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„Tissue reactivity and tissue compatibility of coated collagen patches“

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ABSTRACT

Purpose: The aim of this thesis was to develop a more effective and efficient hemostatic agent based upon Hemopatch by adding a multiarm polyethylenglycol(PEG)-isocyanate surface to the collagen patch and to clarify questions surrounding its stability, adhesion to organs during surgery, and its biodegradability. *Methods:* The stability of isocyanate- and NHS PEG was analyzed by using NMR and FTIR. Its adhesion was tested in preclinical hemostasis models run on heparinized pigs on different organs. Its degradability and influence on the surrounding tissue was investigated through two prospective, randomized, preclinical studies in a rabbit liver lesion model including the appropriate histopathological evaluations. *Results:* The stability test showed that the material lost all or nearly all of its activity after three months of storage due to the presence of water and the transformation of isocyanate group into unstable carbamic acid derivatives. The results of the preclinical tests showed optimal hemostatic efficiency because of the fast and effective adhesion results without the formation of any hematoma. However, the results of the preclinical studies showed that PEG-isocyanate coated pads (Hemopatch Heavy Duty) led to an acute inflammatory reaction, a foreign body response (encapsulation) and the formation of a very strong adhesion formation of the liver lobe to the abdominal wall. In addition, the histopathological results for Hemopatch Heavy Duty showed an acute inflammatory reaction, including the formation of an amorphous mass with a huge number of macrophages and granulocytes.

Conclusion: There is a wide range of positive properties of Hemopatch Heavy Duty. PEG-isocyanate based collagen patches have a shorter application time with a bleeding time of less than 30 seconds in comparison to NHS-PEG-based Hemopatch, with a bleeding time of 1-2 minutes. The adhesive strength to the tissue of the pads based on PEG-isocyanate is higher compared to NHS-PEG pads and isocyanate based collagen sponges showed adhesiveness to all kinds of tested tissue, including tissues where NHS-PEG-coated collagen pads failed. In such cases, Hemopatch Heavy Duty has shown itself to have great potential as a biomaterial and worthy of further investigation. Nevertheless, it needs to be improved due to its instability, adhesion formation and foreign body response.

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INTRODUCTION

1. Biomaterials

Numerous biomaterials and different types of materials are investigated to improve hemorrhage control [1], such as liquid tissue sealants, flowable hydrogels, and hemostatic pads. Currently the following biomaterials for hemorrhage control are used in surgery and can be categorized into three main types of hemostats [1;2]:

- Liquid tissue sealings, such as Tisseel (Baxter Healthcare), Coseal (Baxter Healthcare), Artiss (Baxter Healthcare), Beriplast (CSL Behring), Evicel (Omrix Biopharmaceuticals Ltd.) and Quickseal (Ethicon Inc)

- Flowable hemostatics and gelatin-based tissue glues such as FloSeal (Baxter Healthcare)

- Combined materials or coated collagen patches, composed of collagen and different variants of reactive substances, such as N-hydroxysuccinimidyl-polyethylene glycol (NHS-PEG) in case of Hemopatch (Baxter Healthcare) or different combinations of fibrinogen and thrombin, such as in case of TachoComp and Tachosil (Hafslund Nycomed Pharma AG) [1; 2].

1.1 Liquid fibrin sealants

Liquid tissue sealants, also known as fibrin glues or fibrin adhesives are used to seal a tissue surface to form a physical barrier/seal when applied. To achieve hemostasis, both lyophilized fibrinogen (with or without Factor XIII) and thrombin with calcium chloride are reconstituted separately and mixed together to form fibrin just before application on a bleeding wound. The final steps of coagulation and main mechanism of actions are thus directly activated. The fibrin and thrombin concentrations vary among the ranking [1] commercial fibrin sealants like Tisseel (Baxter Healthcare), Artiss (Baxter Healthcare), Evicel (Omrix Biopharmaceuticals Ltd. NY &Ethicon Inc.), Beriplast P (CSL Behring) and Quixil (Ethicon Inc.) [2; 3]. The components fibrin and thrombin can be applied to a bleeding wound in a liquid or aerosol form by a dual-syringe system, a spraying system, or a spray tip combined with a dual-barrel syringe [1].

Synthetic adhesives like Coseal (Baxter Inc., Deerfield, USA) or Bioglue (Cryolife Inc., Kennesaw, USA) are commonly used in cardiothoracic surgery as alternatives to fibrin-based

glue. Bioglue consists of 45% purified bovine serum albumin and 10% glutaraldehyde and its clinical use has been associated with specific complications and nerve and tissue toxicity from glutaraldehyde [4].

These products are potential adhesives proposed for a wide range of clinical utilities due to its successful control of bleeding in humans with hemophilia or other coagulopathic patients e.g due to traumatic injury [4]. Other properties are the rapid degradation and total biocompatibility when applied onto the bleeding wound due to the fibrinolytic activity, thickness of the sealant layer and the quantity of plasminogen [3].

1.2 Flowable hydrogels

Though fibrin glues play a great role in wound healing and tissue sealing due to the well-known success in control of hemorrhage from various wounds, efforts have been made to improve the effectiveness in three directions. First, the application should be made easier by avoiding mixing the two components shortly before application onto the bleeding wound. Second, a non-human blood origin of the components could result in allergic reaction, viral infection, or the development of antibodies against Factor V and thrombin. Third, it also suffers from low mechanical strength. The development of new application devices and combined materials resulted in different combinations of fibrin sealant components (e.g. thrombin) with other hemostatic products, e.g. oxidized cellulose (Surgicel) or gelatin (Floseal) [1].

Gelatin is a protein produced through hydrolysis of collagen extracted from skin, bones, cartilage, or ligaments and added to water to form a semi-solid colloidal gel. Typical commercial products are Gelfoam (Upjohn, Kalamazoo, MI, USA) which is a gelatin sponge or FloSeal (Baxter Biosurgery), a gelatin-based tissue glue, that contain a gelatin matrix derived from bovine collagen, crosslinked by glutaraldehyde mixed with thrombin before application [1].

1.3 Hemostatic pads

Hemostatic pads are composed of

a) a matrix (e.g. sponge, pad) of a biomaterial like collagen; gelatin, especially cross-linked gelatin; fibrin; chitosan; oxidized cellulose or aldehyde activated dextrans and eventually of a second component e.g. fibrinogen/thrombin (Tachosil) or

b) one hydrophilic polymeric component that has reactive groups susceptible to hydrolysis after contact to water. Polyethylenglycole (PEG) is a comprising polymer and can cross-link blood proteins and tissue surface proteins due to its reactive groups, which are as follows:
   - Electrophilic groups of the hydrophilic crosslinker: groups reactive to the amino-, carboxy-, thiol- and hydroxy- groups of proteins or mixtures thereof
   - Amino group specific reactive groups (e.g. NHS-ester groups, imidoester groups, aldehyde groups, carboxy groups in the present of carbodiimdes, isocyanates, etc.)
   - Carboxy group specific reactive groups (amino groups in the presence of carbodiimides)
   - Thiol group specific reactive groups such as maleiimides or haloacetyls
   - Hydroxy group specific reactive group such as the isocyanate group [5].

Collagen and gelatin products are biopolymers that play an important role in hemostasis, included in wound dressings and as matrices for tissue growth [1; 6]. Collagen provides the water and electrolyte supply for the organism and a wide range of metabolic products have to pass the connective tissue from blood to the organs and vice versa [7]. Collagen exists in fibrillated or fibrous forms that are different in quantity, length, thickness, and arrangements of the collagen fibers depending on the function of the different textures of the body. It interplays with multiple agents like electrolytes (acids, alkalis, salts), molecules (phenols, urea), enzymes and polysaccharides. The ion binding property of collagen is based on the content of acid and alkali amino acids [7]. Collagen thus presents itself as a potential biomaterial in wound healing, as it offers many positive physical, chemical and biological effects: high tensile strength, orientation of fibers, semipermeability of membranes, low-antigenicity, and nontoxic hemostatic properties [6].
Hemostasis is achieved by physical adsorption as dry collagen material concentrates blood. It is commercially derived from porcine or bovine sources and produced as a viscous solution or, more often, as a solid powder by alkaline hydrolysis, enzymatic hydrolysis, or acid hydrolysis [6]. The reaction partner can be adapted in several ways and collagen can thus be converted into and combined with different substances [7].

Examples of hemostatic pads are TachoComb, Tachosil and Hemopatch.
TachoComb is an equine collagen fleece coated with human fibrinogen, thrombin and bovine aprotinin, whereas Tachosil is a collagen patch consisting only of human fibrinogen and thrombin. Both showed good results in hemorrhage control during clinical surgery [1]. They

are important surgical tools especially for complicated surgery procedures as soft tissues and parenchyma organs. TachoComb can be used for prophylactic treatments and for sealing of parenchymatous organs such as lymph or gall bladder [8] and it can be used for treatments for both venous and arterial bleeding [9;10].

Hemopatch is an advanced material for hemostasis and wound healing. It consists of a 2-mm-thick collagen sheet coated homogenously with 14mg/cm^2 of pentaerythritol polyethylene glycol ether tetra-succinimidyl glutarate (NHS-PEG), which is fixed by melting at approximately 70°C for 4 min in a preheated oven. It offers a wide range of advantages, as hemostasis is achieved after 2 minutes' bleeding time and no re-bleeding occurs within 10 minutes. In addition, Hemopatch also succeeds in adhering to the tissue due to the reactive groups of the polymeric component, to its water content of below 2%, and to the collagen layer which is able to absorb fluids when applied to the wound while allowing blood to enter into the sponge [5]. It is a relatively strong biomaterial, as it is easy to apply during surgery and excellent in its biodegradability and biocompatibility to almost all tissues and organs. No hematoma occurred during surgical procedures or afterwards. In addition, it is non-toxic and has the ability to achieve wound healing in an acceptable healing time.

Hemopatch Heavy Duty is a collagen pad coated with 4arm-PEG-isocyanate (see figure 2), 8arm-PEG-isocyanate and 4arm-PEG-ester-isocyanate (see figure 3), respectively. It was initially intended to be a further improvement of Hemopatch by modifying its surface and using isocyanate-PEG as the reactive material instead of NHS-PEG. Isocyanates are very reactive compounds and polyethyleneglycol (PEG) is a protein-reactive cross-linker that promotes hemostasis and strong tissue adherence through binding the pad to proteins in blood and on the tissue surface. Collagen induces clot formation through platelet activation [11].
Organic isocyanates are esters of the isocyanic acid. They conform to the general formula R-N=C=O while polyisocyanates contain two or more isocyanate groups in their structure. They are related to the ketenes and the structural organization of the –N=C=O groups defines the isocyanates as very reactive compounds.
Under dry conditions, the isocyanates can be kept for a long time and they hardly alter. With the entry of an amino group they transform very quickly into insoluble polymerizes and the more isocyanate groups exist the higher the effect of polymerization [12].
Chemical modifications occur in the reaction of interacting functional groups and thus cross-linked compounds may be used to establish active intermediates. Amine-containing molecules

are important functional groups which interact with nearly all protein or peptide molecules. NHS ester is commonly used to create reactive acylation agents [13].

In the presence of water, the isocyanate group is transformed to instable carbonic acid derivatives, from which, by decomposition, an amine and gaseous carbon dioxide results.

Thereby, following reactions take place:

$$R{-}NCO + H_2O \longrightarrow [R{-}NHCOOH]$$

$$[R{-}NHCOOH] \longrightarrow R{-}NH_2 + CO_2$$

The obtained amine can react with isocyanate and urea-derivatives result:

$$R{-}NCO + R'{-}NH_2 \longrightarrow R{-}NHC(O)NH{-}R'$$

Figure 1: Formula taken from https://de.m.wikipedia.org/wiki /Isocyanate, 01 Feb 2018

Polyuria formation occurs by the interacting of a poly-isocyanate and a compound containing two or multiple amine groups. Both poly-isocyanates and polyamines are present when poly-isocyanates are exposed to traces of water (e.g. atmospheric humidity), leading to the formation of insoluble polymers. Therefore, to preserve the chemical reactivity of the isocyanate group, isocyanates have to be protected from moisture during storage [14; 15].

Chemical structure of multiarm coated isocyanate

4arm-PEG-isocyanate (pentaerythritol), MW 10000:

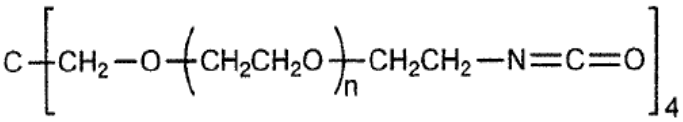


Figure 2: 4arm-PEG-isocyanate (picture taken from Jenkem, Material Datasheet, No. COA-A7106 ENGLISH)

4arm-PEG-ester-isocyanate (pentaerythritol), MW 10000:

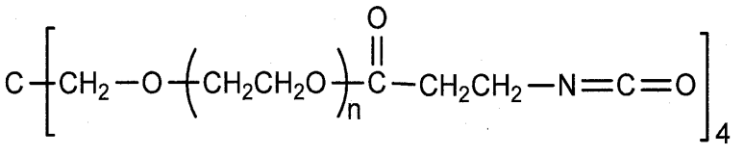


Figure 3: 4arm-PEG-ester-isocyanate (picture taken from Jenkem, Material Datasheet, No. FEDEX-504557060144)

2. Wound Healing

Wound healing occurs as a result of various activated intracellular and intercellular pathways. This process includes activation of the blood coagulation system and the immune system as well as essential steps like inflammation, new tissue formation, and remodeling. These classic stages of wound repair require gene expression changes of various cell types to achieve cell proliferation, differentiation, and mitigation [16]. Typically, the wound healing process is achieved in four overlapping phases: hemostasis, inflammation, proliferation, and remodeling [18; 19] (see figure 4 [17]). Inflammation takes place immediately after tissue damage [16], e.g. in surgical procedures when the epidermis and dermis are damaged and blood vessels opened [20], whereby the first phagocytic neutrophils arrive at the wound site following macrophages, which are important for growth factors [21]. Several processes occur during the proliferative phase: First, the formation of new blood vessels, also called angiogenesis; second, the synthesis of collagen; third, granulation tissue formation; and finally, epithelialization and reorganization of the extracellular matrix. In the final step of wound healing, the remodeling phase, there are increases in the wound's tensile strength along with degradation and remodeling, including formation of scar tissue. This is achieved as fibroblasts produce a collagenous matrix following apoptosis of fibroblasts [21; 2].

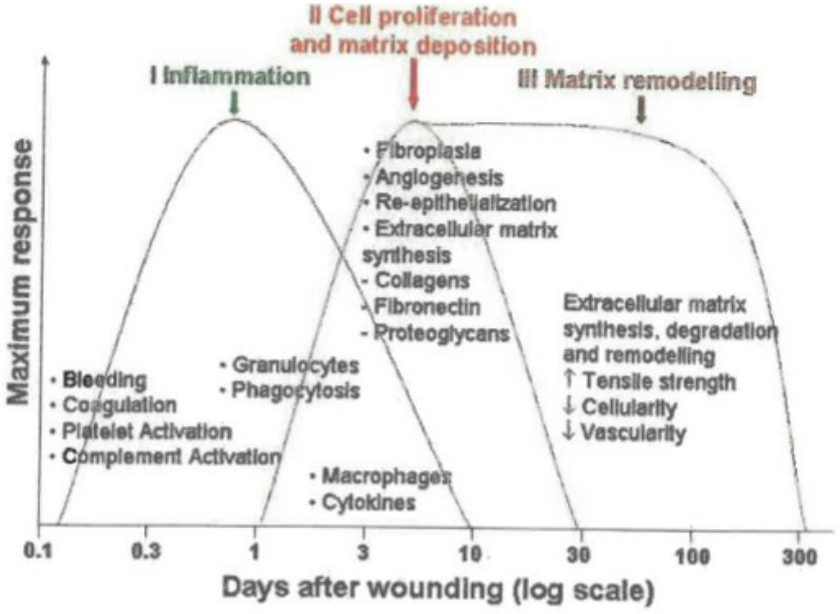


Figure 4: Phases of wound healing (picture taken from https://deref-web-02.de/mail/client/BetbLrt_Su8/dereferrer/?redirectUrl=http%3A%2F%2Fwww.worldwidewounds.com%2F2004%2Faugust%2FEnoch%2Fimages%2Fenochfig1.jpg, 15 Apr 2018)

3. The Extracellular Matrix (ECM)

The formation of the extracellular part of human tissue (ECM) is the precondition for wound healing, growth and fibrosis. It is composed of collagens, adhesive glycoproteins such as fibronectin and laminin and proteoglycans [2].

Normal processing and tissue repair is based on the interaction of specific cell types and its extracellular matrices (ECM) which effect its cellular morphology, growth, differentiation and biosynthetic program [20]. Cell behavior including cellular adhesion, migration, proliferation and differentiation are related to the structure of ECM. Cells interact with molecules of the extracellular matrix via cell surface receptors, called integrins [23]. They are important receptors for cell-cell and cell-matrix interactions, cell adhesion and migration and cell proliferation, programmed cell death (apoptosis) and differentiation [24]. Integrins belong to the key adhesion receptor family [25] and consist of an alpha and a beta subunit which are able to bind extracellular membrane proteins such as collagen I, laminin and fibronectin [24] or adhesive plasma proteins such as fibrinogen, von Willebrand factor, vitronectin and thrombospondin [26]. This is achieved by the ability of integrins to change their conformation in order to bind ligands, cluster integrins and to activate cytoplasmatic plaque proteins such as integrin-linked kinase (ILK). They are able to connect integrins to actin cytoskeleton and to induce adhesion [25]. In addition, the extracellular matrix is necessary for cell migration and adhesion as well as for wound healing, growth and fibrosis [27]. It regulates cell function, migration and angiogenesis by matrix molecules like fibronectin and growth factors such as fibroblast growth factor (FGF).

The ECM consists of collagens, polysaccharide glycosaminogycans covalently linked to fibrous proteins and proteoglycan forming proteins [2; 28]. Collagen consists of three winding upon itself polypeptide chains which form a coil consisting of tropocollagen. They polymerize in the ECM and form fibrils [25]. Collagen filaments aggregate into fibrils and finally form fibers to give strength. [2;30]. Fibronectin represents the glycoprotein content of the ECM and allows interactions between cells and ECM through binding to integrins. Laminin is responsible for cell adhesion via formation of bridges to the basement membrane and glycosaminglycans allow water attraction in order to keep the ECM hydrated [2].

4. Adhesion

Cell adhesion molecules achieve cell-cell or cell-matrix binding and belong to integrins, cadherins, selectins, or the immunoglobulin superfamily, which interfere the ligation of cells or the binding of matrix proteins to immune cells [2; 20].

Three different types of endothelial-leucocyte adhesions exist:
The initial (primary) adhesion is a bond between leucocytes and endothelial cells through adhesion molecules by scrolling of leucocytes on the endothelium. The term "activation phase" defines the activation of leucocytes and endothelial cells, which leads to a strengthening of adhesion molecules. After the damage impulse occurs, p-selectins and other adhesion molecules (e.g. E-selectin, ICAM-I and VCAM-1) are synthesized in the cell membrane that lead to a strong stable adhesion. This activation-dependent adhesion is characterized by additional receptor-ligand-bonds, which allow the strong adherence of leucocytes to the endothelium. Granulocyte LFA-a1-Integrine adhere strongly to the endothelial ICAM-1. Leucocytes slab and the venoles and endothels are filled with leucocytes. Finally, migration of cells takes place amoeboidly [20].

5. Granulomatous Inflammation

Chronic inflammation is often associated with a foreign body and a bacterial-purulent inflammation process. It forms a surrounding barrier to the granulocyte in the form of carbohydrate mucus. Thus, the foreign body protects itself from the organism's defenses. The foreign bodies can originate exogenously, e.g. via surgery, as non-absorbable stitching material, metals, plastic or endogen as necrotic bones in a purulent bone inflammation [20].

Foreign bodies are typically structured as following: [20; 31] (see figure 5)

- Macrophages: responsible for phagocytosis and stimulation of other inflammation cells.
- Epithelial cells: derive from macrophages, responsible for barrier formation around inflamed tissue. They are not able to phagocyte, but form few enzymes and cytokines for the defense process.
- Ordered giant cells (prototype: Langerhans-giant cells): are developed through fusion of macrophages or epithelial cells

- Disordered giant cells (star-shaped structured (asteroid body) or cloud-shaped (conchoids body)
- Damaged tissue

Foreign-body-type granulomas occur through invasion of small materials into the body (crystalline, non-crystalline, foreign, or own-body materials), which are inhaled or absorbed traumatically and cannot be removed. They consist of polycyclic giant cells with disordered aggregation cores. Granuloma activators are often internal substances like urate and cholesterol or foreign bodies like sutures, insect or tick components, splinters of wood, sea urchin barbs etc. [20].

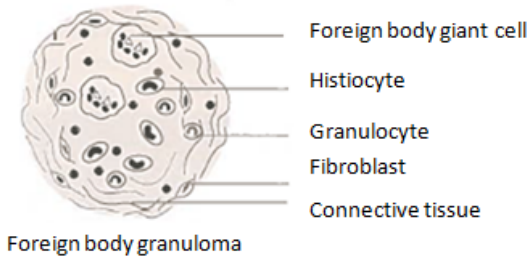


Figure 5: Foreign body granuloma (picture taken from Nennstiel S., 2009, pp. 79)

In the case of implemented biomaterials, foreign body reaction often results in inflammation where monocytes, macrophages, and foreign body giant cells converge at the biomaterial interface [32]. Foreign bodies are often eliminated by macrophages. Small foreign-bodies can be phagocytized following the release of microphagous lysosome enzymes, which leads to the activation of fibroblasts. Larger foreign bodies are eliminated by the activation of T lymphocytes, monocytes, and macrophages in the giant cell [20; 31;32; 33]. The type IV hypersensitivity reaction is a cell-mediated response. Activated T cells destroy target cells on contact and macrophages produce hydrolytic enzymes that transform with intracellular pathogens to multinucleated giant cells [33].

