COMPARATIVE STUDY OF THE COMPETITIVE COMMERCIAL FIBRIN SEALANTS TISSEEL/TISSUCOL (BAXTER), BERIPLAST (AVENTIS BEHRING) AND QUIXIL/CROSSEAL (OMRIX)

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Topic: KK/03.09.16

Keywords: Fibrin sealant; competitive fibrin sealants; Tisseel; Beriplast; Quixil; Crosseal; clot structure; SEM; adhesive strength; tensile strength; fibrin alpha chain cross-linking; FXIII; composition; Fibrinkleber; Konkurrenzprodukte; Reißfestigkeit; Klebefestigkeit; Zusammensetzung

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1 SUMMARY

The present study contains a comprehensive comparative analysis of the main competitive fibrin sealants on the market: Tisseel (Baxter), Beriplast (Avensis-Behring) and Quixil/Crosseal (Omrix). It turned out, after weighing all the advantages and drawbacks of the different commercial fibrin sealants, that Tisseel is the most versatile and reliable product. It is the only product on the market giving physiological fibrin clots (with a demonstrated cell compatibility and supporting wound healing properties). Clots derived from Tisseel have demonstrated highest inner tensile strength. The adhesive strength after gluing (rat skin model) was significant higher after use of Tisseel and Beriplast (which are comparable in this test) compared to gluing by Quixil/Crosseal. Composition analysis revealed that Quixil and Beriplast were develoved focusing on the ease of application (fast dissolution for Beriplast or prolonged storage stability at refrigerator temperature for Quixil). However, Quixil/Crosseal and Beriplast are suited only for the present main applications of hemostasis and sealing and, due to their cytotoxicity, not convenient for ongoing applications such as tissue regeneration and drug delivery. For all these applications Tisseel is an ideal product. Due to the high tranexamic acid content of Quixil/Crosseal its use is contra-indicated in neurosurgery. Further, the deep frozen (Duo) presentation of Tisseel compensate in a large extend the “ease of application” advantages of Quixil/Crosseal and Beriplast,allowing the application of the product in comparable time and with a similar preparation effort.

2 CONTENT

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3 INTRODUCTION

Fibrin sealants mimic the last and most important step in hemostasis, the conversion of blood-fibrinogen into fibrin and the formation of the fibrin network, i.e. the fibrin clot. The conversion of fibrinogen to fibrin is catalyzed by the enzyme thrombin. Therefore, each fibrin sealant contains two components, the sealer protein component (containing fibrinogen) and the thrombin component. These components are applied with the aid of a double syringe, each syringe containing one component. Mixing of the components is carried out during application by simultaneously expelling the contents of the two syringes into a Y-shaped connector. The conversion of fibrinogen to fibrin starts immediately after mixing in the connector and ends with the formation of a fibrin clot at the site of application. Due to the biochemical and mechanical properties of the fibrin clot, fibrin sealants are used for hemostasis, sealing and gluing.

Thrombin catalyzes the cleavage of fibrinopeptides A and B from fibrinogen resulting in the formation of fibrin monomers. Fibrin monomers aggregate electrostatically forming fibrin fibers. The mesh of these fibers represents the "soft clot". The fibers of the soft clot are cross-linked in a slower reaction catalyzed by activated factor XIII (FXIII) resulting in a "hard clot". The activation of FXIII is catalyzed by thrombin and requires the presence of Ca^{2+} ions. A crosslinked hard clot has elastic properties (can be elongated but returns to its initial shape if no force is further applied, like rubber), whereas a soft clot is not elastic at all (can be elongated but remains stretched if no force is further applied, like chewing-gum). A cross-linked fibrin clot has a higher inner tensile strength than a soft clot. Clot elasticity and robustness not only depend on FXIIIa crosslinking, but also on the architecture of the fibrin clot (i.e. the thickness of fibrin fibers and the density of the fiber network) and on fibrin concentration. Further, the architecture of the fibrin clot is influenced by the concentration of certain excipients (e.g. salts, chaotropic agents) during clot formation, i.e. the composition of the fibrin sealant components. White elastic "coarse clots" built by thick fibrin fibers are formed at physiological salt concentration but gelatine-like transparent, brittle "fine clots", composed of thin fibrin fibers, are the result of an non-physiological environment (high salt concentration and/or pH values).
Because commercial fibrin sealants differ substantially in their composition different properties, which probably influence their efficacy, can be expected.

