, probably they were washed down during the procedure of the staining, which requires numerous washing steps (Figure 12).

ImageJ software was used to calculate the percentage of the areas covered by living cells and by the nuclei, and the result is represented in Figure 13. The crosslinker concentration also seemed to have impact on cell adherence: more cells were visible on 5% crosslinker containing gels than on the 2% crosslinker containing ones if they contained fibrin, both in the cases of BDDE and DVS. This observation suggests that 5% crosslinker containing hydrogels may have a more favorable surface structure for cell attachment than 2% DVS and BDDE containing gels.



*Figure 12:* Live-dead staining of MSCs cultured on protein containing HA matrices. A-B: 2% DVS containing gel with SPRF; C-D: 5% DVS containing gel with SPRF; E-F: 2% BDDE containing gel with fibrin; G-H: 5% BDDE containing gel with fibrin; I-J: 2% DVS containing gel with fibrin; K-L: 5% DVS containing gel with fibrin. The nuclei are visible in blue, cells can be observed in green, and no dead cells could be seen on the matrices. Living cells are green and the nuclei are blue (67).



*Figure 13:* Live-dead images were analyzed using ImageJ software and the percentage of the areas covered by the nuclei and by living cells were calculated (67).

#### 3.10. In vivo biocompatibility test

*In vivo* biocompatibility test was conducted on mice to observe *in vivo* degradation, to test if the scaffolds cause inflammation and to examine the ability of our hydrogels to promote remodeling and vascularization.

According to the swelling ratio and enzymatic degradation measurements and cell attachment test the 5% DVS containing gel with fibrin was found to be the most promising scaffold to be tested *in vivo*. The swelling ratio was the smallest; thus, the injected gel does not absorb a large amount of water, and the hyaluronic acid concentration remains relatively high. Slow enzymatic degradation ensures that the implant stays intact until it gets remodeled, and cell attachment test showed that this gel had the most advantageous cell adherence properties. Thus, this matrix was chosen to be homogenized and injected subcutaneously into the abdominal site of the hindleg of five male C57BL/6 mice for *in vivo* biocompatibility testing. As control crosslinked 5% DVS containing gels were injected to the other leg, but without fibrin supplementation.

After 12 weeks, the gels were examined under a light microscope. No inflammation was observed, and the gels were intact and elastic, but hard. Blood vessels could be seen on both kinds of gels, but especially on fibrin containing scaffolds. The gels were harvested, and during this procedure it was noted, that the attachment of the surrounding tissue was stronger in the case of fibrin containing scaffolds. The fibrin supplemented hydrogel seemed to be more interlaced by blood vessels, which was visible, as this gel type was generally more reddish colored (Figure 14). The weight of the

explants was measured and compared to the initial weight of the injected scaffolds, and no weight loss was noticed (data not shown).



*Figure 14:* Homogenized HA scaffolds in black 6 mice 12 weeks after injection. Fibrin containing hydrogels: A and C, and control gels: B and D. A-B: In the leg of the mice, and C-D: harvested (67).

### 3.11. Histological analysis

H-E (hematoxylin-eosin) staining and histological analysis were conducted on the harvested gels to examine remodeling and vascularization (Figure 15). It was observed that the scaffolds were infiltrated with connective tissue. Hyaluronic acid pieces were still visible, and they were surrounded with cells (the cytoplasm is pink, and the nuclei are purple). The number of nuclei was counted with the help of ImageJ software, but no significant difference was found between control and fibrin containing composites (data not shown). However, blood vessels were also visible on the sections with red blood cells, and fibrin containing matrices seemed to contain more of these blood vessel parts, than control gels (Figure 15, B and D, indicated with red arrows).



*Figure 15: Histological analysis of the harvested scaffolds. A-B: fibrin containing and C-D: control HA gels (67).* 

## 4. Discussion

Both blood derivatives and HA based scaffolds are important participants of regenerative medicine. In the present work we aimed to investigate the biological safety of a medical device designed for joint regeneration, besides, we developed a blood derived protein containing crosslinked hyaluronic acid hydrogel for a possible use in soft tissue engineering.

Firstly, the serum produced using HypACT Inject Auto device was compared to manually isolated SPRF to prove clinical equivalence. No significant difference was found between different SPRF samples regarding MSC viability, measured with XTT; thus, isolating SPRF with the HypACT Inject device is a sterile solution, that could be used safely for patient treatment. However, if device derived SPRF contained red blood cells, slightly lower proliferative effect was observed compared to the other serum types, which were free of them, thus the removal of RBCs might improve efficiency. Secondly, SPRF types were compared to FBS, and the result showed that SPRF may be a useful agent as medium supplementation replacing FBS. However, the removal of RBCs is recommended, as well. HSA containing medium seemed to have almost as low proliferative effect as the control medium, which was not supplemented. This result indicates that a protein source is not enough for cell proliferation, growth factors and cytokines are also essential, which are present in human serum and FBS, but not in HSA.