Aim

Numerous biomaterials and different types of materials are investigated to improve hemorrhage control, such as liquid tissue sealants, flowable hydrogels, and hemostatic pads. The aim of this thesis was to develop a ready to use collagen dual mechanism patch for control of high pressure/high volume hemorrhages without applying any other methods to achieve hemostasis. Hemopatch Heavy Duty is a collagen pad coated with 4arm-PEG-isocyanate, 8arm-PEG-isocyanate or 4arm-PEG-ester-isocyanate, respectively. Based on the chemically more reactive coating of Hemopatch Heavy Duty, we hypothesized that it could be a more effective and efficient hemostatic agent than Hemopatch. Therefore, evaluations have been performed to clarify questions surrounding its storage stability, hemostatic activity, and its biodegradability/tissue reaction.

MATERIALS

1.1. Sample Materials

Sample Material	Supplier	Lot Number	Method
4arm-PEG-isocyanate	JenKem Technology	YF111P064	NMR, FTIR
8arm-PEG-isocyanate	JenKem Technology	YF106P181	NMR, FTIR
4arm-PEG-ester-isocyanate	JenKem Technology	YF119P179	NMR, FTIR
4arm-PEG-isocyanate	JenKem Technology	YF111P064	Macroscopic evaluation
8arm-PEG-isocyanate	JenKem Technology	YF106P181	Macroscopic evaluation
4arm-PEG-ester-isocyanate	JenKem Technology	YF119P179	Macroscopic evaluation
Matristypt (2mm thick)	Dr. Suwelack, Cat # 71522-002	L129744	All methods

1.2. Produced Test Items

Test Material	Supplier	Lot Number	Method
Hemopatch HT-4E*	R&D Biosurgery	Hemopatch HT-4E 27.11.12MT (stored at +4°C)	Preclinical testings
Hemopatch HT-4E*	R&D Biosurgery	Hemopatch HT-4E 27.01.13MT (stored at -20°C)	Preclinical testings
Hemopatch HT- 4**	produced : 020212MT	SC 0012012CH-HT4	Macroscopic evaluation
Hemopatch HT- 8**	produced: 020212MT	SC 0012012CH-HT8	Macroscopic evaluation
Hemopatch HT-4E*	produced: 110912MT	SC0012012CH-HT4E	Macroscopic evaluation
Slides study K1204	Baxter Innovations, GmbH	n.a.	Histopathological evaluations, Immunohistochemistry
Slides study K1205	Baxter Innovations, GmbH	n.a.	Histopathological evaluations, Immunohistochemistry
Hemopatch HT-4E***	produced: 260612MT	SC0022012CH	FTIR- extraction with acetone
Hemopatch HT- 4***	produced: 160412MT	n/a	FTIR- extraction with acetone
Hemopatch HT- 8***	produced: 160412MT	n/a	FTIR- extraction with acetone

*** Preparation of Hemopatch HT-4E Pads** (HT4-E defines the 4arm-PEG-isocyanate with an ester bond in its chemical structure). Before sample preparation, the glovebox was switched on and flushed with nitrogen until the humidity dropped to < 3%. The needed amount of powder (10 mg/cm2 of activated PEG each sponge) was transferred from -20°C into the glove box and stored in an exsiccator at room temperature overnight.
A 9x7 cm hemostatic sponge (Matristypt) was placed on an aluminum foil on the balance. 630mg of the powder was scattered with a salt shaker onto the sponge and the amount was controlled by weighting. After powder application the sponge was carefully transferred in a preheated oven where the 4arm-PEG-ester-isocyanate was fixed by melting at 65°C for 4 minutes. The pad was packed into a paper sheet, sealed in an aluminium-pouch with a desiccant inside and stored at +4°C and -20°C, respectively, until use.

**** Preparation of Hemopatch and Hemopatch HT-4 Pads** (HT4 and HT8 represent the 4arm or respectively 8arm –PEG-isocyanate without an ester bond.)
The Hemopatch and Hemopatch HT4 or HT8 samples were produced by coating of Matristypt with 10 mg/cm^2 of the corresponding activated PEG powder. The PEG powder was homogeneously distributed over the surface of 3x3 cm Matristypt, placed on a scale, by using a saltshaker. The amount of powder was controlled by weighing. The fixation of activated PEG on the surface of the collagen sponge was obtained by melting of the activated PEG powder on the surface of the Matristypt collagen sponge. For this purpose, Matristypt coated with the activated PEG powder was introduced in an oven at 65°C for 4 minutes. After cooling each sample was packed into a gas (vapor) impermeable aluminum pouch, a sachet containing 6 g of silica-gel desiccant (Multisorb) was added, and the pouch was sealed. The samples were stored at 2-8°C until use in experimental surgery.

***** Preparation of multiarm Hemopatch extracted with acetone (used for the FTIR analysis)**
Samples were produced by coating of Matristypt with 10 mg/cm^2 of activated PEG according to the above-described method. In addition, the coated pads were introduced in a vial and dried in vacuum in a freeze-drier for 46 h. The freeze-dryer was vented with dry nitrogen and the vials were closed with plugs and capsulated. For the stability study the vials were sealed in gas tight aluminum pouches containing a desiccant and stored at room temperature. For the FTIR analysis of the substitution degree the PEG coating was extracted with acetone, evaporated and the obtained powder was used in the analysis.

1.3. Reference Materials

Reference Material	Supplier	Lot Number	Method
Hemopatch	Baxter	SC0032010CH	Preclinical testings
Hemopatch	produced: 120612MW	M0052012JH	Macroscopic evaluation
Positive Control slides: spleen	Univ. Klinik für Zahn-, Mund- und Kieferheilkunde GmbH, Bernhard Gottlieb Universitätszahnklinik, Sensengasse 2A	n.a.	Immunohistochemistry

1.4. Solutions

Solution	Production data (e.g. Name, Date, ...)	Method
Ketamine Hydrochloride	Dr. E. Gräub AG, Bern	Macroscopic Evaluation
Rompun 2%	Xylazine Hydrochloride	Macroscopic Evaluation
Thiopental Sandoz 1g	Sandoz GmbH, 6250 Kundl	Macroscopic Evaluation
Ringer's Solution	Mayrhofer, 4021 Linz	Macroscopic Evaluation
Natriumchlorid 0.9%	Mayrhofer, 4021 Linz	Macroscopic Evaluation
Buprenovet	Bayer Vital GmbH, 51368 Leverkusen	Macroscopic Evaluation
Betaisodona-Solution	Mundipharma GesmbH, Wien	Macroscopic Evaluation
Lactated Ringer's Solution	Fresenius Kabi, 8055 Graz	Macroscopic Evaluation
Baytril	Bayer Austria, Herbststraße 6-10, 1160 Wien	Macroscopic Evaluation
T-EDTA buffer diluted 1:10with Aqua dd Citrate Buffer 6,0 (10x) diluted 1:10	Fresh prepared	Macroscopic Evaluation

1.5. Antibodies and antibody diluent

Antibody 1

Antibody Type	Supplier, Order Number	Lot Number	Method
Monoclonal Mouse Anti-Human CD3, Clone F7.2.38	AbD Serotec, Cat.# MCA 7254	n.a.	Immunohistochemistry
Monoclonal Mouse Anti-Human CD8, Clone C8/144B	Dako Cytomation, Cat. M7103	n.a	Immunohistochemistry
Mouse Anti-Human CD18 (activation epitope), Clone MEM-148	AbD Serotec, Cat.# MCA 2086	n.a.	Immunohistochemistry
Goat Anti-Human Pi-3-Kinase p110 Subunit Alpha	AbD Serotec, Cat.# AHP 1898	n.a.	Immunohistochemistry

Antibody 2

Antibody Type	Supplier, Order number	Lot Number	Method
Antibody2 for CD3, CD8 and CD18: Labelled polymer, Anti-Mouse	Dako Envision+System-HRP, Labelled polymer, Cat.#, REFK4001	10068246	Immunohistochemistry
Antibody2 for pi3K: ImmPRESS Reagent, Peroxidase, Anti-Goat lg	Vector, MP-7405	Y0224	Immunohistochemistry

Antibody diluent for all antibody types

Antibody Type	Supplier, Order number	Lot Number	Method
Anti-Mouse Antibody diluent Dilution buffer	Dako REALTM S2022, 250ml, Cat.#, REFK4001	00028133	Immunohistochemistry

1.6. Chemicals

Name	Supplier, Order Number	Lot number	Method
Hydrogen Peroxide 30%	Roth, Cat.# 80701	092182272	Immunohistochemistry
T-EDTA Buffer	Zytomed, Cat.# ZUC029-500	I454	Immunohistochemistry
Citrate Buffer PH 6,0 (10x)	Zytomed, ZUC 028-500	H312	Immunohistochemistry
Proteinase K	Dako Proteinase K, Cat.# S3020	10057914	Immunohistochemistry
Tris wash buffer (20x), TBS	Zytomed, Cat.# ZUC052-500	H850	Immunohistochemistry
IMMPACT NovaRED Peroxidase Substrat Kit	VectorLabs, Cat.# SK-4805	n.a.	Immunohistochemistry
Acetone	Merck, 100299.0161	1557699039	FTIR

1.7. Equipment/ Disposables

Equipment/ disposables	Supplier, Order Number	Method
Cleerpeel Alu-pouches (170x280 mm)	Sengewald, REF 3000306	Preclinical Testings
Minipax dessicant	Multisorb Technologies, 02-00039AG46	Preclinical Testings
Salt Shaker	n.a.	Preclinical Testings
DIN A4-sheets	n.a.	Preclinical Testings
Scale Sartorius LP6200	LP6200	Macroscopic evaluation
Refrigerator	Liebherr Profiline	Macroscopic evaluation
Incubator	Ehret 54769	Macroscopic evaluation
Scale, Sartorious BP2100	Sartorius	Immunohistochemistry
Steam sterilizer	Melatronic 23, Melag Medizintechnic	Immunohistochemistry
Polylysine slides	Thermoscientific, Cat # J2800AMNZ	Immunohistochemistry
Shandon Coverplates	Thermo Scientific, Cat#72110017	Immunohistochemistry

1.8. Instruments

Instrument, Model	Manufacturer	Unique Identifier (e.g. Serial Number, Inventory Number)	Method
Pouch sealer	Polystar 245	BS 2127	Preclinical Testings
IR-Heater	Krelus AG	BS2166	Preclinical Testings
Glove box Type ITA 14	Intertec AG	SN 095636	Preclinical Testings
Analytical balance, AT200	Sartorius	SN 102347	Preclinical Testings
Coverslipper	Thermo Scientific CTM6 Objektträger-Eindeckautomat	125632	Immunohistochemistry
Microscope	Olympus XXX, Program Olympus Olyvia Vs26	125874	Immunohistochemistry

METHODS

1. Stability of PEG-isocyanate - Determination of the Substitution Degree of multiarm PEG-isocyanates

1.1 FTIR Spectroscopy

Isocyanate (NCO) groups can be easily determined by using the method of IR-spectroscopy. FTIR-method was developed to determine the NCO content of a multi-arm polyethylene glycol raw material and to assess the extent of its hydrolysis. This FTIR method was suitable for the analysis of the NCO-PEG as it showed an intensive band at 2270 cm^{-1} that typically represents the isocyanate group. FTIR-analysis was performed using an ATR-FTIR spectrometer. According to the user manual of Bruker, solid samples as well as liquids were measured by direct application onto the ZNSE-ATR-crystal. During light entry into the sample a total reflection occurred which radiance was measured and could be transformed into an IR spectrum by fourier transformation [34;35]

In order to locate isocyanate group in the FTIR spectrum and to evaluate the substitution degree, the respective absorption (peak area) of the infrared radiation at a wave number of 2270cm^{-1} was measured which indicates the isocyanate peak proportional to the number of isocynate (NCO) groups. In addition, a standard peak was defined which was stable and not changing over time. Peak 1 in the FTIR spectrum of isocyanate-PEGs does not change during the storage of isocyanate-PEGs and served as an internal standard. Thus, the ratio of the peak areas of the isocyanate peak (2270cm-1) to peak 1 (2881cm-1) was determined to determine the substitution degree of 4- or 8arm isocyanate-PEGs. The advantage of the FTIR-method was that it was very easy to perform and highly reproducible. In addition, it could be used to determine the isocyanate decrease in the raw material as well as extracted with acetone.
In addition to the FTIR-method, NMR determined the isocyanate substitution degree of the raw material as well (see chapter 1.3).

1.2 Stability of Hemopatch Heavy Duty extracted with acetone

In order to evaluate the stability of Hemopatch Heavy Duty different samples were produced by coating Matristypt with 10 mg/cm^2 of 4arm-PEG-isocyanate, 8arm-PEG-isocyanate and 4arm-PEG-ester- isocyanate, respectively. They were stored at room temperature and at 4°C. The PEG coating was extracted with acetone, evaporated and the obtained powder was used for FTIR-analysis at different time points (0, 6, 14, 38 and 76 days).

The method of FTIR for determining the NCO content was reproducible in the appearance of the intensive NCO band at 2270cm^{-1}. The significance of the FTIR method was suitable for the analysis of the powder in a solid state, as well as extracted from a collagen sponge coated with 10mg/cm^2 multi-arm NCO-PEG. The substitution degree was obtained based on integration of standard and isocyanate peaks following the respective calculation (see formula results, chapter 1.1.2)

1.3 NMR Spectroscopy

The measurement of NMR spectrum was carried out by Susanne Felsinger from the department of Organic Chemistry at the University of Vienna. A small sample was dissolved in 0.5ml of solvent and filled into a long, thin glass tube that was subsequently placed in a strong magnetic field. To find out the position of the molecules in the magnetic field, the sample glass tube was turned around its longitudinal axis. The change in intensity was recorded by a computer and converted into „intensity frequency data“[34;35]. Dr. Lothar Brecker interpreted the results of the NMR spectrum.

2. Biodegradability of Hemopatch Heavy Duty

2.1 Preclinical testing of 4arm-PEG-ester-isocyanate coated pads

Samples coated with 4arm-PEG-ester-isocyanate, stored at 4°C and -20°C, respectively, were tested in a liver lobe abrasion model, in a spleen resection model as well as in a lung and heart-sealing model to evaluate the efficacy to stop bleeding. In the same course 4arm-PEG-ester-isocyanate coated Hemopatch was compared to Hemopatch coated with NHS-PEG.

2.1.1 Testing of Hemopatch 4arm-PEG-ester-isocyanate coated pads (stored at 4°C) – liver lobe and spleen abrasion model

A circular bleeding wound (1.8 cm diameter) was created on the surface of the spleen and liver lobe of a heparinized pig, respectively, by using a flat, round, rotating abrasion tool. A pad with a size of 3x3 cm was applied in its dry state onto the bleeding wound and hold in place by exerting slight pressure with a dry gauze for 30 seconds.

The efficacy to stop bleeding was evaluated after 30 seconds. In order to test the adherence of the pad on the tissue after 30 seconds, slight tangential force was applied with the lateral part of the forceps.

2.1.2 Testing of Hemopatch 4arm-PEG-ester-isocyanate coated pads (stored at -20°C) – liver lobe and spleen resection model

A respective part of the liver and the spleen of a heparinized pig, respectively, was resected using a scalpel. A pad with an appropriate size was applied in its dry state onto the bleeding wound and hold in place by exerting slight pressure with dry gauze for 30 seconds.
The efficacy to stop bleeding was evaluated after 30 seconds as well as the adherence of the pad to the tissue.

2.1.3 Testing of Hemopatch 4arm-PEG-ester-isocyanate coated pads (stored at 4°C) – gall bladder sealing model

An incision into the gall bladder was created with a needle. A pad with an appropriate size was immediately placed onto the hole. It was held in place for 30 seconds and the adherence was tested.

2.1.4 Testing of Hemopatch 4arm-PEG-ester-isocyanate coated pads (stored at 4°C) – lung sealing model

A circular wound (1.8 cm diameter) was created on the surface of the lung of a heparinized pig using a flat, round, rotating abrasion tool. A pad with an appropriate size was applied in its dry state onto the bleeding wound and held in place by exerting slight pressure with a dry gauze for 30 seconds.

2.1.5 Testing of Hemopatch 4arm-PEG-ester-isocyanate coated pads (stored at 4°C) – heart sealing model

A small incision on the heart muscle was created with a flat, round, rotating abrasion tool. A pad with an appropriate size was immediately placed onto the hole. It was held in place for 30 seconds and the adherence was tested.

2.2 Preclinical studies

2.2.1 Macroscopic evaluation

The biodegradation of PEG-isocynanate coated collagen patch (Hemopatch Heavy Duty) in relation to Hemopatch (NHS- PEG coated collagen patch) was investigated in two prospective, randomized, preclinical studies in a rabbit liver lesion model.
The first study was performed over 1, 2, 7, 12, 30, 45 and 60 days and the biodegradability of 4arm-PEG-isocyanate coated collagen Hemopatch Heavy Duty and 8arm-PEG-isocynate coated Hemopatch Heavy Duty was evaluated over a period of 60 days.

The second study differed to the first in the test item which now included an ester bond in its structure. 4arm-PEG-ester-isocyanate was used as the test material compared to NHS coated Hemopatch and the study was performed over 30, 45 and 60 days.
Histopathological evaluations of test item persistence and differences in tissue reaction have been performed at each of the time points for both studies (see chapter 2.2.2).

2.2.2 Histological Evaluation

A histological evaluation of the hematoxylin and eosin (H&E) stained histological slides at every time point was carried out by Dr. Wolfgang Öhlinger. From each paraffin block, serial paraffin sections were performed.
He evaluated the sample slides by using the following scoring system for the categorization of residual material, necrosis, granulocytes, macrophages, foreign body reaction:
0=negative, 1=minimal change, 2=moderate change, 3= major change (see table 9 and 10)
Mary-Anne Lastro performed documentation in digital photomicrographs.

2.2.3 Immunohistochemistry

The immunhistochemical evaluation served as an additional analysis to the histopathological part (see chapter 2.2.2). The slides used for this analysis were identical with those used for the histopathological evaluation with the difference not being HE stained.

In order to block the endogenous peroxidase all samples were pretreated by incubation with hydrogen peroxide following a steaming procedure. Afterwards, various antibodies (CD3,CD8, CD18 and pi3K) were applied to the slides following the coat of an appropriate labeled polymer for binding the first antibody as well as to exposure binding sites to the staining molecules. Thus, detection was achieved with two staining techniques, first with Vector NovaRed to locate the investigated cells and second with Mayer's Hemalaun a nucleus counter staining to obtain an overview picture. Finally, all slides were permanently mounted [20].

Method in brief:

<u>Analysis of pi3K, CD3,CD8 and CD18 antibodies:</u>

- Dry slides for 30min at 60°C or overnight at 40°C in the drying chamber
- Paraffinize according to the laboratory's manual using following program steps:

- 2 x 10 min xylol
- 2 min ethanol ≥ 99,8 %
- 1 min ethanol ≥ 96 %
- 1 min ethanol ≥ 70 %
- 1 min ethanol ≥ 50 %
- Aqua dd.