Baxter offers two variants of fibrin sealant, which differ by their FXIII-concentration: Tisseel/Tissucol, further called Tisseel (EU), with 10-15 IU FXIII, is available in the most countries. The second variant, Tisseel VH, further called Tisseel (US), with less than 1 IU FXIII, is approved and distributed only in the US, UK and Puerto Rico. Tisseel (EU) is offered in a freeze-dried (Kit) as well as in a deep frozen (Duo) presentation. The latter consists of both components filled into two syringes and mounted ready for application. Tisseel (US) is only available in the Kit presentation. Beriplast (Aventis-Behring) is offered only as freeze-dried presentation in vials and Quixil (Omrix) as frozen presentation in vials. Crosseal is identical in all aspects to Quixil.

4 MATERIALS

4.1 TEST MATERIALS

Chemicals and solutions
The chemicals and solutions used in this study are prepared according R&D Biosurgery prescriptions and listed in Book 2 of Margarete Szekely.

Test materials
The fibrin sealants Tisseel VH STIM3 1 ml Kit (EU), Tisseel VH STIM4 1 ml Kit (US), Beriplast (Aventis Behring), Quixil/Crosseal (Omrix) were purchased, stored and reconstituted according to directions from manufacturer. The following lots were used for this study.

Table 3: Tisseel STIM3 EU 1ml Kit

Lot	Expire date	Reconstitution	Tests with date
K00403A	11.2004	21.10.03	α-Kinetics, BCA Fibrinogen, pH and conductivity (21.10.03), BCA HSA (28.10.03)
K00403A	11.2004	18.11.03	α-Kinetics (18.11.03), SDS-PAGE, Laemmli Thrombin component (20.11.03)
K00403A	11.2004	27.11.03	Clot for electron microscopy (27.11.03), FXII-Test (09.12.03)
K00403A	11.2004	07.01.04	U-SDS Electrophoreses (5 % AA) (21.01.04), BCA Fibrinogen (26.01.04)
K00403A	11.2004	29.03.04	U-SDS Electrophoreses (5 % AA) (24.09.04)

The Tisseel STIM3 EU Kit consists of a sealer protein- and a thrombin component, both lyophilized. The components are prepared by dissolving with aprotinin-, respectively with calcium chloride solution. The Kit K00403A contains Tisseel 08P0103A (Exp. Date 12.2004), Thrombin 78H3202L (Exp. Date 11.2004), aprotinin 76B0702GA (Exp. Date 07.2005) and calcium chloride 810202AA (Exp. Date 01.2007)

Table 4: Tisseel STIM4 US 1ml Kit

Lot	Expire date	Reconstitution	Tests with date
Not available	29.02.04	18.11.03	α-Kinetics (18.11.03), Laemmli Thrombin component (20.11.03)
Not available	29.02.04	27.11.03	Clot for electron microscopy (27.11.03), FXII-Test (09.12.03)
Not available	29.02.04	03.12.03	α-Kinetics with adding of FXIII (03.12.03), U-SDS Electrophoreses (5 % AA) (21.01.04), BCA Fibrinogen (26.01.04)
Not available	29.02.04	16.03.04	U-SDS Electrophoreses (5 % AA) (24.09.04)

The Tisseel STIM3 EU Kit consists of a sealer protein- and a thrombin component, both lyophilized. The components are prepared by dissolving with aprotinin-, respectively with calcium chloride solution. The vials were delivered separately in bags for research only. The four components were individually combined; they did not fit to any kit lot number. Fibrin sealant 08P7402K (Exp. Date 31.10.04), thrombin 78H2203H (Exp. Date 31.07.2005), aprotinin 76B0401C (Exp. Date 29.02.04) and calcium chloride 811102J (Exp. Date 30.09.07) were used.

Table 5: Beriplast 1ml Kit

Lot	Expire date	Reconstitution	Tests with date
604242A	01.2005	21.10.03	α-Kinetics, BCA Fibrinogen, pH and conductivity (21.10.03), BCA HSA (28.10.03), Laemmli Thrombin component (20.11.03), FXII-Test (09.12.03), BCA Fibrinogen (26.01.04)
604242A	01.2005	27.11.03	Clot for electron microscopy (27.11.03), AA-Determination, Plasminogen (Elisa, Herr Kren) (21.01.04), U-SDS Electrophoreses (5 % AA) (21.01.04),
604242A	01.2005	16.03.04	U-SDS Electrophoreses (5 % AA) (24.09.04),