Sodium hyaluronate is the base material of a biocompatible and biodegradable scaffold, which was crosslinked to assure its longer presence after implantation. Two well-known crosslinker reagents were chosen, which are already used in many HA hydrogels in the literature (71, 101), and in existing products (98, 100), BDDE and DVS in 2 and 5% concentrations were used to examine the physical and biological properties of these differently prepared matrices. Swelling ratio was measured to get information about the water absorbing capacity of the hydrogels and to indirectly conclude the strength of crosslinking. As it was reported previously (109), we also noted, that increasing crosslinker concentration decreases swelling ratio. Besides, DVS gels were less swollen than BDDE gels. Summarily, the concentration and the type of crosslinker also affects the strength of crosslinking.

Ideal scaffolds are biodegradable, as their objective is to be implanted or injected into the body, attract the surrounding cells, which adhere to the implant and start to digest and parallelly remodel them. Consequently, the aim is to produce a hydrogel, which is present in the body until new tissue is formed. *In vitro* enzymatic degradation was tested to compare the different matrices to each other, and it was found, that 5% crosslinker containing gels were more resistant against enzymatic degradation than the hydrogels, which contained only 2% BDDE or DVS. Therefore, the 5% crosslinker containing gels were considered to be better candidates for *in vivo* application.

The disadvantage of crosslinked high molecular weight hyaluronic acid as a scaffold is that it does not induce cell adhesion and thus it does not promote tissue remodeling. However, cell adhering capacity can be increased if hybrid scaffolds are prepared, for example with collagen, chitosan, or silk fibroin (69, 88, 92) or if the HA matrix is supplemented with protein (62). As blood derivatives were found to have regenerative and tissue healing effects (13, 20, 110, 111), two kinds of blood derived proteins were combined with crosslinked HA to get promising composites for soft tissue regeneration.

Chemical structures of the crosslinked gels were compared to sodium hyaluronate using FTIR and newly formed bonds were observed, which referred to relevant changes. After fibrin coating new peaks appeared at 1390 cm<sup>-1</sup>, at 1643 cm<sup>-1</sup>, and at 1533 cm<sup>-1</sup>, which indicate the presence of new amide groups from the blood derivatives, also present in fibrin (112). Native HA also has a peak indicating the amide related carbonyl group around 1606 cm<sup>-1</sup>, however, 1643 cm<sup>-1</sup> and 1533 cm<sup>-1</sup> are characteristic peaks for the absorbance of fibrin, thus they shall not be confounded (113). At 1030 cm<sup>-1</sup> the peak decreased after fibrin supplementation, which may be because fibrin provided a coating on HA. The smaller peaks around 1300 cm<sup>-1</sup> were probably caused by the presence of both DVS (70) and the ether linkages from BDDE, or by CH stretching (106), however, fibrin also has smaller peaks around 1300 cm<sup>-1</sup>, thus they may overlap in the case of fibrin containing derivatives (113). In the case of SPRF supplementation the differences to the initial crosslinked matrices were less remarkable, thus we concluded that the SPRF crosslinking method into the gels was not efficient enough.

Protein containing gels were visualized by scanning electron microscopy to analyze the visible changes caused by protein linking to HA matrices. The protein addition did not change the porosity of the structure, both after SPRF and fibrin supplementation the cross-section of the hydrogel remained porous, and the pore sizes were around 50-200  $\mu$ m, which is comparable to other existing HA hydrogels in the literature (61, 94). However, the inner surface looked diverse in both cases. In the case of SPRF a protein layer could be observed, which seemed to be connecting weakly, maybe only physically, or via secondary bonds. In contrast, fibrin polymers seemed to be an integral part of the HA matrix. Like in the case of other fibrin containing scaffolds, individual fibrin chains were visible (96), the filaments seemed to be strongly attached to the hyaluronic acid scaffold.

Cytotoxicity was measured to make sure, that the hydrogels do not contain any excess of unreacted crosslinker reagent. As these materials are toxic, it is important to wash them out from the scaffolds, and to prove the safety of the implants before application. Thus, the hydrogels were washed in several steps after crosslinking and subsequently MSCs were cultured in the same environment with them. Viability measurement showed no significant difference between the viability of these cells and control cells; showing that the toxic reagents were successfully removed, and it was safe to further examine these scaffolds on cells *in vitro* and with mice *in vivo*.