- Block of the endogenous peroxidase (3% H_2O_2 in aqua dd., 10 min)
- Wash with aqua dd.
- Pretreat sample slides according to pretreatment 1 or 2:

Pretreatment 1: applicable to Pi3K: Steam 20 min with citrate buffer pH 6,0 (20 min preheating of steamer and sample container), 20 min cooling

Pretreatment 2: applicable to CD3, CD8 and CD18: Incubate 8 min with proteinase K

- Transfer slides on Shandon coverplates: Set one drop TBS directly on the coverplate. Slope sample slide onto the drop and avoid any air bubbles. Store the coverplate in a fixed glass container with aqua dd.
- Flush 5 min with TBS
- Dilute antibody 1 for CD3, CD8 and CD18 1:25 and antibody 1 for pi3K 1:150 respectively in Antibody diluent
- Applicate 100 µl of diluted antibody 1, incubate for 1 hour at room temperature or at 4°C
- Wash 5 min with TBS
- Applicate 100 µl of antibody 2, incubate for 30 min at room temperature
- Antibody 2 for CD3 and CD 18: labeled polymer, anti-mouse
- Antibody 2 for pi3K: anti-goat Ig peroxidase
- Wash 5 min with TBS

Detection:

- Applicate 2-3drops of Vector NovaRed and incubate for 6 min
- Stop reaction with aqua dd. (do not store longer than 10 min in water)
- Stain with program “Nova red”
- Mount permanently

RESULTS

1. Stability of Hemopatch Heavy Duty

1.1 FTIR analysis

1.1.1 FTIR spectra of multiarm-PEG-isocyanates

FTIR spectrum of 4 and 8arm-PEG-isocyanates as powder (day 0, room temperature)

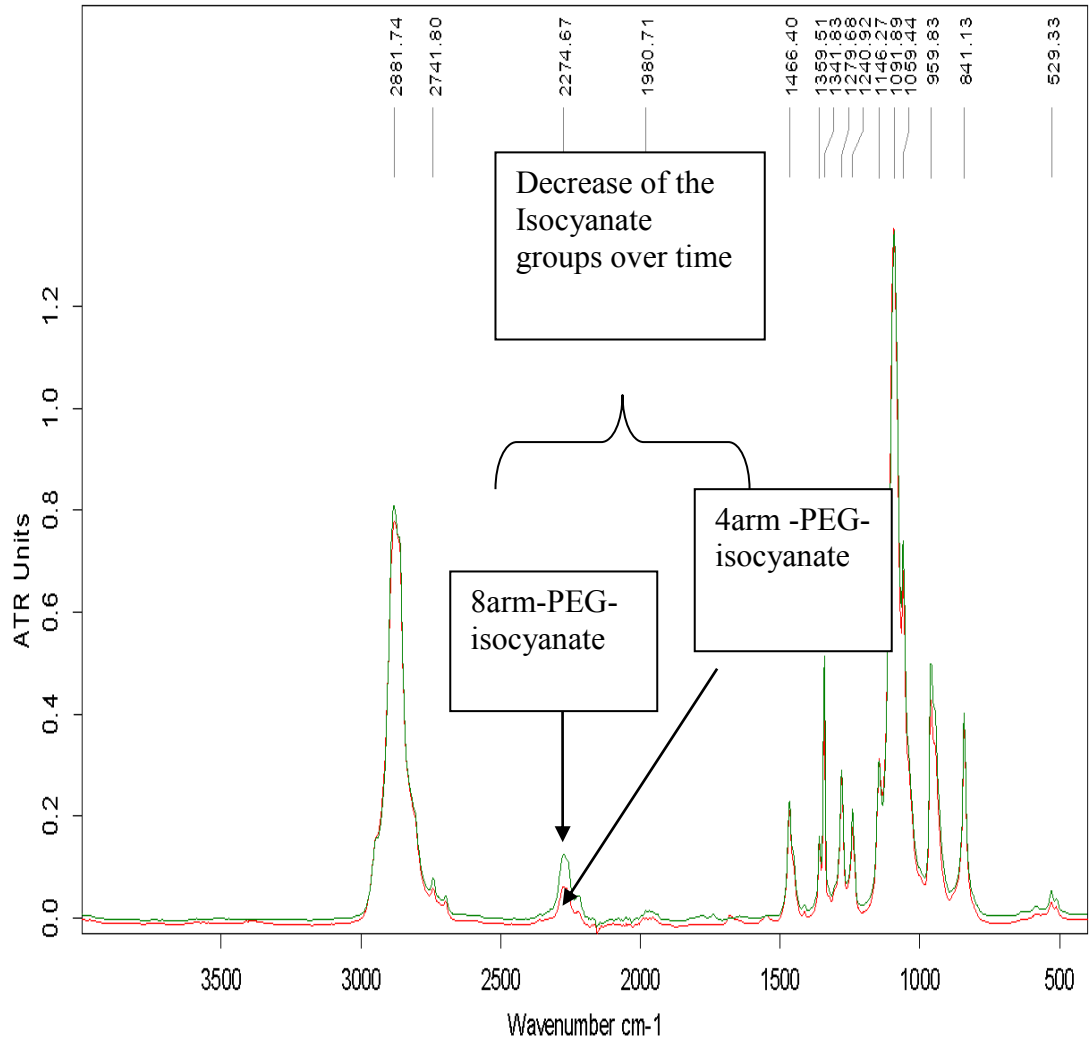


Figure 6: FTIR spectrum of 4arm-PEG-isocyanate and 8arm isocyanate-PEG immediately after removal from the freezer (before storage at room temperature and ambient humidity).

The isocyanate group can be associated to the intensive band at 2270cm^{-1}(figure 6). The isocyanate group was not stable during storage over 6 days at -20°C which can be seen by the complete disappearance of the isocyanate associated band and the appearance two bands at 1700-1800cm^{-1}. These new bands can be explained by the transformation of isocyanate groups into unstable carbamic acid derivatives and the formation of new carboxy groups. (C=O, see figure 7).

FTIR spectrum of 4 PEG-isocyanates as powder (day 6, room temperature)

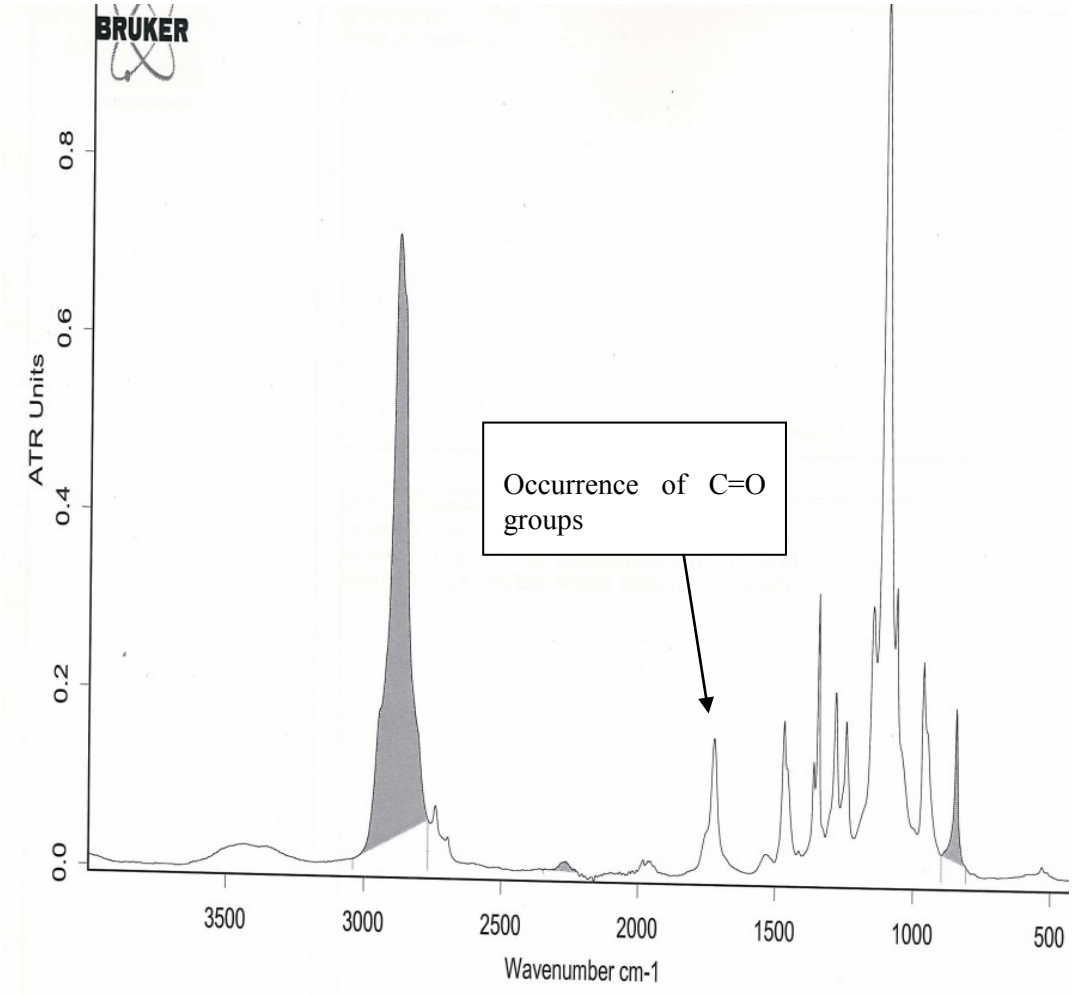


Figure 7: FTIR Spectrum of 4arm-PEG-isocyanate after storage as a powder at room temperature and ambient conditions of humidity after 6 days.

FTIR-Spectrum of 4arm-PEG-ester-isocyanate as powder

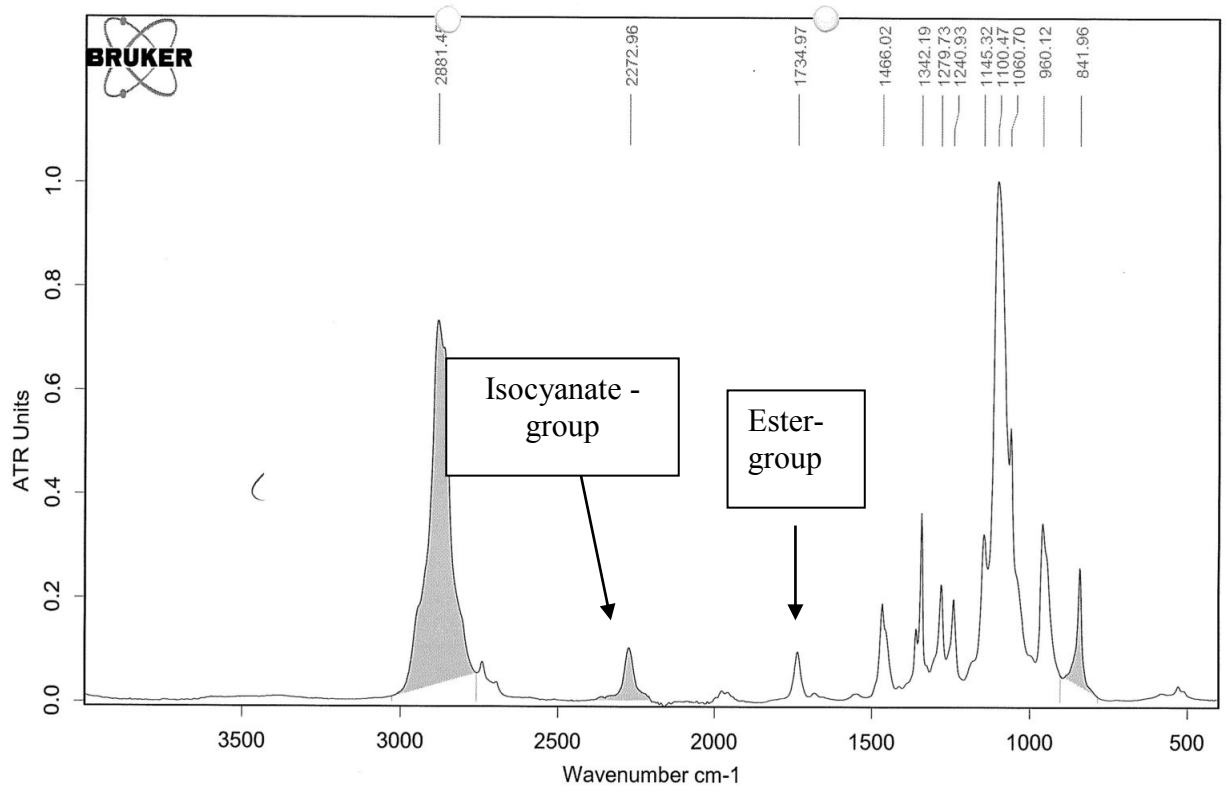


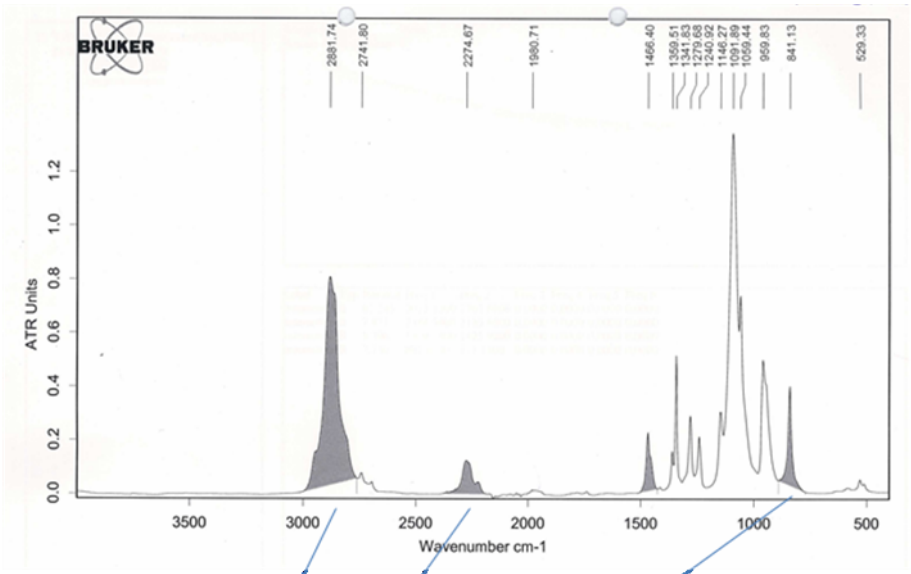
Figure 8: FTIR Spectrum of 4arm-PEG-ester-isocyanate immediately after removal from the freezer (before storage at room temperature and ambient humidity).

The spectrum of the 4arm-PEG-ester-isocyanate is similar to the 4arm-PEG-isocyanate except the appearance of the C=O group at 1734cm^{-1}.

1.1.2 Integration of multiarm PEG-isocyanates

In order to determine the substitution degree of the isocyanate groups a ratio between three integrated peak areas was obtained (figure 9).

The peaks at 2861cm^{-1} and 841 cm^{-1} were used as internal standards because they did not change neither in their quantity nor in their quality. They were first put in proportion to the isocyanate peak at 2273cm^{-1} to determine the decrease of the isocyanate groups. Secondly, they were put in relation to each other for control purposes.



Label	Typ	Resultat	Freq 1	Freq 2	Freq 3	Freq 4	Freq 5	Freq 6
Interactive	B	61.245	3013.1300	2761.6900	0.0000	0.0000	0.0000	0.0000
Interactive	B	7.921	2384.5400	2185.4800	0.0000	0.0000	0.0000	0.0000
Interactive	B	5.396	1509.7400	1425.9200	0.0000	0.0000	0.0000	0.0000
Interactive	B	7.730	891.6200	771.1400	0.0000	0.0000	0.0000	0.0000

Figure 9: Integration model of 4arm-PEG-isocyanate, Day 0.

The substitution degree of isocyanate-PEG is calculated by the formula:

$$\text{Substitution degree} = \frac{\frac{[ANCO]t}{[AP1]t}}{\frac{[ANCO]0}{[AP1]0}} \times 100 \quad (1)$$

Where: [ANCO]t, [AP1]t, [ANCO]0, [AP1]0 are the peak areas of the isocyanate peak [ANCO], or of peak 1 [AP1] in the test sample after storage [t] or in the initial sample taken from the freezer, before storage [0].

In formula (1) the substitution degree of the initial fresh sample was considered to be 100%. By this formula only the percentage of the decrease of substitution degree in comparison to the initial value was calculated. In order to calculate the absolute substitution degree of an isocyanate-PEG sample a calibration of the method by NMR was necessary. From the same sample the substitution degree was determined by NMR and the ratio [ANCO]0/[AP1]0 by FTIR. The corrected substitution degree was calculated according to the formula:

$$\text{Substitution degree [\%]} = \frac{[A_{NCO}]_t}{[A_{P1}]_t} \times \frac{SG\ (NMR)}{\frac{[A_{NCO}]_0}{[A_{P1}]_0}} \qquad (2)$$

Formula 2: SG (NMR) represents the substitution degree (in %) determined by NMR (see chapter 1.3). The second fraction of formula 2 has to be determined only once, and can be used as a constant in all the later calculations. In order to obtain the right constant, it is very important that the samples determined by NMR and FTIR have the same substitution degree.

Example for the calculation see following example from the stability raw data of 4arm PEG-isocyanate as powder at room temperature over time:

4arm-PEG-isocyanate

Day	**Internal Standard peak**	**Isocyanate peak**	**Int. Standard : ICN**	**Substitution degree [%]**
0	61.52	7.921	0,13	97*
1	62.983	8.37	0.13	100
5	59.057	4.808	0.08	61
6	59.68	4.043	0.07	51
7	58.75	2.943	0.05	38

Table 1: Stability of 4arm-PEG-isocyanate over time.

*97% purification was determined by NMR

1.1.3 Stability of multiarm-PEG-isocyanates raw material as powder over time

Stability of 4arm-PEG-ester-isocyanate at room temperature

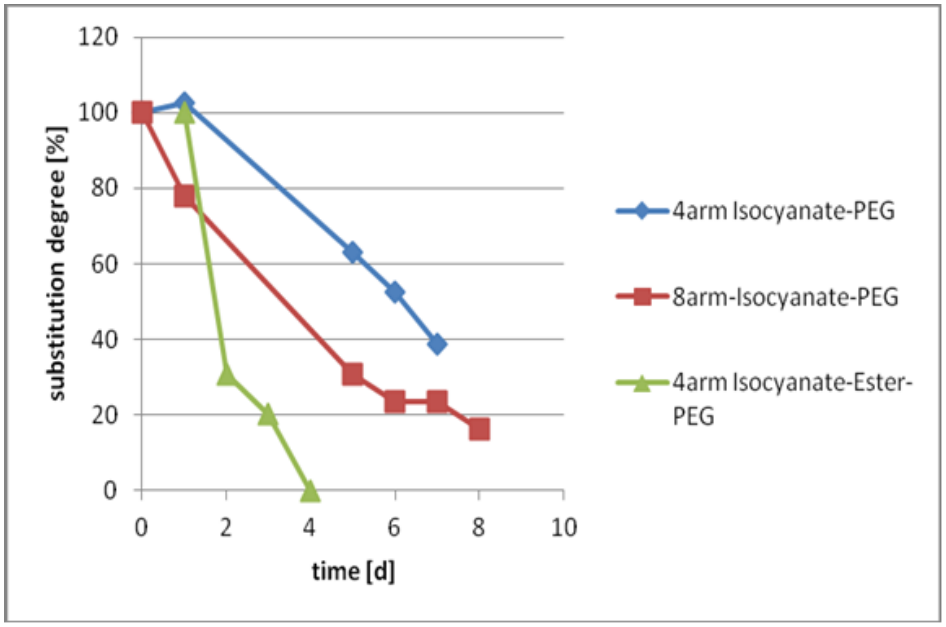


Figure 10: Stability of multiarm-PEG- isocyanate raw material as powder over time. The samples were stored as a powder in an open glass bottle. Every day 0.2 g of the powder was taken out and placed on the surface of the ZnSe-ATR-crystal of the FTIR instrument. The substitution degree was determined.

1.2 Stability Hemopatch Heavy Duty (PEG-isocyanate coated collagen patch) extracted with acetone

1.2.1 Hemopatch coated with 4arm PEG-isocyanate (HT4), 8 arm PEG-isocyanate (HT8) and 4arm PEG-ester-isocyanate (HT4-E) at RT

Data given in figure 11 show a rapid decrease of Hemopatch Heavy Duty at room temperature.

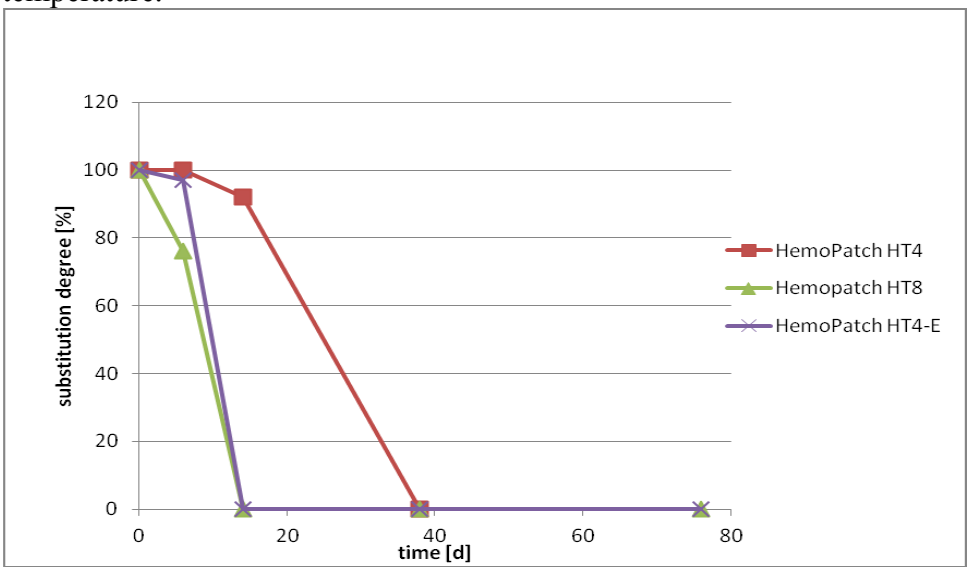


Figure 11: Stability of Hemopatch Heavy duty variants at room temperature.

1.2.2 Stability of Hemopatch /HT4/HT8/HT4-E at 4°C

Data given in Fig 12 show a rapid decrease of Hemopatch Heavy Duty at 4°C.

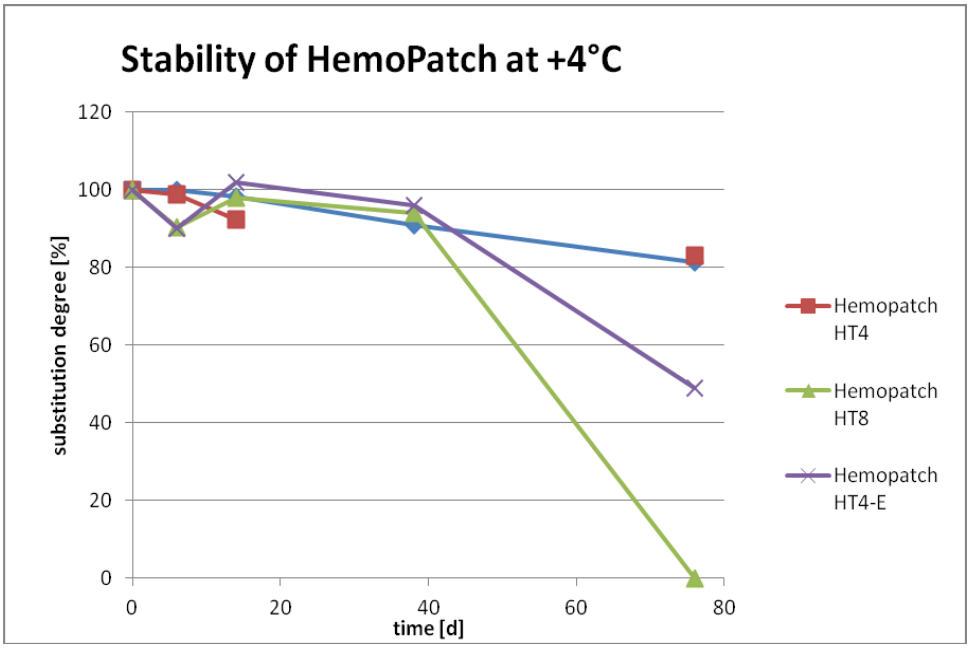


Figure 12: Stability of Hemopatch Heavy duty variants at 4°C. HT4 and HT8 represent the multiarm (4 or 8arm) –PEG-isocyanate without an ester bond. HT4-E defines the 4arm-PEG-ester-isocyanate with an ester bond in its chemical structure. The PEG coating was extracted with acetone, evaporated and the obtained powder was used in the analysis.