The Beriplast Kit consists of a sealer protein- and a thrombin component, both lyophilized. The components are prepared by dissolving with aprotinin-, respectively with calcium chloride solution. The Kit 604242A contains sealer protein 25663614 (Exp. Date 11.2005) and thrombin 33460511 (Exp. Date 01.2005)

Table 6: Quixil 1ml Kit

Lot Kit	Expire date	Thawing	Tests with date
F51332	10.2003	21.10.03	α-Kinetics, BCA Fibrinogen, pH and conductivity (21.10.03), BCA HSA (28.10.03), Laemmli Thrombin component (20.11.03)
G26112C	04.2004	27.11.03	Clot for electron microscopy (27.11.03), Acetate-Test (10.12.03)
G26112C	04.2004	03.12.03	α-Kinetics with adding of FXIII (03.12.03), FXII-Test (09.12.03), U-SDS Electrophoreses (5 % AA) (21.01.04), Na^+ determination QC (14.01.04), AS determination QC (20.01.03)
G26112C	04.2004	21.01.04	Plasminogen (Elisa, Herr Kren) (21.01.04), BCA Fibrinogen (26.01.04), U-SDS Electrophoreses (5 % AA) (24.09.04)

The Quixil Kit consists of a frozen BAC (sealer protein) and thrombin component. The Kit F51332 contains BAC F46152 and thrombin F47122 both with expire date 10.2003. The Kit G26112C contains BAC G24072 (Exp. Date 05.2004) and Thrombin G22062 (Exp. Date 04.2004).

Lots used for tensile strength testing:

Tisseel kit with FXIII (Tisseel EU)
TS.LYO, 2ml Lot 08P4301F
Aprotinin Lot 76B1200JB
Thrombin 4IU, 2 ml Lot 78H0501B
$CaCl_2$ (40 mmol) Lot 810401CA

Tisseel (US)
TS.LYO BAY.St4 ; USA 1ml Lot 08P4300E
Aprotinin Lot 76B0899K
Thrombin 4IU, 1 ml Lot 78H3401I
$CaCl_2$ (40 mmol) lot 810400E

Beriplast P-Combi- Set
Fibrinogen, 1ml Lot 24063611
Aprotinin Lot 02819711
Thrombin, 500 IU Lot 33260511 (diluted to 4 IU/ml)
$CaCl_2$ (40 mmol) lot 210011

Quixil
BAC, 1 ml Lot F46152 ;G24072
Thrombin, 1000 IU Lot 22062 (diluted to 4 IU/ml)

5 METHODS

Preparation of the syringes for application

All products were prepared for application according the instruction leaflet/package insert. The necessary time for preparation was observed and compared with the company instructions. Any remarkable occurrences during preparation were noted as well.

Conductivity and pH

The conductivity and the pH of both components of fibrin sealants were measured. The samples were diluted 1:10 with bi-distilled water. The same test solution was used for measuring first the pH and afterwards the conductivity.

Determination of protein content

The protein concentrations of both components were determined by using BCA protein assay kit according to the protocol “BCA-Test, version Oct.2003” (R&D BioSurgery). Fibrinogen (DS050501F) was used as standard protein for the sealer protein component and human serum albumin (St. 03, DP, book 1, pg. 29) for the thrombin component. The appropriated diluted standards (1,6- 0,05 mg/ml Protein) and the samples (in TBS buffer) are placed in the wells of a 96-well microtiter plate. After adding of the premixed reagent (Pierce) and incubating for 30 minutes at 37 °C the optical density is measured at 550 nm using an EL 808 ELISA reader (BIO-TEK, Winooski, VT)

Determination of FXIII Content

The concentration of FXIII in the sealer protein component was determined according to the prescription “FXIII-Test, enzymatic, optical Test with Casein/GIDH version 01/95” (R&D Biosurgery). The FXIII activity was assayed by monitoring the oxidation of NADH at 340 nm. Glycine ethyl ester and β-Casein are used as substrate in the absence of fibrinogen. The substrates are converted to peptide-glycylethyl ester and ammonium by FXIII, which is activated by Thrombin and Ca^{2+}. In the successive NADH dependent reaction, α-ketoglutarate is reduced to glutamate in the presence of ammonium by glutamate dehydrogenase. The FXIII activity refers to the consumption of NADH.

Determination of acetate content

The concentration of acetate in the Thrombin component of Quixil was determined according to the SOP BM4631.02 (QCAS-PA-CH/PSN). This enzymatic, spectrophotometric test is based on the formation of $NADH+H^+$, measured at 340 nm. Briefly, acetate is converted to acetyl-CoA in the presence of ATP and CoA by acetyl-CoA synthetase. In the successive reaction, acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthase. The oxaloacetate required for the previous reaction is formed from L-malate in a L-malate dehydrogenase-catalyzed reaction where also reduced $NADH+H^+$ results.