Cell attachment capacity of the hydrogels was examined, MSCs were cultured on the scaffolds, and they were visualized by live-dead staining. More cells were observed on fibrin containing gels, which can be explained by the higher protein content, proved by FTIR spectroscopy. Additionally, cell adherence occurred on the gels, which were crosslinked using DVS, both if SPRF or fibrin was linked to them, while in the case of BDDE cell attachment was less efficient, MSCs attached only onto fibrin containing hydrogels. This effect may be caused by the smooth structure of BDDE crosslinked gels, which seems to be less favorable for cells than the rougher surface of DVS gels. However, in each case preferred regions were observed on the gels and cell density was inhomogeneous on their surfaces. Moreover, cell attachment was also affected by the crosslinker concentration: in the case of fibrin containing gels 5% crosslinker containing gels had more cells on their surfaces than 2% crosslinker containing gels. Fibrin polymerization into the gels seemed to be a more effective protein linking method than SPRF crosslinking according to FTIR analysis and SEM visualization, and cell attachment test supported this theory. The weaker effect of SPRF gels may be explained by the sensitiveness of the blood derived proteins. The crosslinking step requires highly alkaline pH values, but because the proteins in serum are sensitive to pH change and chemical reagents, the conditions of crosslinking were tried to be optimized. However, the lower alkaline pH value may not have supported strong crosslinking, and the proteins could have been washed out. The other explanation is that even at these lower alkaline pH values DVS damaged the proteins of the serum.

According to the cell attachment experiments fibrin containing gels were chosen for *in vivo* testing. Besides, grounded in swelling ratio and *in vitro* enzymatic degradation tests 5% crosslinker containing gels were preferable, as they were the most resistant against degradation and swelling.

Based on the previous experiments 5% DVS containing and fibrin supplemented scaffolds were chosen for *in vivo* biocompatibility, vascularization, and remodeling testing. The hydrogels were homogenized to get an injectable composite and implanted subcutaneously into mice. After 12 weeks it was observed that the gels did not cause inflammation and they were not resorbed, their structure remained intact, relatively hard but elastic, which seems to be an ideal combination for a soft tissue implant. In a similar *in vivo* degradation study, where the same amount of hydrogel was injected to the dorsum of nude mice, and no significant tissue growth occurred, but the hydrogels shrank in volume after 15 and 36 weeks (71). In contrast, in our case weight measurement did not show weight loss, which probably indicates, that parallelly with degradation tissue growth occurred. When harvesting the gels, we noticed that the fibrin containing gels were more difficult to remove from the mice than the control gels, which indicated that surrounding tissues attached stronger to protein supplemented gels. Blood vessels could be observed on both kinds of gels, but especially on fibrin containing scaffolds, besides, generally they had a more reddish color, indicating that their structure was infiltrated with more vessels.

H-E staining and histology analysis revealed that HA fragments were still present in the scaffolds, and they were surrounded with connective tissue indicating remodeling. The whole implants were interlaced with connective tissue; however, cell quantification did not show significant difference between fibrin containing and control hydrogels. This phenomenon may be because the fibrin content could have been the first material of the gel to be utilized by remodeling cells, thus fibrin's role in remodeling could not be estimated after 12 weeks of implantation. Histological analysis indicated the blood vessels inside the scaffolds, even red blood cells were visible. More blood vessels were observed in fibrin containing gels than in controls, which anticipates that fibrin may had an impact on vascularization.

# 5. Conclusions

We attempted to examine the effects of a serum product isolated by a new medical device, HypACT Inject on mesenchymal stem cell viability, and additionally, we aimed to develop a scaffold for soft tissue engineering based on crosslinked hyaluronic acid. Our conclusions were the following:

The MSC supporting characteristics of different serum products were compared: one of them was manually isolated SPRF, the other type of SPRF was isolated by a new medical device, and the third one was also isolated using the device, but with an additional centrifugation step. According to cell viability tests they had the same proliferative effect; thus, it was concluded that SPRF isolated with the medical device is safe to be injected intraarticularly to osteoarthritic patients if serum treatment is indicated. Moreover, each serum kinds were found to be able to replace FBS in *in vitro* experiments with MSCs, however residual RBC removal is beneficial in the case of the device derived SPRF.

Crosslinked hyaluronic acid derivatives are important candidates for regenerative medicine based on their biocompatible, non-immunogenic, and biodegradable nature. In this study two crosslinkers were tested in different concentrations to prepare hydrogels for a possible application in soft tissue engineering. Their physical and biological properties were found to be promising, especially in the case of the 5% DVS containing hydrogel, as it had relatively low swelling ratio, slow degradation and the rougher surface made it more capable of promoting cell attachment than the smooth surface of BDDE containing gels. However, even a beneficial surface structure cannot induce cell adherence and thereby initiate remodeling of the implanted hydrogel, as high molecular weight HA alone was found to be bioinert. Therefore, the crosslinked hyaluronic acid matrices were further modified using human blood products.