4arm-PEG-ester-isocyanate at RT and +4°C

Data given in figure 13 show a rapid decrease of Hemopatch Heavy Duty at 4°C.

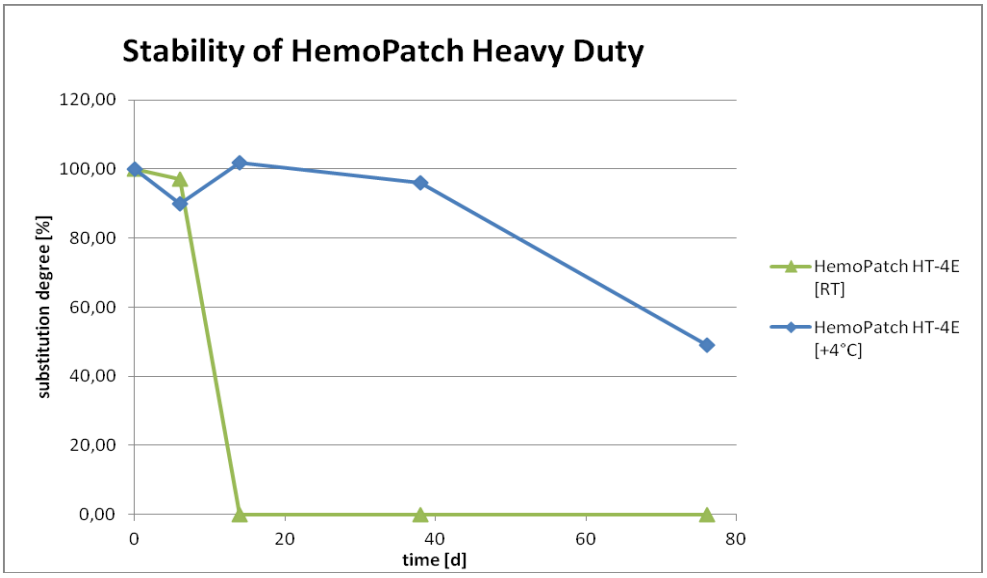


Figure 13: Stability of Hemopatch Heavy Duty at 4°C and RT (4arm-PEG-isocyanate contains an ester bond additionally). The PEG coating was extracted with acetone, evaporated and the obtained powder was used in the analysis.

1.3 NMR analysis of multiarm PEG-isocyanates

In order to determine the decomposition of multiarm-PEG-isocyanate samples NMR spectra within the period of two weeks were recorded.

The ^{1}H-NMR spectroscopic analysis spectrums identified a large signal that refers to the PEG groups of the polymer chain and contains a range of equivalent protons due to its molecular weight of 10 000Dalton. Its signals overlay resulting in an enormous signal without any further splitting. The ^{1}H-NMR signals of the two neighboring CH_2 groups of the terminal glycol unit refer to the assumption that the isocyanate groups are terminally located. Apart from the isocyanate groups there is a contamination which is in the sample but which is low in comparison to the remainder molecule (2-3%) and a degree of purity of 97% can be assumed.

Whereas the ^{1}H NMR signals of the terminal glycol unit progressively decreased until their disappearance after 2 weeks another glycol unit could be detected which takes over the terminal position of the converted product. Its chemical shifts and linkings leave to the conclusion of a simultaneous primary amine appearance in terminal position. (see figure 14 b and c)

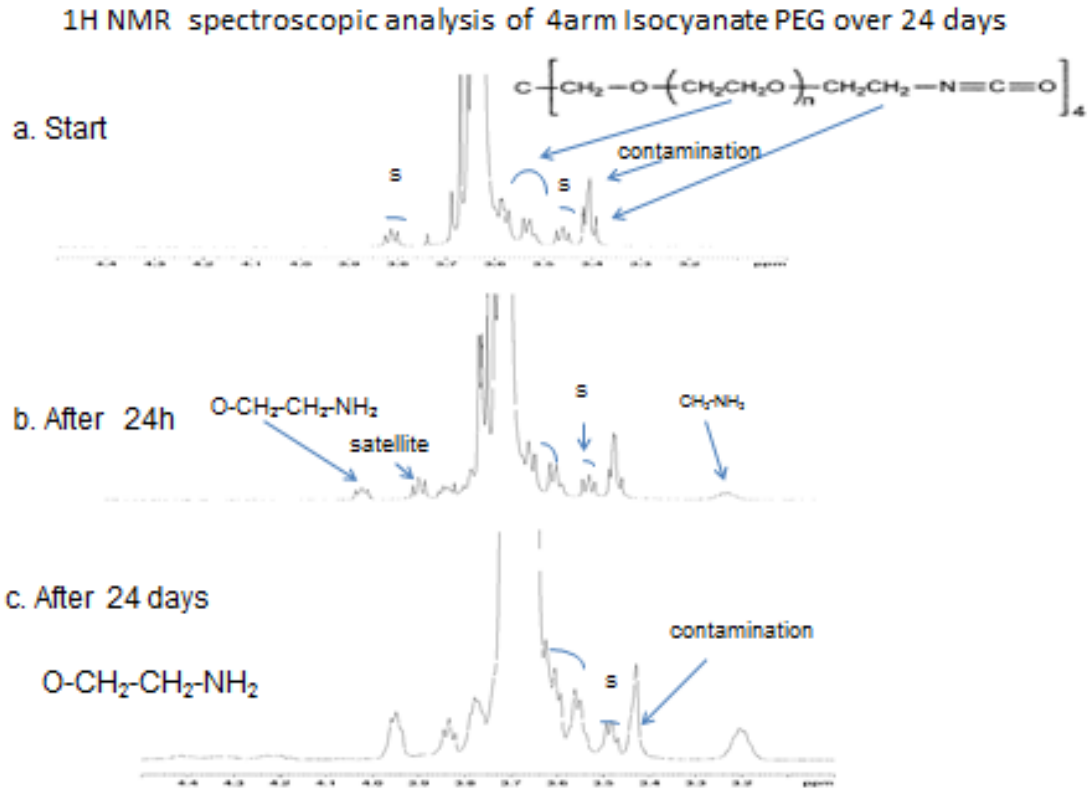


Figure 14: ^{1}H-NMR spectroscopic analysis of decomposition of 4arm-PEG-isocyanate at the beginning, after 24 hours and after 24 days. At the start point, the PEG chain is intact and further signals allow interpretation of the availability of terminal isocyanates. During analysis period the PEG chain remains stable whereas the signals of the terminal glycol group change. Their chemical shift refers to the degradation of the isocyanate groups and development of a primary amine.

The 13C spectra (see figure 15 and 16) allow further location of the assumed isocyanate groups. Due to the assumption of amine formation during time and low water concentration in the sample following reaction takes place:

$$R\text{-}N{=}C{=}O + H_2O \rightarrow [R\text{-}NH\text{-}COOH] \rightarrow RNH_2 + CO_2$$

The NMR spectra align with the results of the FTIR spectra (see results chapter 1.1.) and show significant occurrence of carboxyl bands over time.

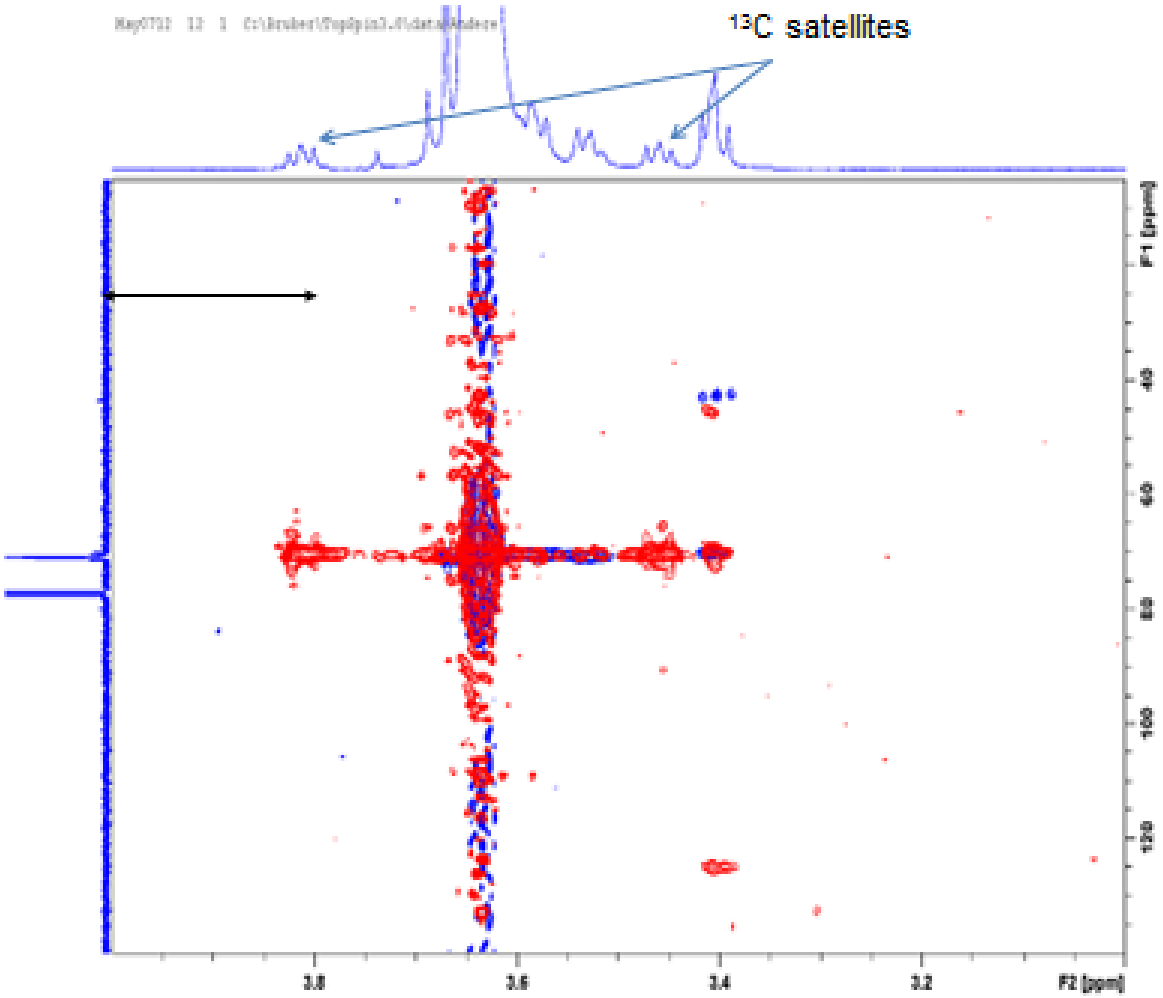


Figure 15: Heteronuclear 2D NMR spectrum (HSQC in blue and HMBC in red) of fresh prepared 4arm PEG-isocyanate sample. The detected $^{1}J_{C\text{-}H}$, $^{2}J_{C\text{-}H}$ and $^{3}J_{C\text{-}H}$ detected linkings confirm to the structure reflected in the 1H NMR spectrum.

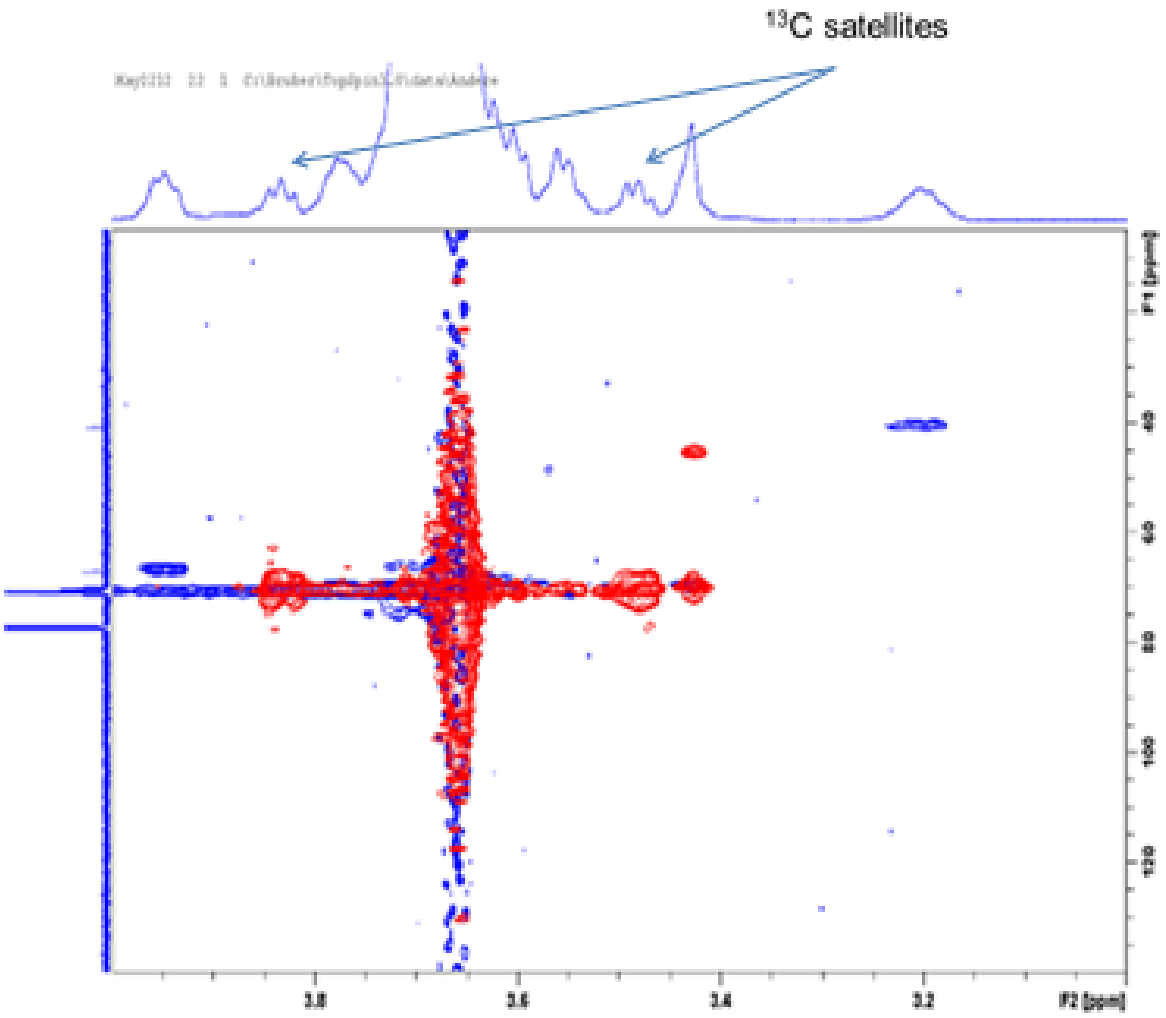


Figure 16: Heteronuclear 2D NMR spectrum (HSQC in blue and HMBC in red) of the 4arm-PEG- isocyanate sample stored for 14 days. The detected $^{1}J_{C\text{-}H}$, $^{2}J_{C\text{-}H}$ and $^{3}J_{C\text{-}H}$ detected linkings confirm to the structure reflected in the 1H NMR spectrum.

4arm-PEG-ester-isocyanate

In order to determine the decomposition of the 4arm-PEG-ester-isocyanate sample NMR spectra within the period of 4 days have been recorded. Analysis proved that ester-isocyanate groups fully decompose at day four.

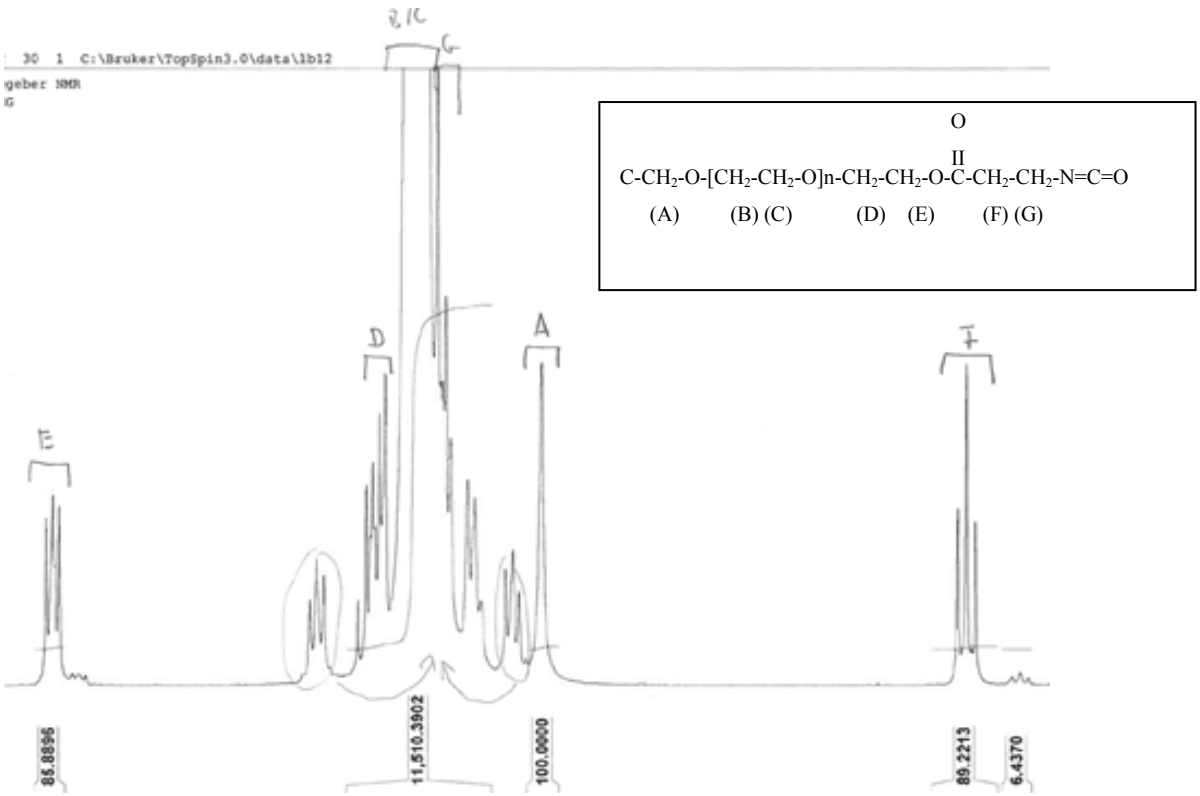


Figure 17: 1H NMR spectrum of 4arm PEG-ester-isocyanate. To facilitate their description, the signals were denoted alphabetically and in capital letters and refer to the existing H of the sample. The sample fully decomposed at day 4.

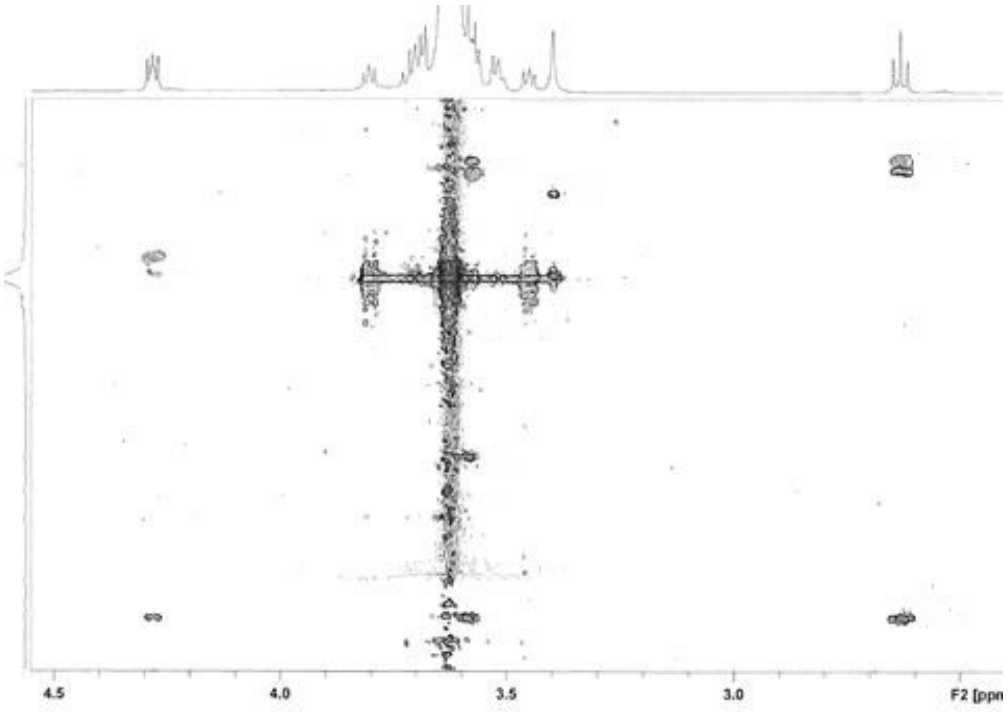


Figure 18: Heteronuclear 2D NMR spectrum (HSQC and HMBC respectively) of fresh prepared 4arm-PEG-ester-isocyanate sample. The detected $^{1}J_{C\text{-}H}$, $^{2}J_{C\text{-}H}$ and $^{3}J_{C\text{-}H}$ detected linkings confirm the structure reflected in the 1H NMR spectrum.