Discontinuous SDS Electrophoresis

The protein composition of the thrombin component was analyzed according to Laemmli by discontinuous SDS/PAGE (5 % stacking gel and 12,5 % separation gel) using a minigel-twin electrophoresis apparatus (Biometra, Germany). Furlan (Coomassie Brilliant Blue R250) staining of protein bands was performed. Samples were diluted with 0,9 % NaCl solution to a

concentration of approx. 0,8 mg/ml. 10 µl of sample were mixed with 10 µl sample buffer and heated at 70 °C for 10 minutes. 20µl of each sample was applied on the gel. The gel was run according to Biometra Minigel-Twin Manual (Oct 1994). Immediately afterwards the gel was stained and destained according “SDS/PAGE: Staining with Coomassie-Blue R250 after M. Furlan (Version 07/97/SE)” (R&D Biosurgery).

Continuous urea/SDS electrophoresis

The protein composition of the sealer protein components were analyzed by UREA/SDS electrophoreses (5 % acryl amid separation gel) on a DESAGA electrophoreses system (Sarstedt-Gruppe). Furlan (Coomassie Brilliant Blue R250) staining of protein bands was performed. The reduced and non-reduced samples were prepared and the gel was run according “Urea/SDS Electrophoresis continues 5% polyacryl amid for DESAGA system (Version 01/91)” (R&D Biosurgery). Staining and destaining was performed according “SDS/PAGE: Staining with Coomassie-Blue R250 after M. Furlan (Version 07/97/SE)” (R&D Biosurgery). The amounts of α-chain, β-chain, γ-chain, fibronectin and albumin were determined by densitometry according to “Determination of fibrin fractions by densitometry after U/SDS electrophoreses under non-reduced and reduced conditions” (R&D Biosurgery). On the lanes of the reduced samples the peaks of fibronectin, α-chain, β-chain + albumin monomer and γ-chain were determined. On the lanes of the non-reduced samples the peak of albumin monomer is determined. For densitometry the edges of the gel pockets are excluded from quantification. In the case of the non-reduced lane the edge of the gel pocked include the fibronectin peak. Therefore it is necessary to correct the amount of albumin read out from the non-reduced lane. The β-chain is calculated by subtracting the real albumin amount (non reduced lane) from the β-chain + albumin peak (reduced lane). The other components can be read out directly.

Fibrin α-chain cross-linking

The fibrin sealants Tisseel VH STIM3 1 ml Kit (EU), Tisseel VH STIM4 1 ml Kit (US), Beriplast (Aventis Behring), Quixil/Crosseal (Omrix) were reconstituted according to the manufacturer. Analysis was performed according to according the protocol “Kinetic of fibrin-α-chain cross linkage (R&D BioSurgery, version 07/94)”. After mixing the fibrinogen and thrombin component in the ratio 1:1, the mixture was incubated at 37°C. After a reaction time of 0, 15, 30, 60 and 120 min, the reaction was stopped by addition of a denaturant sample buffer and heating at 70°C for 10 min. The clots were left overnight for dissolution in the sample buffer at 37°C. 15 µg protein/lane were loaded under reducing conditions on a homogeneous 5 % polyacrylamide/ureea electrophoresis-gel. Electrophoresis, staining and destaining were carried out as described above. The degree of α-chain cross-linking was analyzed quantifying the reduction of the α-chain-band in comparison to the band containing the fibrin-ß-chain and albumin (the latter two not affected by factor XIII).

Electron microscopy

From each fibrin sealant, using the original device of each manufacturer, a clot was prepared within the lid of a 1,5 ml Eppendorf tube. After one-hour incubation at room temperature the clot with the lid was transferred into a fixation solution (0,088 mol/l sodium cacodylate, 3 % glutare aldehyde, 20 g/l glucose). After fixation, the clot was sent to the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology/Vienna for electron microscopy investigation.

Determination of Plasminogen (ELISA)

The determination of plasminogen was carried out by R. Kren, department A. Weber (Product & Process Development), according to KVAAGLP.14

Amino acid determination

The concentrations of amino acids and tAMCHA in Quixil (Crosseal) were determined by the QC-department (Baxter AG, Vienna) by amino acid analysis using HPLC according to SOP BM4015, adapted for the specific analysis of Quixil. All components were identified by their retention times and quantified using calibration with the pure substances at known concentrations.