Blood derived proteins are also in widespread use in regenerative medicine; thus, they were chosen to supplement HA hydrogels and increase their cell attachment and remodeling capacity. Firstly, we examined a serum product, SPRF, which was already tested for tissue regeneration by our research group (13). SPRF was aimed to be crosslinked to HA matrices to increase the regenerative effect of the hydrogel. However, the crosslinking of SPRF to hyaluronic acid was not effective enough, as we have

observed on the FTIR spectra and cell attachment tests. Secondly, we implemented fibrin immobilization into the matrices, where instead of protein crosslinking the natural polymerization process of plasma products can be utilized. FTIR analysis and cell attachment test showed, that this method was more effective than SPRF linking.

The most promising gel type, 5% DVS crosslinked fibrin containing gels were homogenized and tested for *in vivo* biocompatibility and remodeling, and the results showed safety and biocompatibility, incipient remodeling, and vascularization. Thus, we concluded that the fibrin containing HA derivatives are promising future scaffolds for soft tissue replacement after further investigation and development. Our long-term aim is to have an authorized tissue engineering product, which can be used as a drug or medical device, and to achieve this, detailed biological evaluation is required.

There are already authorized hyaluronic acid based soft tissue fillers on the market, for example Hylaform of Genzyme or Juvederm of Allergan (99), however, these products have a different indication from us, as they are used in aesthetics. In addition, beside soft tissue replacement, HA scaffolds may be used in the fields of wound healing, like the Hyalo4 family of Fidia, or treating musculoskeletal disorders, like the products called Cingal and Monovisc of Anika Therapeutics (114). These products do not contain protein. Our developed scaffold might be able to compete with the existing products because of the additional regenerating effects of the blood derived protein components, however blood derivatives make authorization quite challenging, and the effectiveness of our hydrogel as wound dressing or intraarticular injection is yet to be proven.

## 6. Summary

In the present work biological application was investigated of a serum product, SPRF to be used as medium supplementation or treatment for osteoarthritis. A medical device was developed, which can produce SPRF, while maintaining sterility. Clinical equivalence was examined using human MSCs to prove safety and effectiveness of the device derived SPRF. Device derived SPRF was found to be equivalent to SPRF isolated in the traditional, open-cast way, that has been approved for *in vitro* regeneration (13, 20).

Hyaluronic acid hydrogels may be used as scaffold components in soft tissue engineering based on their advantageous biological and physical properties. In the present study butanediol diglycidyl ether and divinyl sulfone, two known crosslinkers in HA products on the market, were used in different concentrations to find the most advantageous initial material for scaffold development. Crosslinked HA was supplemented with blood derived protein components, SPRF and fibrin were linked to the matrix to facilitate cell attachment. The aim was to create a scaffold, which can be used as a soft tissue implant promoting new tissue formation, while degrading parallelly with remodeling. Physical characteristics were measured of the crosslinked hydrogels, and 5% crosslinker containing ones were found to have lower swelling ratio and slower degradation rate, than 2% crosslinker containing matrices. Additionally, SEM and FTIR analyses have revealed that fibrin linking was more efficient than SPRF crosslinking to HA hydrogels. Cytotoxicity test proved the biological safety of each gel, and cell attachment test showed that the surface of DVS containing HA and fibrin linking supports cell attachment the most. Thus, 5% DVS containing gel with fibrin coating was found to be the most promising candidate, thus it was homogenized, injected, and examined in mice for 12 weeks. Biocompatibility of the hydrogel was proven in vivo, as no inflammation was found, and the harvested gels were found to be elastic and intact. Small blood vessels could be observed especially on the surfaces of fibrin containing gels. Initial remodeling and vascularization could be observed by histology analysis on both control and fibrin containing gels. The remodeling and vascularization potential of the tested hydrogels along with their biocompatibility indicated its possible future usability as soft tissue implant in human.

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# 8. Bibliography of the candidate's publications

Publications related to the thesis:

Hinsenkamp A, Ézsiás B, Pál É, Hricisák L, Fülöp Á, Besztercei B, Somkuti J, Smeller L, Pinke B, Kardos D, Simon M, Lacza Z, Hornyák I. (2021) Crosslinked Hyaluronic Acid Gels with Blood-Derived Protein Components for Soft Tissue Regeneration. Tissue Eng Part A, 27: 806-820, IF: 3.845.

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