2. Biodegradability of Hemopatch Heavy Duty

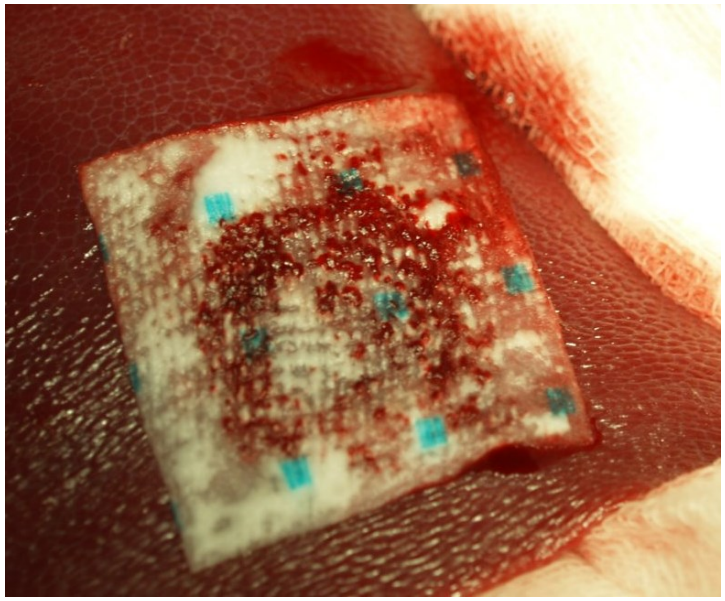
2.1 Preclinical testing of 4arm-PEG-ester- isocyanate coated Hemopatch Heavy Duty in comparison to NHS-PEG coated Hemopatch

Item	**Test model**	**Mechanical test passed/not passed**	**Results animal test**
Hemopatch	Liver abrasion	Passed	Stopped bleeding
Hemopatch Heavy Duty, stored at +4°C		Passed	Stopped bleeding very quickly
Hemopatch Heavy Duty, stored at -20°C	Liver abrasion	Passed	Stopped bleeding very quickly, stuck strongly to the liver lobe
Hemopatch Heavy Duty, stored at -20°C	Liver resection	Passed	Stopped strong bleeding very quickly, no hematoma, stuck strongly to the liver lobe
Hemopatch	Spleen abrasion	passed in one of two	Stopped bleeding, but hematoma
Hemopatch Heavy Duty stored at 4°C		Passed	Stopped bleeding very quickly, no hematoma
Hemopatch	Spleen resection	n.a.	n.a.
Hemopatch Heavy Duty		Passed	Stopped bleeding

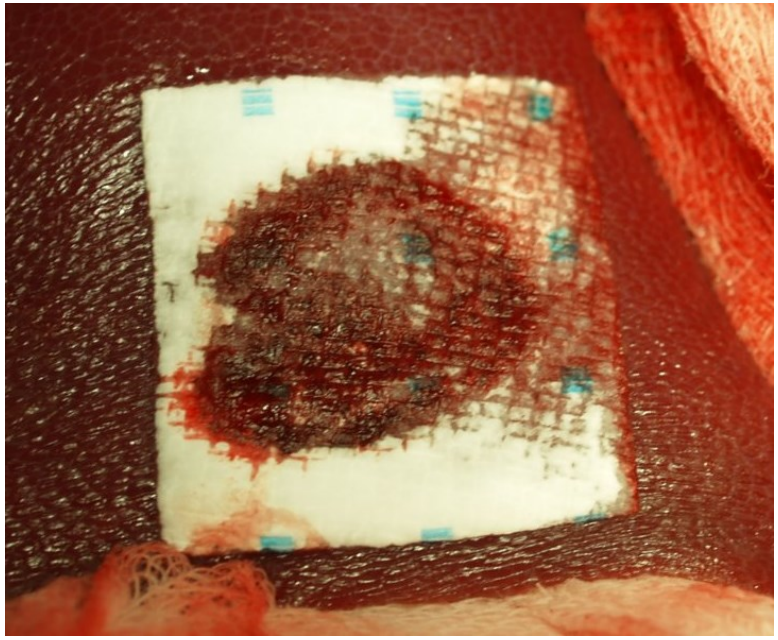
stored at -20°C			
Hemopatch	Lung sealing, afterwards filling with water	not passed	failed
Hemopatch Heavy duty		Passed	Good sealing
Hemopatch	Gall bladder incision	not passed	failed
Hemopatch Heavy Duty		Passed	Good adherence
Hemopatch	Heart sealing	N/A	N/A
Hemopatch Heavy Duty		Passed	Good adherence

Table 4: Summary of all results of the preclinical testing of 4 arm-PEG-ester- isocyanate coated Hemopatch Heavy Duty in comparison to NHS-PEG coated Hemopatch.

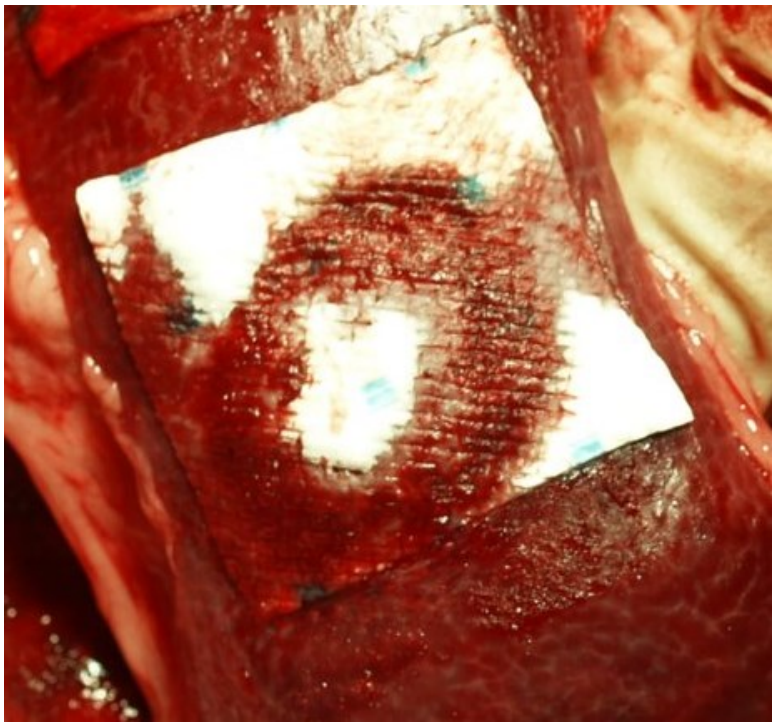
2.1.1 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch Heavy Duty – liver lobe abrasion model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) Liver abrasion model	Fleece size 3x3cm. Wet application showed no adhesion, bleeding from one edge. After 2 minutes reapplication with dry gauze (see photo 2).  **Photo 1: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyante coating) after 30 seconds on the liver lobe.**

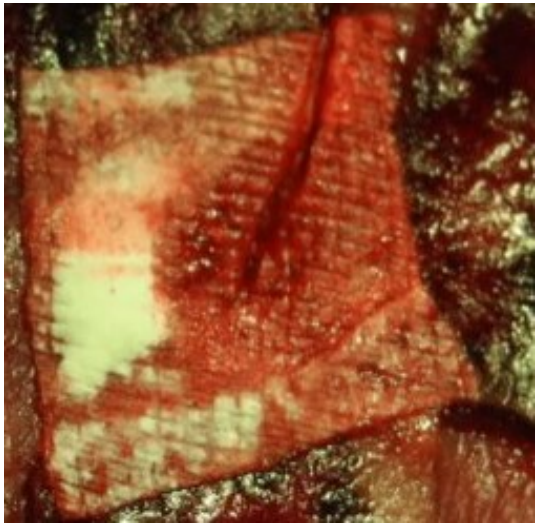
2.1.1 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch – liver lobe abrasion model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) liver abrasion model	Fleece size 3x3cm. Dry application showed very good adhesion, no bleeding, after 1 minute not possible to remove  **Photo 2: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coating) after 30 seconds on the liver lobe. Reapplication with dry gauze.**

2.1.1 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch – spleen abrasion model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) spleen abrasion model	Fleece size 3x3cm. Dry application showed very good sealing, very strong adhesion  **Photo 3: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) on the spleen after 30 seconds.**

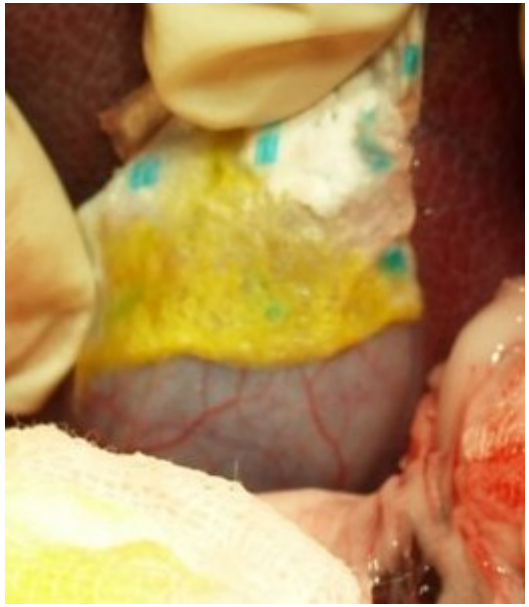
2.1.2 Testing of Hemopatch 4arm–PEG-ester-isocyanate coated pads (stored at -20°C) – liver lobe resection model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at -20°C) liver lobe resection model	A 2x2 cm piece was cut out of the liver lobe and a lot of blood was bleeding out of the wound. The wound was closed with Hemopatch Heavy Duty. The observation showed good adhesion after 30 second. It stuck strongly to the liver lobe and there was no hematoma.  **Photo 4: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) after 30 seconds on the liver lobe.**

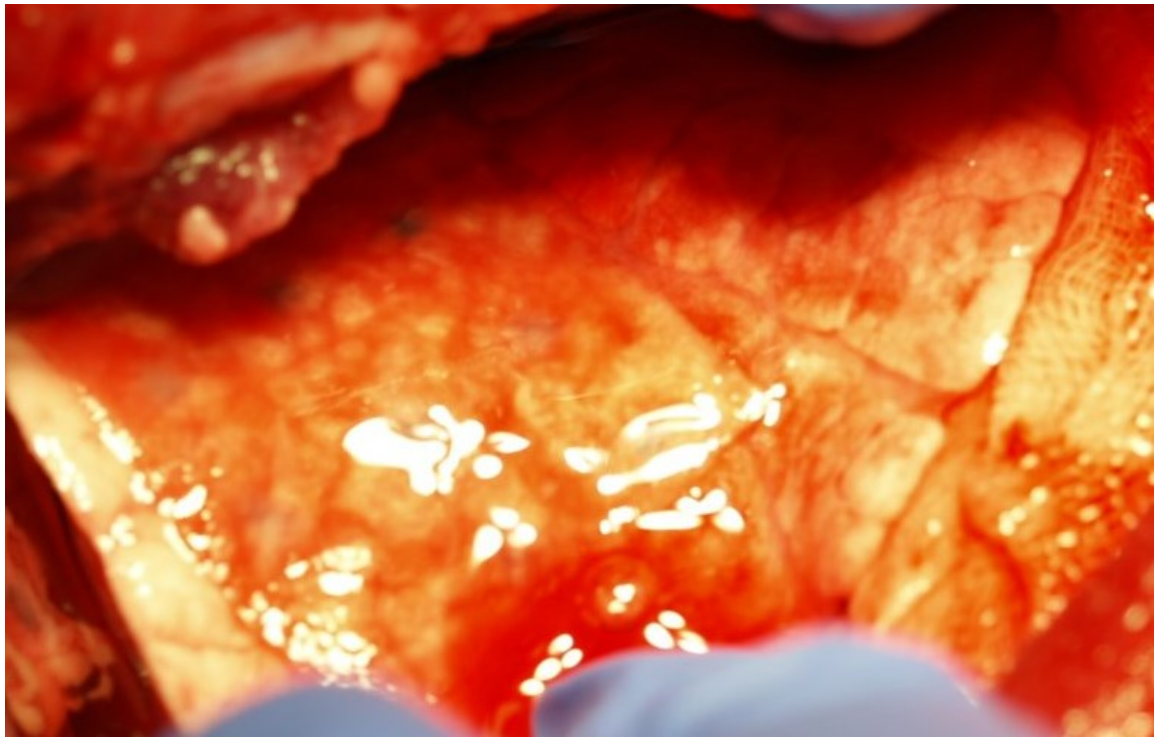
2.1.2 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch (stored at -20°C) – spleen resection model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) spleen resection model	Fleece size 3x3cm. Dry application showed very good sealing, very strong adhesion, stopped strong bleeding wound after 30 seconds moderate contact pressure with dry gauze.  **Photo 5: Top view of Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) 30 minutes after spleen resection.**

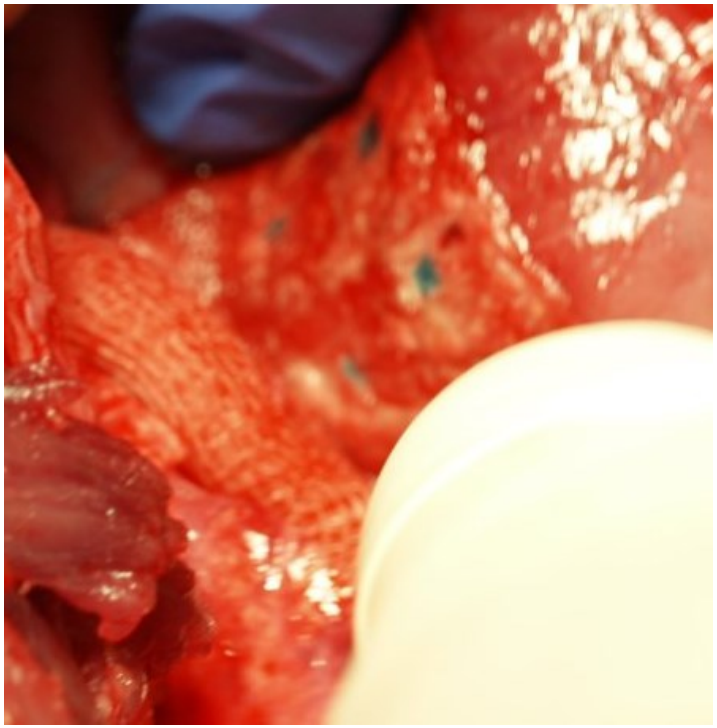
2.1.3 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch (stored at 4°C) – gall bladder sealing model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) gall bladder incision	Fleece size 3x3cm. Dry application showed very good adhesion and a very good sealing after 30 seconds.  **Photo 6: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) on the gall bladder.**

2.1.4 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch (stored at 4°C) – lung sealing model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) lung sealing model	Fleece size 3x3cm. Dry application showed very good adhesion and a very good sealing after filling the lung with water.  **Photo 7: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) on the lung.**

2.1.5 Testing of 4arm-PEG-ester-isocyanate coated Hemopatch (stored at 4°C) – heart sealing model

Test item	Description	Animal test results
Hemopatch Heavy Duty	Matristypt coated continuously with 10mg/cm^2 4arm PEG-ester-isocyanate (stored at **+4°C**) heart sealing model: small incision on heart muscle	Fleece size 3x3cm. Dry application showed that the pad completely stuck to the heart and could not be moved or removed.  **Photo 8: Top view of Hemopatch Heavy Duty (4arm-PEG-ester-isocyanate coated patch) on the heart muscle.**

2.2 Preclinical studies

The results of the histopathological evaluations referred to an acute inflammatory reaction of isocyanate coated pads. In both studies the surface of the injured liver lobe coated with the material stuck to the fat tissue of the abdominal wall in a strong lesion. The adhesion areal contained macrophages with lipid inclusions in the cytoplasm.
Hemopatch coated with 4arm-PEG-isocyanate and 8arm-PEG-isocyanate as well as coated with 4arm-PEG-ester-isocyanate showed a foreign body response with encapsulation 60 days postoperatively in a rabbit liver model, while Hemopatch completely degraded within 60 days following a normal wound healing process.

2.2.1 Macroscopic evaluation of Hemopatch Heavy Duty

Hemopatch Heavy Duty coated with 4arm-PEG-isocyanate and 8arm-PEG-isocyanate, respectively, application site in situ at necropsy 12/30/49 and 60 days after surgery

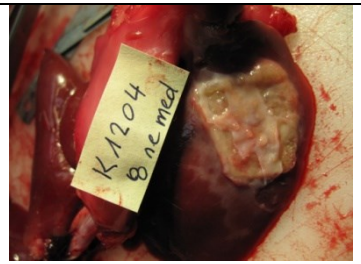
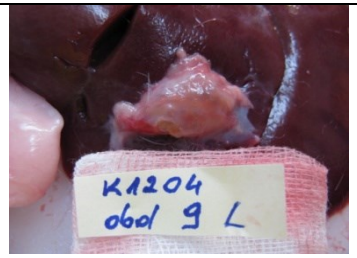
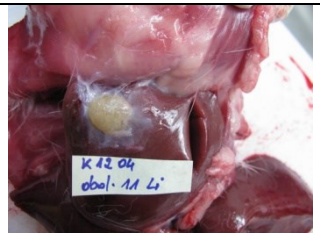
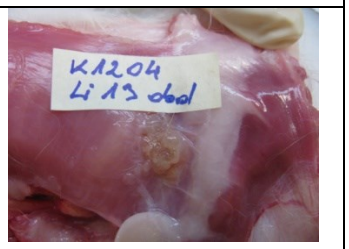
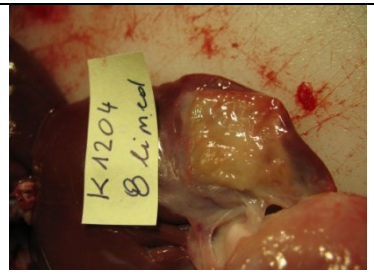
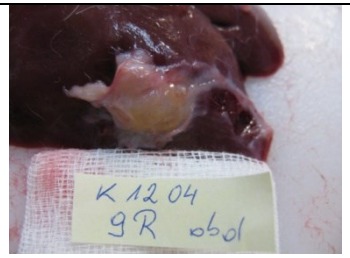
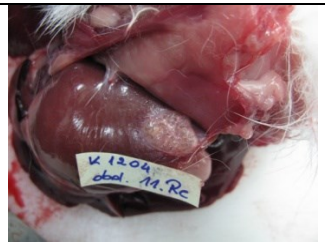
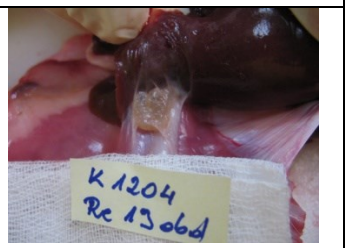
	0 days	12 days	30 days	49 days	60 days
Results Hemopatch Heavy Duty coated with 4arm-PEG-isocyanate	Not applicable				
Results Hemopatch Heavy Duty coated with 8arm-PEG-isocyanate	Not applicable				

Table 5: Macroscopic results of Hemopatch Heavy Duty coated with 4arm-PEG-isocyanate in comparison to Hemopatch coated with 8arm-PEG-isocyanate. Application site in situ at necropsy 12/30/49 and 60 days (with support of Sonja Reisinger).

	0 days	12 days	30 days	49 days	60 days
Macroscopic results Hemopatch Heavy Duty coated with 4arm-PEG-isocyanate		right medial liver lobe with yellow mass (25x30x3mm), in situ, soft, swollen, liver capsule with white discoloration	left medial liver lobe with yellow-white mass (20x13x5mm), in situ, soaked with fluid, low vascularization	left medial liver lobe with yellow mass (12x12x4mm), in situ, soft, low vascularization, low strength adhesion formations from item to peritoneum	Material detached from the liver and adheres to the abdominal wall
Macroscopic results Hemopatch coated with 8arm-PEG-isocyanate		left medial liver lobe with soft yellow mass (20x20x2mm), in situ	right medial liver lobe with yellow-white, compact-elastic mass (16x12x5mm), in situ, low vascularization	right medial liver lobe with yellow white mass (15x15x2mm), low vascularization, no adhesion formations with test item, 2 adhesion formations from liver to peritoneum	right medial liver lobe with compact elastic yellow-brown mass (10x4mm) and white discoloration (12x7x4mm), adhesion formations from item and diaphragm, Material detached from the liver and adheres to the abdominal wall

Table 6: Description of macroscopic results obtained in situ at necropsy (with support of Sonja Reisinger).