Na^+ and Ca^{2+} determination

Na^+ and Ca^{2+} concentrations of the thrombin component of Quixil were determined by the QC-department (Baxter AG, Vienna) using inductively coupled plasma optical emission spectroscopy (ICP-OES).

Inner tensile strength and clot elasticity

These experiments were carried out in the BioSurgery/Preclinicals department. First a butterfly-shaped clot was cast in a corresponding latex-mould (figure 7 and 8). For this the original sealer protein components of the competitive fibrin sealants were used. Because only low thrombin concentrations allow the casting of homogeneous fibrin clots into the mold, in the case of Tisseel the commercial 4 IU/ml thrombin (component of Tisseel-kit and Duo 4) was used for casting the clots. The thrombin components of Beriplast (500 IU/ml) and Quixil (1000 IU/ml) were diluted with a specific, adequate dilution buffer to 5 IU/ml. The specific composition of these thrombin dilution buffers ensures that the salt and additives concentrations (that could influence the properties of the clots) remain unchanged compared to the clots obtained with the original Quixil- and Beriplast-thrombin solutions.
Quixil thrombin component dilution-buffer: 0.6% human serum albumin, 2 % mannitol, 20 mM Na-acetate, 5.9 mg/ml $CaCl_2 \cdot H_2O$, 77 mM NaCl, pH 6.7. Beriplast thrombin component dilution-buffer: 4.8 mg/ml NaCl, 3 mg/ml Na_3-citrate$\cdot 2H_2O$, 5.9 mg/ml $CaCl_2 \cdot 2H_2O$, pH 6.13. After casting, the clots were incubated for 30 min at 37°C in a moist chamber and subsequently tested.
The measurements of maximum inner tensile strength and elongation at clot rupture were performed with an Istron-machine. The butterfly-shaped clot was clamped over its broad edges between the two arms of the machine. The clot was stretched with 5 mm/min. The maximum applied force and the elongation at clot rupture were recorded.

Rat skin adhesive strength

Two strips of excised, cleaned rat skin from male Wistar rats (400-600g) were cut to a size of 3 x 1 cm. These skin strips were glued together with 100 µl fibrin glue, of which the thrombin component was diluted to 4 IU/ml using the specific dilution buffers described previously. The overlapping area was 1 cm^2. The overlapping area was pressed together using a 50 g weight for 30 seconds, and then the whole assembly was incubated for 30 min at 37°C in a humid incubator.
Following this sample preparation, the skin strips were clamped at their respective free ends, and the breaking strength under tensile load was measured on a custom-built tensile tester at 0.3 mm/sec. 12 repeats were carried out for each data point, avoiding to use one rat for more than two data points for each measurement to minimize individual differences between rats

6 RESULTS AND DISCUSSION

6.1 EASE OF APPLICATION

For comparability the 1 ml package size (1 ml sealer protein + 1 ml thrombin) have been used in this study for all tested commercial fibrin sealants. All products were reconstituted and applied according to the enclosed instruction leaflets. New device developments (e.g. Baxter´s EasyPrep) have not been used. Always the fastest method of preparation was used in case different options were offered in the instructions.
The preparation time of each fibrin sealant, starting from taking the sample out of the freezer / refrigerator until the sealant is ready for application, was examined. The time needed to complete the task differed significantly for the tested products (figure 1).
Tisseel / Tissucol frozen formulation was prepared in approximately 12 minutes, which is the shortest preparation time of all tested products. As it is a frozen and not a freeze-dried product, it does not have to be preheated. After thawing, the pre-filled syringes have only to be unwrapped.

Quixil is a frozen product as well. However, after thawing of the solutions in the vials, they have to be transferred air bubble free into the syringes. For this Omrix provides a quite convenient system, so the transfer needs only a couple of minutes. However, this additional step is prone to handling mistakes that could compromise a successful application. Further, the preparation device needs bigger packages, more storage space and causes more litter in comparison to the other products.