Hemopatch and Hemopatch Heavy Duty coated with 4arm-PEG-ester-isocyanate application site in situ at necropsy 12/30/49 and 60 days after surgery

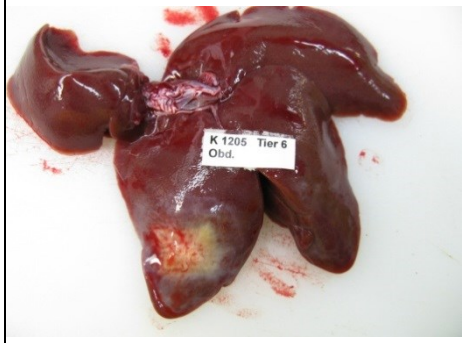
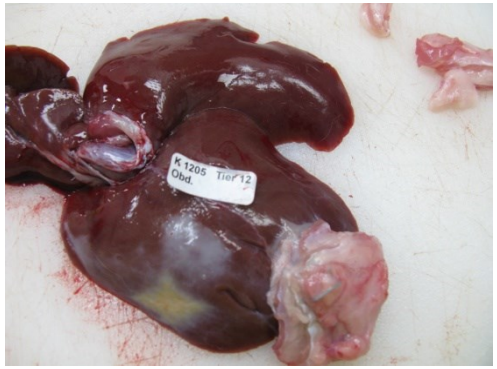
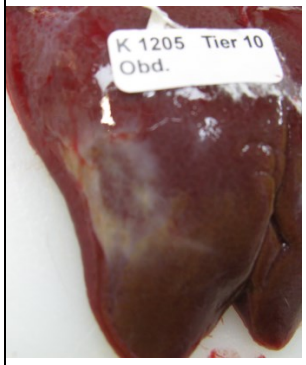
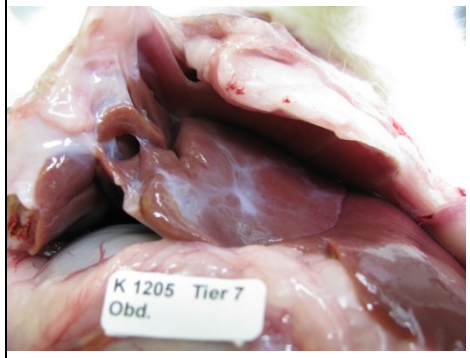
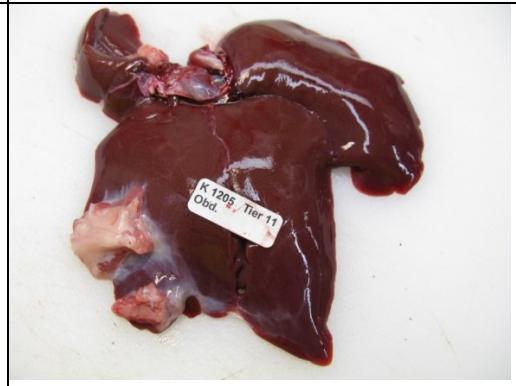
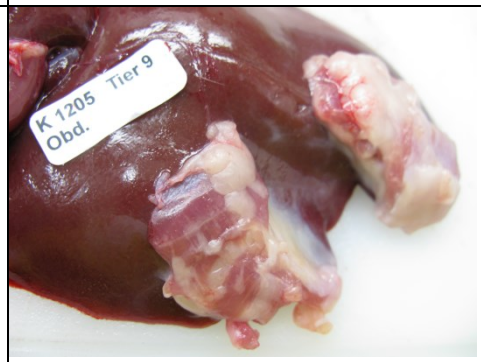
Hemopatch	**30 days**	**49 days**	**60 days**
			
Hemopatch HT-4E			

Table 7: Hemopatch and Hemopatch Heavy Duty coated with 4arm-PEG-ester-isocyanate in comparison. Application site in situ at necropsy 12/30/49 and 60 days after surgery (with support of Sonja Reisinger and Alexandra Schiwitz).

	30 days	49 days	60 days
Hemopatch	right medial liver lobe with yellow mass, in situ, soft, high vascularization in area of the yellow mass	right medial liver lobe with yellow mass, in situ, soft, diffuse white discoloration of the right medial liver lobe, no adhesion formations with item, adhesion formation of the right top of medial liver lobe, left top of medial liver lobe and the peritoneum	right medial liver lobe with yellow-brown mass, in situ
Hemopatch Heavy Duty (coated with 4arm-PEG-ester-isocyanate	diffuse adhesion formation of the right medial liver lobe with peritoneum, diffuse white discoloration of the left medial liver lobe	one adhesion formation from item to peritoneum, one adhesion formation from top of right medial liver lobe to peritoneum	one adhesion formation of the right medial liver lobe with peritoneum in area of liver lesion, one adhesion formation of the left top of medial liver lobe with peritoneum

Table 8: Description of macroscopic results obtained in situ at necropsy (with support of Sonja Reisinger and Alexandra Schiwitz).

2.2.2 Histopathological evaluation

In order to answer the question of biocompatibility/degradability to Hemopatch Heavy Duty`s in comparison to Hemopatch's histopathological evaluations of each animal at each time point have been performed. The following images represent the results from the histopathological evaluation of slides indicating sections of liver specimen with the respective test material isocyanate coated Hemopatch or Hemopatch, respectively. The images show overviews as well as detailed digital photomicrographs.

Hemopatch Heavy Duty (Hemopatch HT-4E - Collagen pad coated with 4arm-PEG-ester-isocyanate)

Timepoint 30 days

The test material 4arm-PEG-isocyanate coated collagen patch is located between the liver lobe and fat tissue. The semiquantitive evaluation indicates a score of 1 for granulocytes and a score of 2 for macrophages. The appearance of foamy macrophages in the adhesion area point to an active inflammation reaction and a strong adhesion zone between the fat tissue and liver lobe.

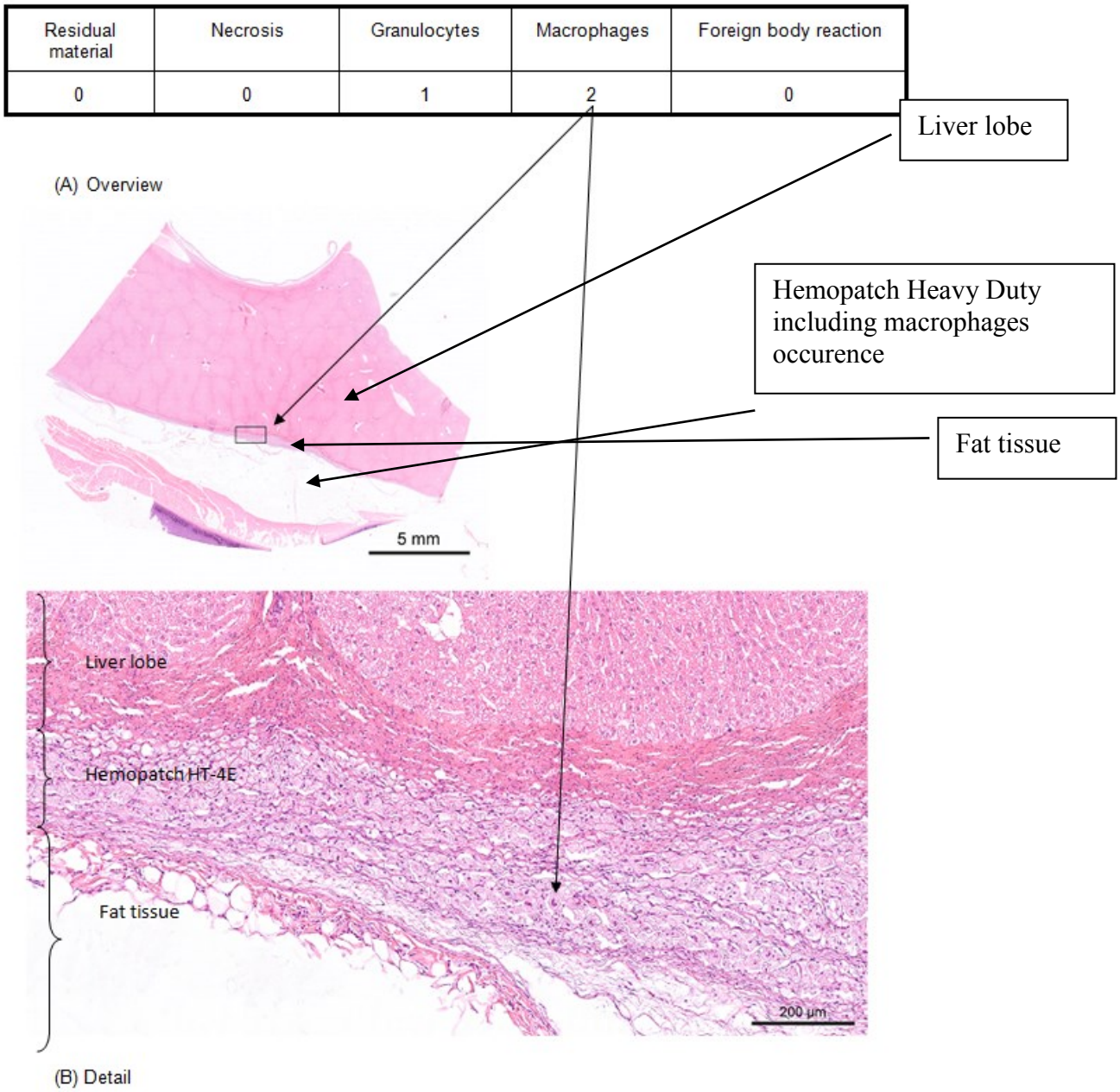


Figure 19: (A) and (B) demonstrate example of histological scoring at day 30 after Hemopatch Heavy Duty application. A strong adhesion zone occurs between fat tissue and liver lobe including aggregates of foamy macrophages.

Hemopatch (Collagen pad coated with NHS-PEG)

Timepoint 30 days

The reference material Hemopatch is located between the liver lobe and fat tissue. The semiquantitive evaluation indicates a score of 0 for residual material, necrosis, granulocytes and macrophages. Therefore, neither inflammation nor adhesion occurs. Only few foreign body giant cells appear around birefringant particles (hair).

Residual material	Necrosis	Granulocytes	Macrophages	Foreign body reaction
0	0	0	0	1

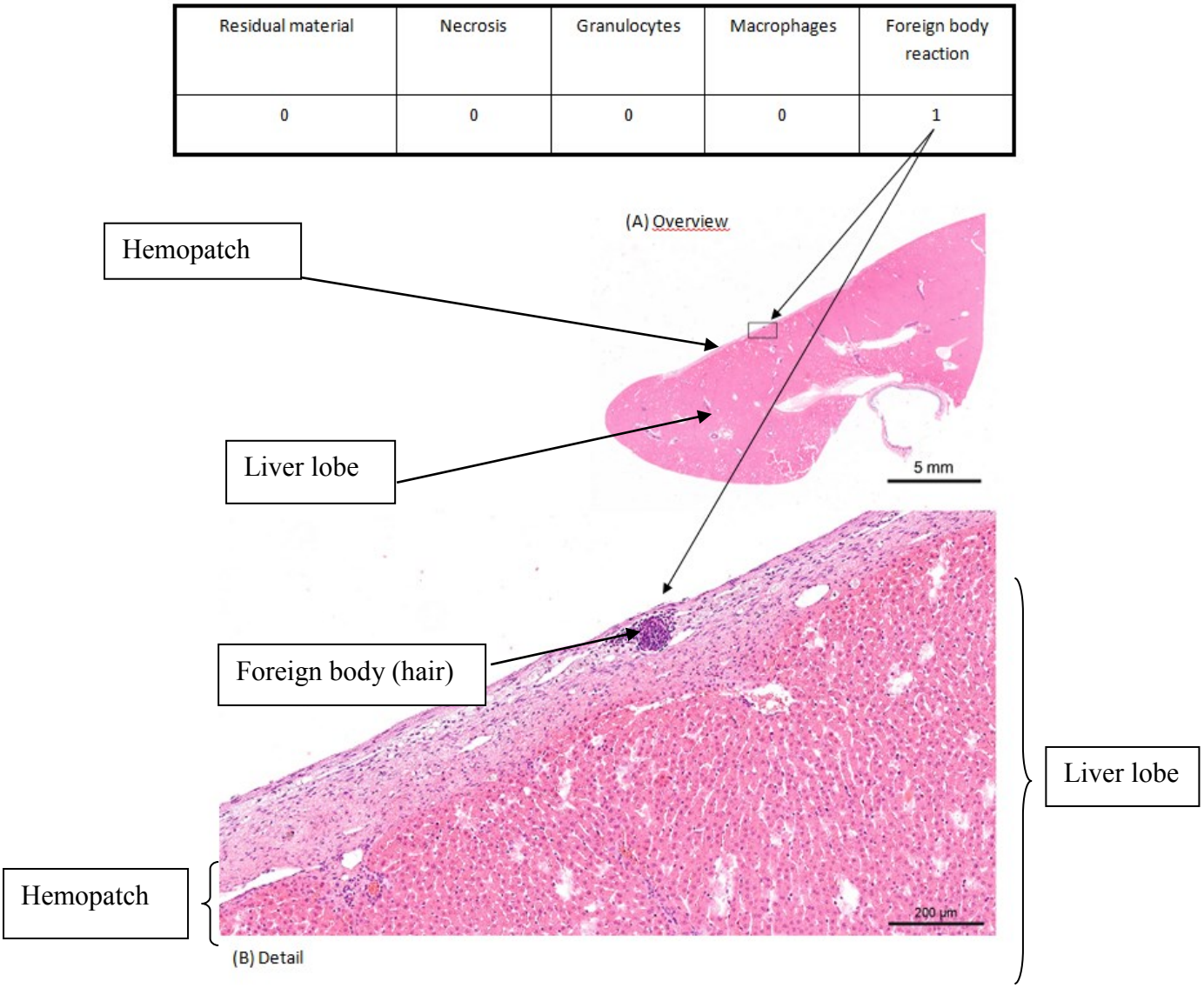


Figure 20: (A) and (B) demonstrate example of histological scoring at day 30 after Hemopatch application. The wound healing process finished completely, a bright scarring, no adhesion, few isolated granulocytes occur, few foreign body giant cells around birefringant particles (hair) inside scar area of former lesion.

Hemopatch Heavy Duty (Heamopatch HT-4E - Collagen pad coated with 4arm-PEG-ester-isocyanate)

Timepoint: 45 days

Implant formed an amorphous mass with many macrophages inside, granulocytes enclose a hole with a capsule inside, macrophages are busy to resorb the material. In areas of adhesion, aggregates of foamy macrophages are found. A hole contains residua of patch material and foamy macrophages. Few foreign body giant cells are located around birefringent particles.

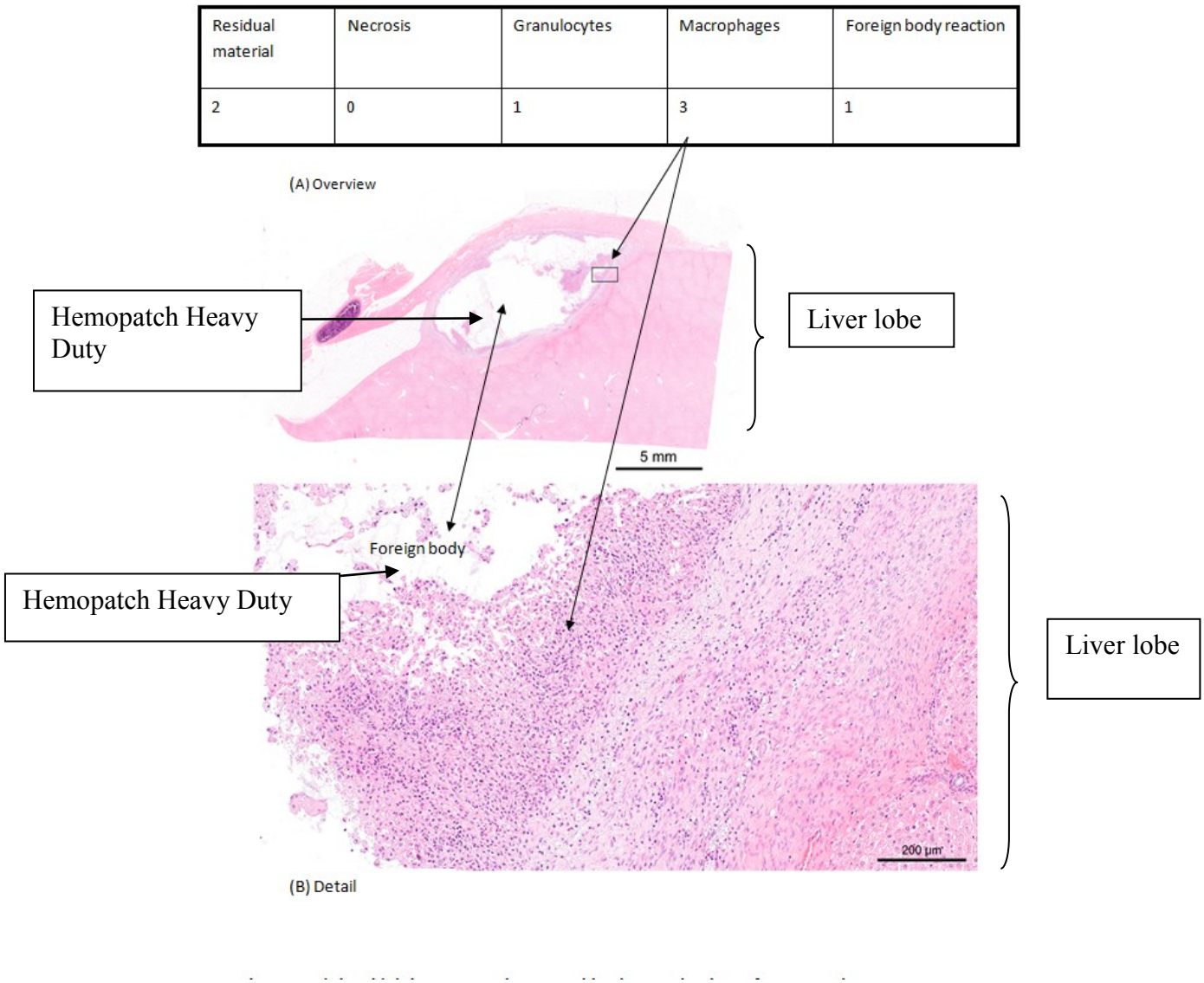


Figure 21: (A) and (B) demonstrate examples of histological scoring at day 45 after Hemopatch Heavy Duty (Collagen pad coated with 4arm-PEG-ester-isocyanate) application. Foreign body formation due to test material that is hard to resorb (capsule). Occurrence of strong adhesion between fat tissue and liver lobe.

Hemopatch (Collagen pad coated with NHS-PEG)

Timepoint: 45 days

No adhesion occurred. Good biocompatibility of Hemopatch and only little test material is remaining.

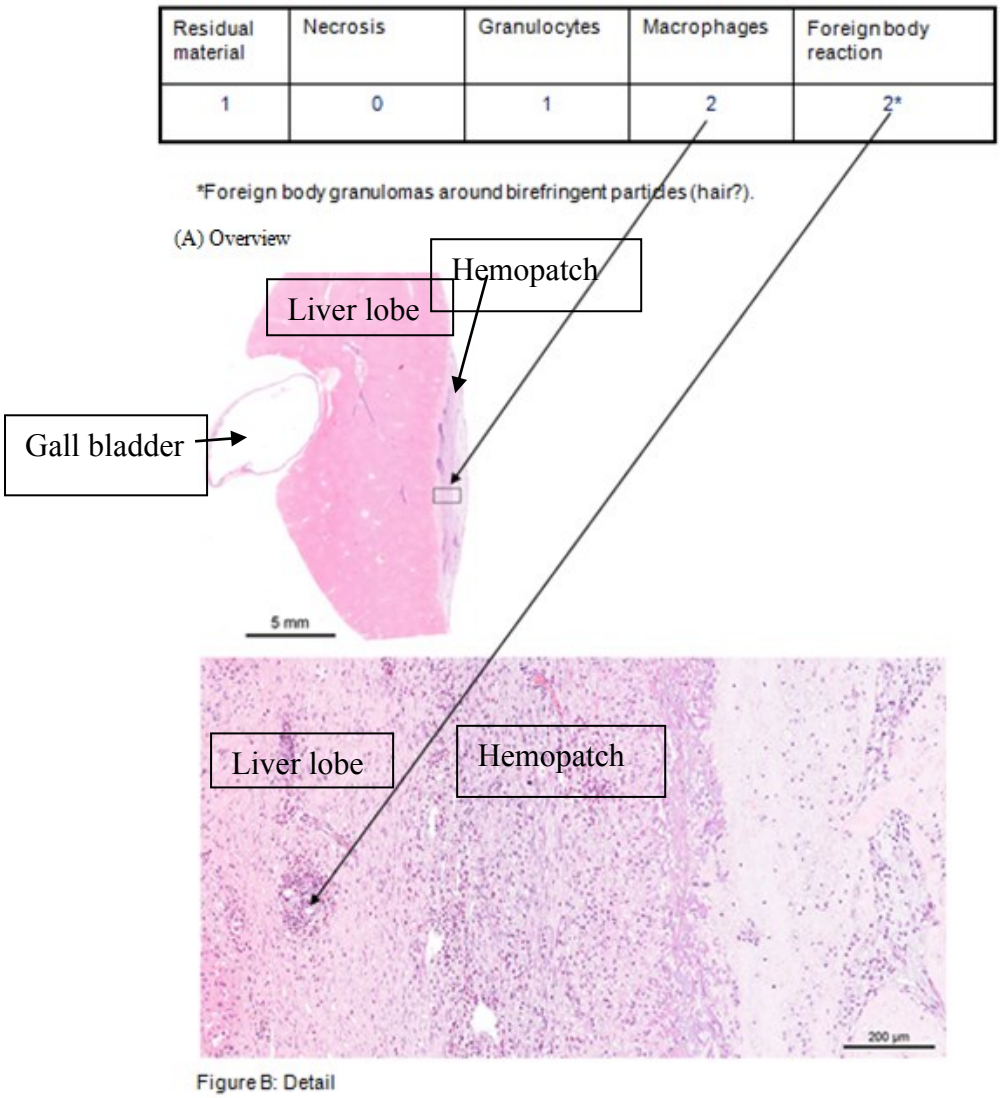


Figure 22: (A) and (B) demonstrate examples of histological scoring at day 45 after Hemopatch (Collagen pad coated with NHS-PEG) application.

Hemopatch Heavy Duty (Hemopatch HT- 4E -Collagen pad coated with 4arm-PEG-ester-isocyanate)

Timepoint 60 days:

An adhesion area with aggregates of foamy macrophages and seroma like space containing residua of patch material is still available. A few foreign body giant cells around birefringent particles are noticed.

Residual material	Necrosis	Granulocytes	Macrophages	Foreign body reaction
0	0	0	3	0

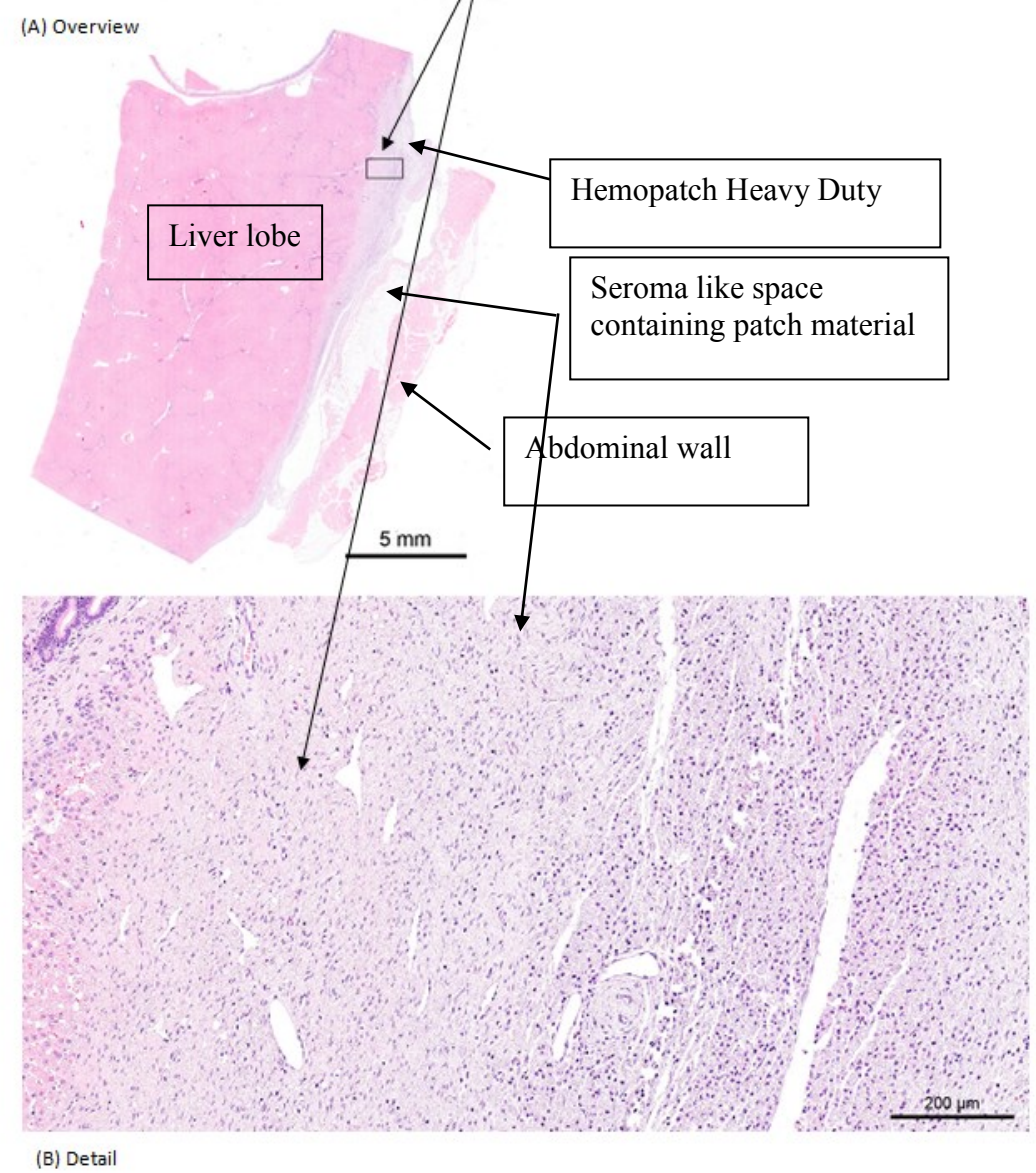


Figure 23: (A) and (B) demonstrate examples of histological scoring at day 60 after Hemopatch Heavy Duty (Collagen pad coated with 4arm-PEG-ester-isocyanate) application.

Hemopatch (Collagen pad coated with NHS-PEG)

Timepoint: 60 days

Wound healing was successful following formation of normal liver cells in damaged area.

Residual material	Necrosis	Granulocytes	Macrophages	Foreign body reaction
0	0	1	1*	0

*Scarred broadening of liver capsule with few granulocytes and aggregates of foamy macrophages. No adhesion.

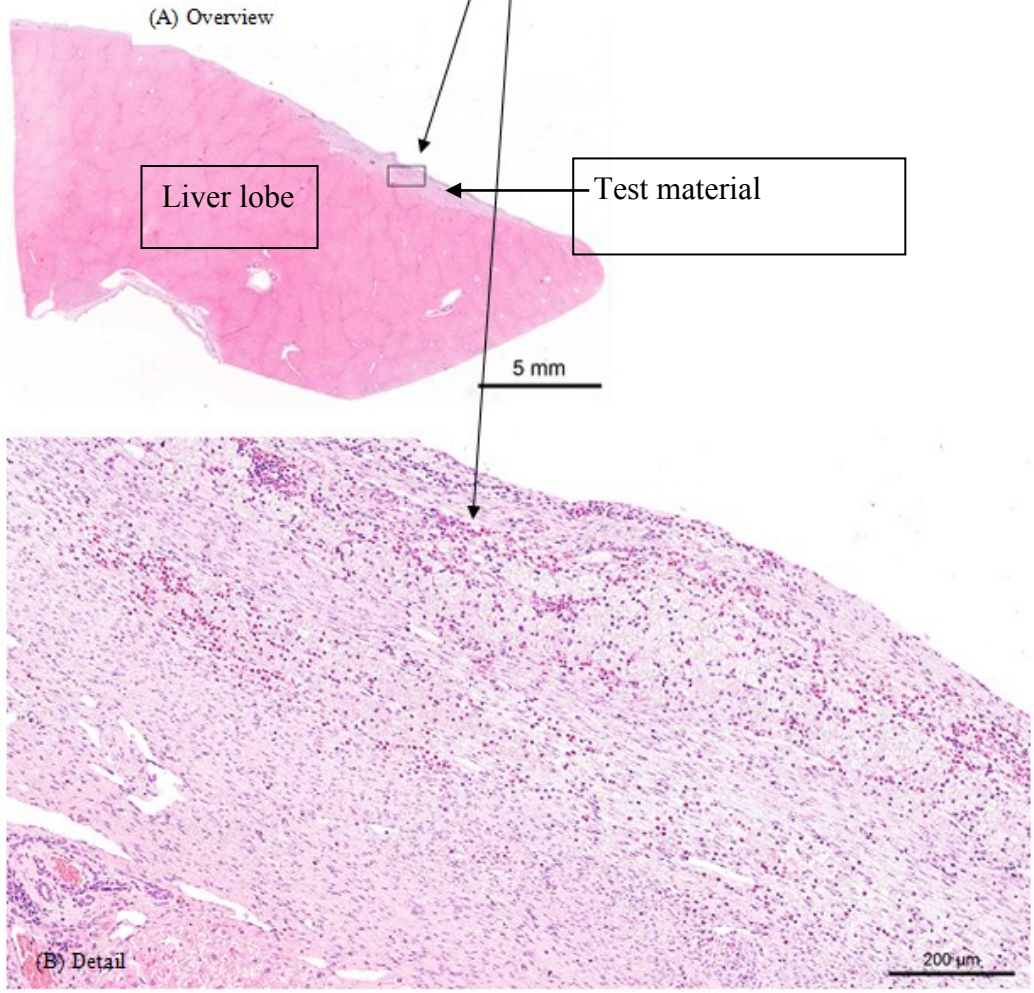


Figure 24: (A) and (B) demonstrate examples of histological scoring at day 60 after Hemopatch (Collagen pad coated with NHS-PEG) application. Formation of normal liver cells in damaged area.

2.2.2 Overview histological scoring

0=negative, 1=minimal change, 2=moderate change, 3= major change (table 9 and 10)

Animal number	Timepoint [d]	liver lobe	Test item left liver lobe	Residual material	Necrosis	Granulocytes	Macrophages	Foreign body reaction
Animal 1	1	left	Hemopatch HT-4	3	2	3	0	0
Animal 1	1	right	Hemopatch HT-8	3	2	3	0	0
Animal 2	1	left	Hemopatch HT-8	3	2	2	0	0
Animal 2	1	right	Hemopatch HT-4	3	2	3	0	0
Animal 3	2	left	Hemopatch HT-4	3	3	3	0	0
Animal 3	2	right	Hemopatch HT-8	3	3	2	0	0
Animal 4	2	left	Hemopatch HT-8	3	2	2	1	1
Animal 4	2	right	Hemopatch HT-4	3	2	3	1	0
Adhesion area liver				0	2	1	2	0
Animal 5	7	left	Hemopatch HT-4	3	1	3	3	2
Animal 5	7	right	Hemopatch HT-8	3	1	1	2	3
Animal 7 (adhesion)	12	left	Hemopatch HT-8	3	1	1	3	3
Animal 7 (adhesion)	12	right	Hemopatch HT-4	3	2	3	3	3
Animal 10	30	left	Hemopatch HT-4	2	1	1	3	3
Animal 10	30	right	Hemopatch HT-8	2	0	1	3	3

Table 9: Overview histological scoring. A median laparotomy was performed and the right and left medial lobe of the liver were exposed. A superficial circular lesion with a diameter of 2 cm and a depth of 1 mm was created with scissors. Test items 4arm-PEG-isocyanate and 8arm-PEG-isocyanate Hemopatch Heavy Duty, respectively, were applied to the wound.

Animal number	Timepoint [d]	Test item	Residual material	Necrosis	Granulocytes	Macrophages	Foreign body reaction	Comments [*]
1	60	Hemopatch HT-4E	0	0	1	2*	0	Adhesion area with aggregates of foamy macrophages
2	60	Hemopatch NHS-PEG	0	0	0	0	1*	Few. Foreign body gigant cells around birefringant particles (hair?) inside scar area of former lesion. No adhesion. Normal liver and gallbladder.
3	60	Hemopatch HT-4E	1	0	0	2*	0	Adhesion area with aggregates of foamy macrophages
4	60	Hemopatch NHS-PEG	0	0	0	1	1*	
5	30	Hemopatch HT-4E	2	0	1	3*	1	Adhesion area with aggregates of foamy macrophages. Seroma like space continuing residua of patch material and foamy macrophages. Few foreign body giant cells around birefringant particles.
6	30	Hemopatch NHS-PEG	1	0	1	2	2*	Foreign body granulomas around birefrigent particles (hair?)
7	30	Hemopatch HT-4E	1	0	1	2	0	Adhesion area with aggregates of foamy macrophages around seroma like cavity with residua of implant material.
8	30	Hemopatch NHS-PEG	2	0	1	2*	2	Adhesion area with aggregates of foamy macrophages around seroma like cavity with residua of implant material.
9	45	Hemopatch HT-4E	0	0	0	3*	0	Adhesion area with aggregates of foamy macrophages
10	45	Hemopatch NHS-PEG	0	0	0	0	0	
11	45	Hemopatch HT-4E	0	0	0	2*	0	Adhesion area with aggregates of foamy macrophages
12	45	Hemopatch NHS-PEG	0	0	1*	1*	0	Scarry broadened liver capsule with few granulocytes and aggegates of foamy macrophages. No adhesion. Normal liver.

Table 10: Overview histological scoring. Two animals per group per time point were used. A median laparotomy was performed and the right medial lobe of the liver was exposed. A superficial circular lesion with a diameter of 2 cm and a depth of 1mm was created with scissors. The lesions were treated with Hemopatch 4arm-PEG-ester-isocyanate or Hemopatch NHS-PEG, respectively.

2.2.3 Immunohistochemistry

The results of the histological analysis of the isocyanate treated Hemopatch as described before indicated an acute inflammation during the whole period of wound healing. This conclusion was verified with the method of immunohistochemistry using a CD18 antibody. CD 18 recognizes leucocytes that can be identified by the use of the staining techniques as described in the method part (chapter 2.2.3). Leucocytes are localized by binding anti-CD18 antibodies and staining them red.

Unfortunately, the method was not successful for the other applied antibodies as the reaction failed in the application of CD3, CD8 and pi3K. However, CD18 showed binding capacities to the rabbit liver tissue.

Hemopatch Heavy Duty (Hemopatch HT4 – collagen patch coated with 4arm-PEG-isocyanate)

Timepoint 1 Day

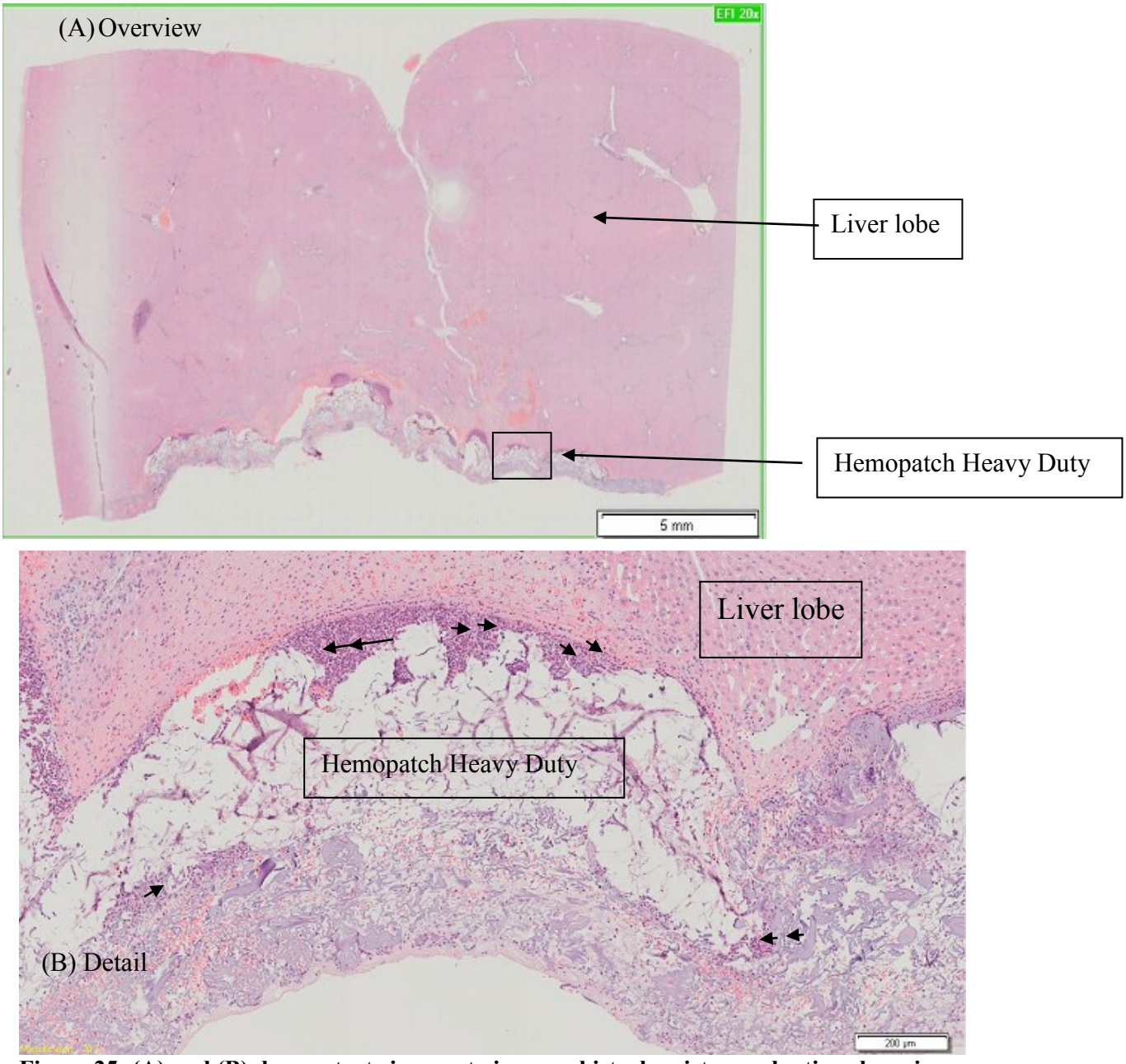


Figure 25: (A) and (B) demonstrate images to immunohistochemistry evaluations by using antibody CD18 of Hemopatch Heavy Duty (4arm-PEG-isocyanate coated collagen pad) at day 1. A strong adhesion area with aggregates of macrophages and seroma like space containing residua of patch material. CD18 showed good binding capacities to the rabbit liver tissue (red dyed cells).

Hemopatch Heavy Duty (Hemopatch HT8 – collagen patch coated with 8arm-PEG-isocyanate)

Timepoint 1 Day

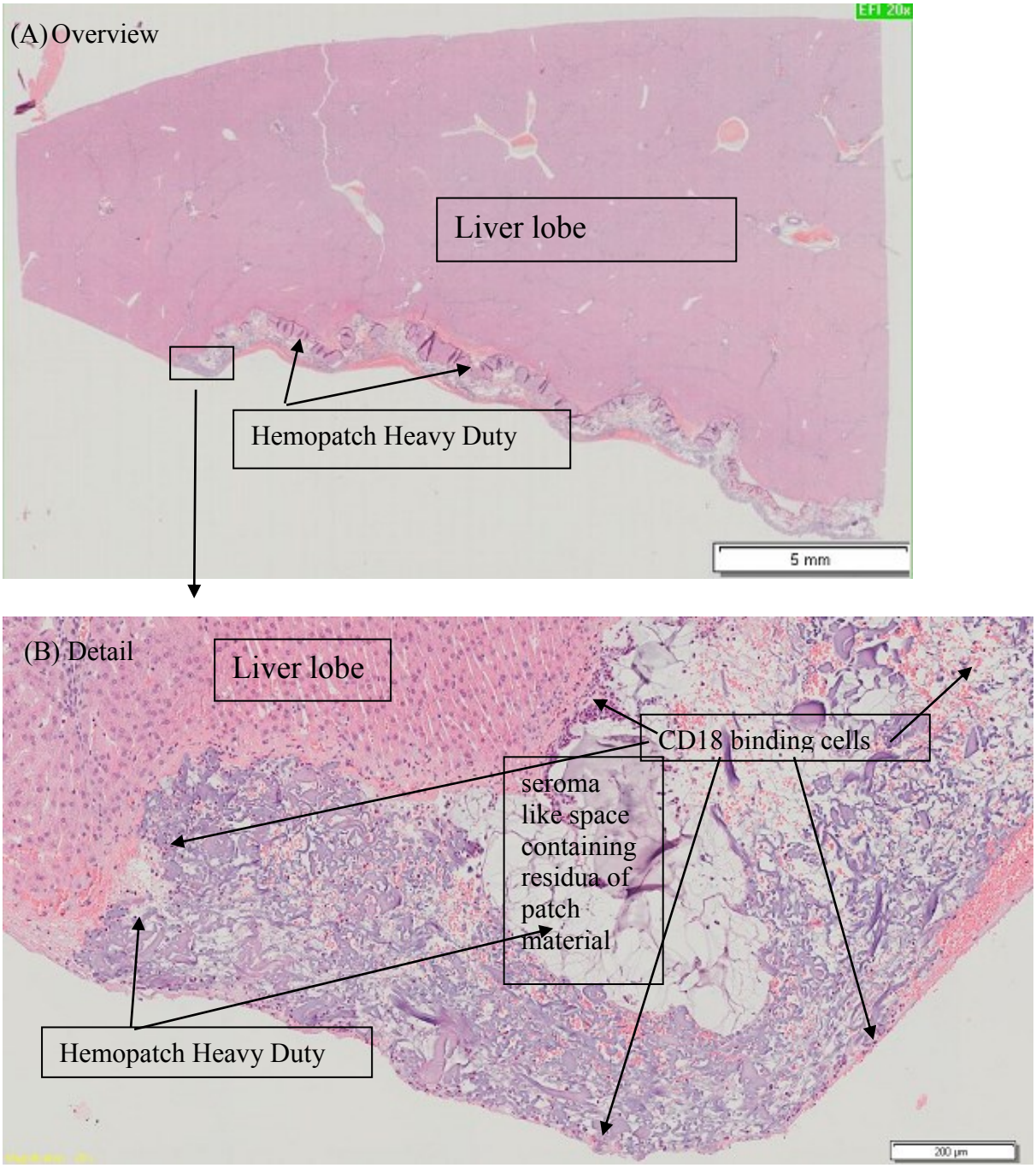


Figure 26: (A) and (B) demonstrate images to histology evaluations of Hemopatch Heavy Duty (Hemopatch HT8 – 8arm-PEG-isocyanate coated collagen patch) application site in situ at necropsy 1 day after surgery. A strong adhesion area with aggregates of macrophages and seroma like space containing residua of patch material occurred. CD18 binding leucocytes were stained in red.

Example for test item Hemopatch HT4 (collagen patch coated with 4arm-PEG-isocyanate) using CD18

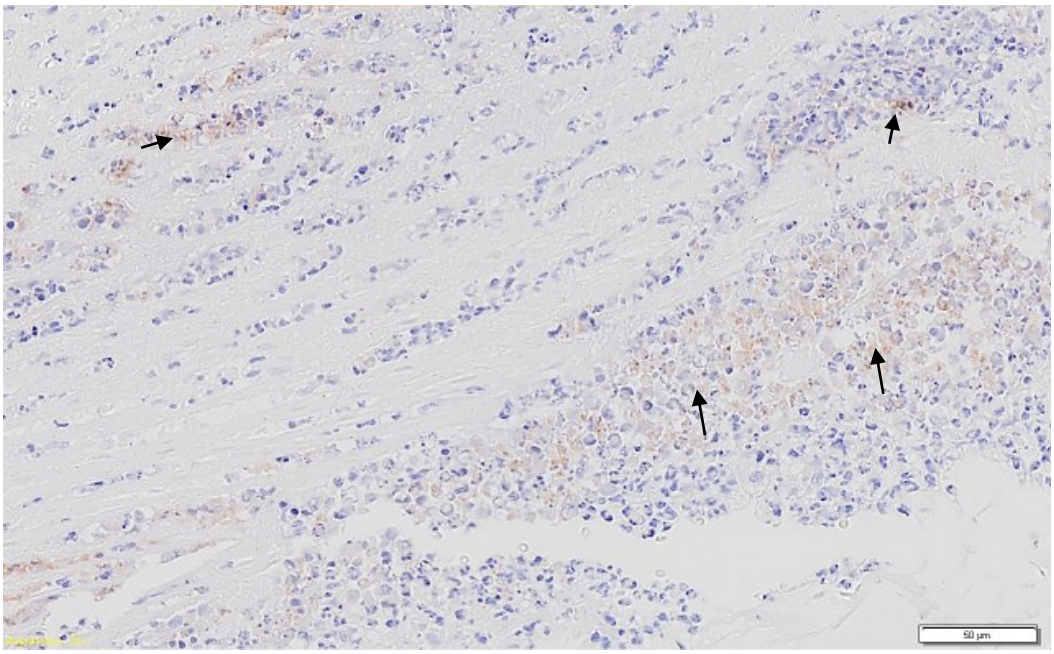


Figure 27: Immunohistochemistry: Evaluation of Hemopatch HT4 (4arm-PEG-isocyanate coated Hemopatch) application site in situ at necropsy 1 day after surgery. The arrows indicate CD18 bearing leukocytes.

Example for test item Hemopatch HT8 (collagen patch coated with 8arm-PEG-isocyanate) using CD18

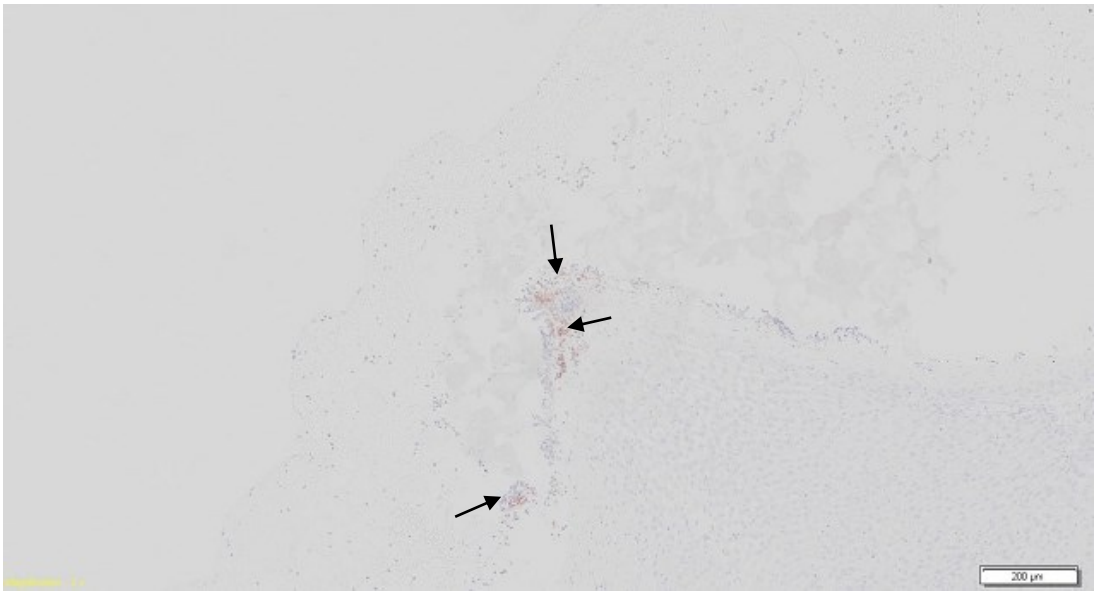


Figure 28: Immunohistological evaluation of Hemopatch HT8 (8arm-PEG-isocyanate coated Hemopatch) application site in situ at necropsy 1 day after surgery. The arrows indicate CD18 bounded leucocytes.

Example for test item Hemopatch HT4 (collagen patch coated with 4arm-PEG-isocyanate), using CD18 antibody

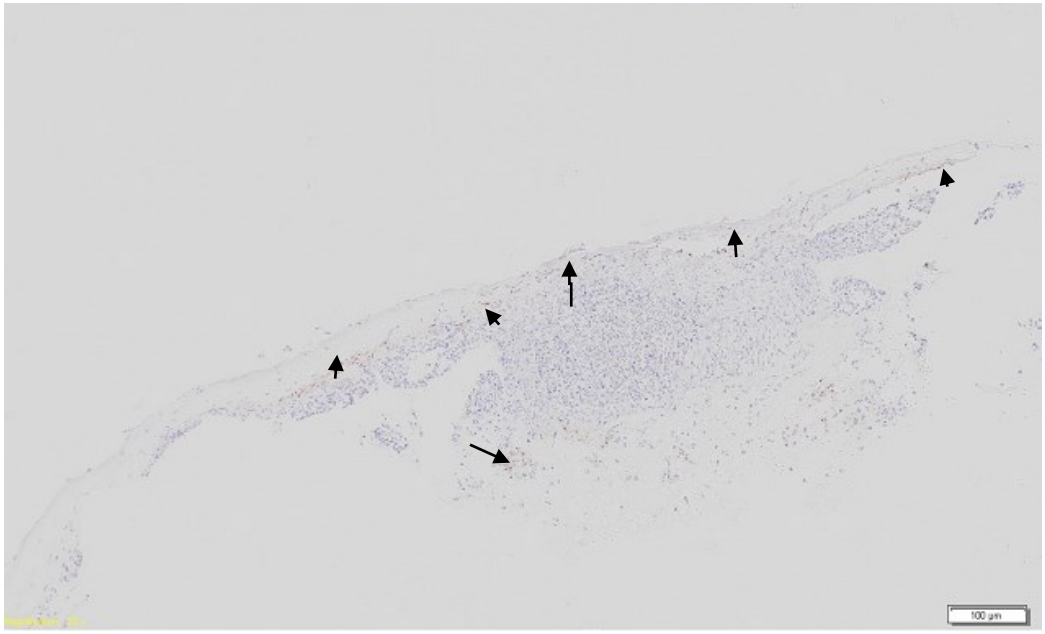


Figure 29: Immunohistological evaluation of Hemopatch HT4 (4arm –PEG-isocyanate coated Hemopatch) application site in situ at necropsy 2 days after surgery. The arrows indicate CD18 bounded leucocytes.

Example for test item Hemopatch HT8 (collagen pad coated with 8arm PEG-isocynanate), CD18 antibody

Figure 30: Immunohistological evaluation of Hemopatch HT4 (4arm-PEG-isocyanate coated Hemopatch) application site in situ at necropsy 2 days after surgery. The arrows indicate CD18 bound leucocytes.

DISCUSSION

The question about the stability of multiarm PEG-isocyanate and multiarm PEG-ester-isocyanate coated collagen patches (Hemopatch Heavy Duty) was investigated through analysis of the isocyanate raw material and through extraction techniques with acetone from the patch.

The FTIR and NMR methods proved to be suitable techniques for determining the isocyanate content of various multiarm polyethylene glycol raw materials and its hydrolysis over time. First, NMR allowed the evaluation of the powder's purity. Low contamination of 2-3%, and thus a purity of the original substitution degree of 97% could be established. In addition, the FTIR spectra recorded a significant band at 2270 cm^{-1}, which was reproducible throughout all measurements and suitable to identify multiarm-PEG-isocyanates as powder as well as extracted from collagen sponges. Furthermore, both methods allowed conclusions on the quantification of the degradation of PEG-isocyanate and PEG-ester-isocyanate over time. In general, isocyanates require dry conditions for stable constitutions [12]. Chemical modification occurs in the reaction of interacting functional groups and thus cross-linked compounds [13]. Our investigations proved first that isocyanate bonds are very reactive groups and, second, that they are quickly prone to hydrolysis. The FTIR results of the solid powder impressively showed that isocyanate groups change into carboxy groups and completely degrade and loose their activity within six days at room temperature and air moisture of 45%. This occurs even faster with 4arm-PEG-ester-isocyanate. The stability test of samples that were produced by coating of Matristypt with 10 mg/cm^2 of activated PEG showed that the material lost all or nearly all of its activity after three months of storage due to the presence of water and the transformation of isocyanate group into unstable carbamic acid derivatives [15].

Based on the results obtained from the chemical part of this diploma thesis, it can first be concluded that all of the PEG-isocyanate coated material had very low stability. Thus, at room temperature, 4arm-PEG-isocyanate coated collagen pads lost all their reactivity after 38 days, 8arm-PEG-isocyanate coated collagen pads after 14 days, and 4arm-PEG-ester-isocyanate containing an ester bond after just 6 days. Second, there was no great improvement of the stability at 4°C. After 76 days, a substitution degree of 4arm-PEG-isocyanate of 49% was determined and for 4arm-PEG-ester-isocyanate about 83%. Third, 4arm-PEG-ester-isocyanate powder in a solid state lost all its stability even after only 3 days. Finally, the storage of

Hemopatch Heavy Duty is an important stability factor. It is more stable at lower temperature. Nevertheless, samples that were stored in a well-sealed aluminum pouch with a desiccant inside lost all or nearly all of their reactivity after 3 months of storage.

The second question that raised our interest through this investigation was the influence of isocyanate-coated pads on surrounding tissue over time once applied.

Hemostatic performance of 4arm-PEG-ester-isocyanate coated patches, stored at +4°C and -20°C respectively, was tested in heparinized pigs. Hemopatch samples with a substitution degree of approximately 65% as well as Hemopatch coated with 4arm-PEG-ester-isocyanate samples with a high substitution degree (~ 97%) were applied to the liver or the spleen, the lung, gall bladder and the heart through the abrasion model or the spleen resection model. The results of the preclinical tests showed optimal hemostatic efficiency because of the fast and effective adhesion results without the formation of any hematoma. Once the pad was applied, it could not even be removed after 30 seconds so that it passed all the test methods without any complications. The application was performed with dry gauze because wet application showed no efficacy. An explanation for this observation is the strong reaction with water caused by the strong reactivity of the isocyanate groups with water. The fast uptake of fluids was an advantage when the bleeding was very strong or when the application was difficult, in the heart or gall bladder sealing model. In these cases, Hemopatch with a lower substitution degree was not able to stop the bleeding or to stick to the wound, but 4arm-PEG-ester-isocyanate coated Hemopatch closed the wound immediately after application.

These positive results from the preclinical tests raised our interest in the tissue compatibility of multiarm-PEG-isocyanate coated collagen pads in long-term studies. We therefore performed two prospective, randomized, preclinical studies in a rabbit liver lesion model. The aim of our investigation was to evaluate the hemostatic efficacy, absorption, histocompatibility, and biodegradation of PEG-isocyanate coated Hemopatch Heavy Duty in relation to NHS-PEG coated Hemopatch in vivo. The expectations for the outcome were to achieve rapid hemostasis and wound healing through the replacement of damaged cells with liver cells within 60 days.

The conclusion of the results of both studies was that PEG-isocyanate coated pads led to an acute inflammatory reaction, which resulted in a foreign body response (encapsulation) and the formation of a very strong adhesion formation of the liver lobe to the abdominal wall. The adhesion was formed shortly after application and resulted in a tight connection of tissue components. 4arm and 8arm-PEG-isocyanate coated pads formed an encapsulation of the

material that was barely resorbable. 4arm-PEG-isocyanate containing an ester bond led to a hole formation between the foreign body and the abdominal wall and could be resorbed within 60 days. However, the strong adhesion formation could not be removed.

In contrast wound healing by use of Hemopatch provided excellent results in contrast to isocyanate coated Hemopatch. Hemopatch coated with NHS-PEG offered positive properties due to an ideal hemostatic material. It was easy to apply in surgery, no thromboembolic complications occurred, and hemostasis could be achieved within minutes. Its biocompatibility was excellent, as it provided a rapid degradation with no pathological signs of tissue reaction. The wound healing process was rapidly initiated and followed the normal mechanisms of reparation and regeneration. Basically, the healing process defines the stepwise tissue healing that occurs from the bottom up or from outside to inside until the defect is closed and healed [36]. Similar wound healing also took place in the case of Hemopatch. Within the first 30 days, Hemopatch could stimulate an inflammatory reaction and replacement of cells. The wound healing process resulted in mild scar formation, but without any adhesion formation. After 60 days, the Hemopatch material was completely degraded and normal liver tissue established.

The histopathological results for Hemopatch Heavy Duty also showed an acute inflammatory reaction, including the formation of an amorphous mass with a huge number of macrophages inside and granulocytes enclosing a hole with a capsule inside. The foreign body is the result of PEG-isocyanate coated Hemopatch's hardly absorbable material. Every foreign body occurrence prevents wound healing, as the normal body response is modulated [37] due to inadequate oxygen supply to single wound parts. A delay in the wound healing process thus follows [36]. In the case of PEG-isocyanate coated Hemopatch, this effect is shown within the first 30 days, when the material reacts with protein adsorption onto the biomaterial surface following the constitution of a blood-based provisional matrix surrounding the implanted biomaterial [32]. In addition, a strong adhesion area with aggregates of foamy macrophages and a seroma-like cavity with residuals of implant material between the fat tissue and liver lobe was evaluated. This effect was recorded until the end of the studies (60 days). It is assumed that the adhesion would probably have been dissolved in future. Nevertheless, it could be concluded that PEG-isocyanate coated Hemopatch showed bad results in its degradability including enormous inflammation occurrence, which does not make it suitable as a useful and efficient biomaterial.

Furthermore, investigations using anti-CD18 antibodies proved that CD18 is present within 30 days for both materials due to the leukocyte accumulation in the inflammatory reaction. However, with Hemopatch CD18 sufficiently decreased over time and final slides of the endpoint (60 days) showed an intact liver surface due to the nearly complete replacement of damaged cells by liver cells. On the other hand, isocyanate-coated Hemopatch showed positive results for CD18 over the whole study period. This means that wound healing was difficult to achieve and the hardly absorbable material led to intensive inflammation/adhesion resulting in continuous detection of CD18. In addition, the foreign body reaction led to the formation of a giant hole and leucocytes could be found on its surface. The conclusion of histopathology and immunohistochemistry with regards to PEG-isocyanate coated Hemopatch proved that Hemopatch Heavy Duty has failed with regard to biodegradability.

The aim of this thesis was to develop a more effective and efficient hemostatic agent to Hemopatch and to widen the application field in surgery. There is a wide range of positive properties of Hemopatch Heavy Duty. PEG-isocyanate based Hemopatch has a shorter application time with a bleeding time of less than 30 seconds in comparison to NHS-PEG-based Hemopatch, with a bleeding time of 1-2 minutes. The adhesive strength to the tissue of the pads based on PEG-isocyanate is higher compared to NHS-PEG pads and PEG-ester-isocyanate based collagen sponges showed adhesiveness to all kinds of tested tissue, including tissues where NHS-PEG-coated collagen pads failed (e.g. gall bladder). In such cases, Hemopatch Heavy Duty has shown to have great potential as a biomaterial and worthy of further investigation. Nevertheless, it needs to be improved due to adhesion formation, foreign body response, and its instability.

ABBREVIATIONS

4arm PEG-NCO	4arm-PEG- isocyanate (pentaerythritol), MW 10000
8arm PEG-NCO	8arm-PEG- isocyanate (tripentaerythritol), MW 10000
4 arm PEG-E-NCO	4arm-PEG-ester-isocyanate (pentaerythritol), MW 10000
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATR-FTIR	Attenuated total reflection- Fourier transform infrared spectroscopy
BrdU	Bromdesoxyuridin
CD3	Cluster of Differentiation, receptor 3
CD8	Cluster of differentiation, receptor 8
CD18	Cluster of differentiation, receptor 18
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
ENCO	Ester-isocyanate
FTIR	Fourier-transform infrared spectroscopy
GDP	Guanosindiphosphat
G.I.	Growth inhibition
GTP	Guanosintriphosphat
H_2SO_4	Sulphuric acid
HAS	Human serum albumin
HBSS	Hank's Balanced Salt Solution
HP	Hemopatch
H&E	Hematoxylin and Eosin (H&E)
Hemopatch HT4	Hemopatch Heavy Thin (or Heavy Duty), Bovine collagen pad coated with 4arm PEG, MW 10000
Hemopatch HT8	Hemopatch Heavy Thin (or Heavy Duty), Bovine collagen pad coated with 8arm PEG, MW 10000
Hemopatch HT-4E	Hemopatch Heavy Thin (or Heavy Duty), Bovine collagen pad coated with 4arm PEG-ENCO
IU	International Units

IV	Intravenous
NaOH	Sodium hydroxide
NCO	Isocyanate group (MW 10 000)
NCS	Newborn Calf Serum
NHS	N-Hydroxysuccinimide
NHS-PEG	Pentaerythritol polyethylene glycol ether tetra-succinimidyl glutarate
PBS	Phosphate-Buffered Saline
PEG	Polyethylenglycol
Pi3K	Phosphoinositid-3-Kinase
POD	Peroxidase
SQ	Subcutaneous
TBS	Tris wash buffer (20x),
TCM	Tissue culture medium
T-EDTA	Ethylenediaminetetraacetic acid
TMB	Tetramethyl-benzidine
WFI	Water for injection
ZNSE-ATR-crystal	Zinc selenide attenuated total reflection crystal

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ZUSAMMENFASSUNG

Das Ziel dieser Arbeit war es Hemopatch zu verbessern und ein effektiveres und effizienteres blutstillendes Mittel zu entwickeln. Dies sollte dadurch erreicht werden, dass das Kollagenpatch mit einer mehrarmigen hochreaktiven PEG-Isocyanatoberfläche beschichtet wurde. Damit sollte es als Hämostat für starke Blutungen beziehungsweise an schwer zugänglichen Organen anwendbar werden. Daher war das Hauptaugenmerk dieser Arbeit Fragen zur Hemopatch Stabilität, Adhäsion an Organen während der Operation und seiner biologischen Abbaubarkeit/Biokompatibilität. *Methoden:* Die Stabilität von Isocyanat- bzw. NHS-PEG wurde mittels NMR und FTIR analysiert. Gewebeadhäsion wurde in präklinischen Hämostasemodellen getestet, die in heparinisierten Schweinen an verschiedenen Organen durchgeführt wurden. Die Abbaubarkeit und der Einfluss auf das umliegende Gewebe wurde in zwei prospektiven, randomisierten, präklinischen Studien an einer Kaninchenleberläsion histopathologisch untersucht. *Ergebnisse:* Die Stabilitätsstudien zeigten, dass das Material nach einer dreimonatigen Lagerung aufgrund der Anwesenheit von Wasser und der Umwandlung der Isocyanatgruppe in instabile Carbaminsäurederivate seine Aktivität fast vollständig verlor. Die Ergebnisse der präklinischen Tests zeigten, dass zwar eine optimale hämostatische Effizienz aufgrund der schnellen und effektiven Adhäsion ans Gewebe ohne jegliche Bildung von Hämatomen erreicht werden konnte, aber die darauffolgenden präklinischen Studien belegten, dass PEG-Isocyanat-beschichtete Patches zu einer akuten Entzündungsreaktion, einer Fremdkörperreaktion (Verkapselung) und der Bildung einer sehr starken Adhäsion des Leberlappens an der Bauchdecke führten. Die histopathologischen Ergebnisse für Hemopatch Heavy Duty zeigten weiters die Bildung einer amorphen Masse mit einer großen Anzahl von Makrophagen und Granulozyten, die in einer Kapsel eingeschlossen waren.

Fazit: Es gibt eine Vielzahl positiver Eigenschaften von Hemopatch Heavy Duty. PEG-Isocyanat basierendes Hemopatch hat eine kürzere Applikationszeit mit einer Blutungszeit von weniger als 30 Sekunden im Vergleich zu NHS-PEG-basiertem Hemopatch mit einer Blutungszeit von 1-2 Minuten. Die Haftfestigkeit an das Gewebe der Patches auf der Basis von PEG-Isocyanat ist höher im Vergleich zu NHS-PEG-, und PEG-Isocyanat-basierte Kollagenschwämme zeigten auch eine hohe Haftfähigkeit an Organen, bei denen NHS-PEG-beschichtete Kollagenpatches versagten. Hemopatch Heavy Duty hätte vielleicht großes Potenzial als Biomaterial in der Chirurgie, jedoch sind weitere Verbesserungen hinsichtlich seiner Instabilität, Adhäsionsbildung und Fremdkörperreaktion erforderlich.