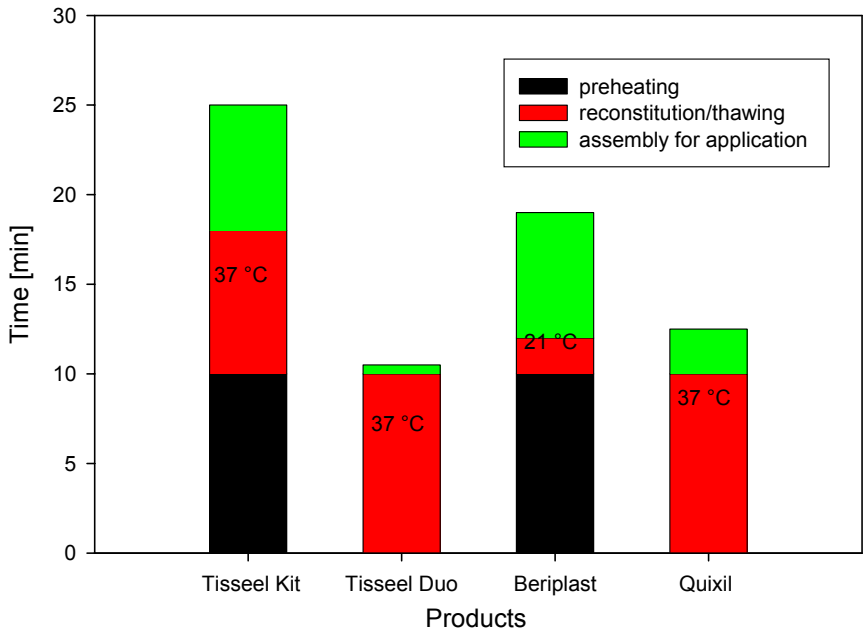

Figure 1: Preparation time of the fibrin sealants Tisseel Kit and Duo from Baxter, Beriplast (Aventis Behring) and Quixil (Omrix), from taking the samples out of the freezer / refrigerator until they are ready for application.

Tisseel Kit has the longest preparation time of approximately 25 minutes. As it is a freeze-dried product stored in the refrigerator, vials have to be preheated to 37 °C before

reconstitution. Due to the physiological low salt content, the dissolution of the sealer protein concentrate is slow and is speeded up by carrying it out at 37°C in the Fibrinotherm (a combined heating and stirring device). The air bubble-free transfer of the solutions into the syringes takes quite a lot of praxis as there are exactly 1 ml in the vials. The assembly of the filled syringes in the Duploject also takes a couple of minutes.
In comparison, the fibrin sealant Beriplast, which is also purchased as a freeze-dried product, needs approximately 20 minutes for preparation. The timesaving compared with the Tisseel Kit comes from an easier transfer of the aprotinin and $CaCl_2$ solutions to the vials containing sealer protein and thrombin and the faster dissolution of the sealer protein component. Whereas in the case of Tisseel Kit the transfer of the solutions is carried out with syringes and needles, the Beriplast kit contains two pairs of vials, each pair in a sterile bag. One pair contains the sealer protein lyophilizate and the aprotinin solution, the other the thrombin lyophilizate and the $CaCl_2$ solution. The two vials in a bag are placed plug-to-plug one over the other. By pushing the two vials through the unopened bag one against the other, the barrier between the vials is penetrated by a spike and the solution transferred into the vial containing the lyophilizate. The dissolution of both components is carried out at room temperature within 2 minutes. The much faster dissolution time of the sealer protein in Beriplast Kit compared to Tisseel Kit is achieved by the high salt- and arginine concentration in Beriplast. These ingredients, on the other hand, are responsible for the non-physiological clot structure and the cytotoxicity of Beriplast. After dissolution the transfer of the solutions into the syringes is carried out in a similar way to the Tisseel Kit. However the vials are filled with a little bit more than 1 ml, which allows an easier air-bubble-free transfer.

6.2 STABILITY IN THE READY TO USE FORM

The following stability data are extracted from the product leaflets according to the manufacturers and are summarized in figure 2.
As the Tisseel Duo and Quixil are frozen products they have to be stored at –20 °C. However, after thawing Quixil can be stored in the refrigerator up to 30 days and is ready to use only in a couple of minutes. Tisseel Duo can only be used within 36 h (in some coutries 48 h) after thawing, if kept at room temperature and inside the sterile plastic cover. Both freeze-dried preparations, Tisseel Kit and Beriplast, have to be stored in a refrigerator. After reconstitution Beriplast can be kept for 24 h at room temperature, however the Tisseel Kit only for 4 hours. In the case of Tisseel Kit, during its reconstitution, the sterility barrier is destroyed. Resulting sterility issues are the cause of the comparable short storage periods after reconstitution. The preparation system of Beriplast enables the reconstitution and storage under sterile conditions (protection through the sterile bag) and longer storage times in the ready-to-use form.

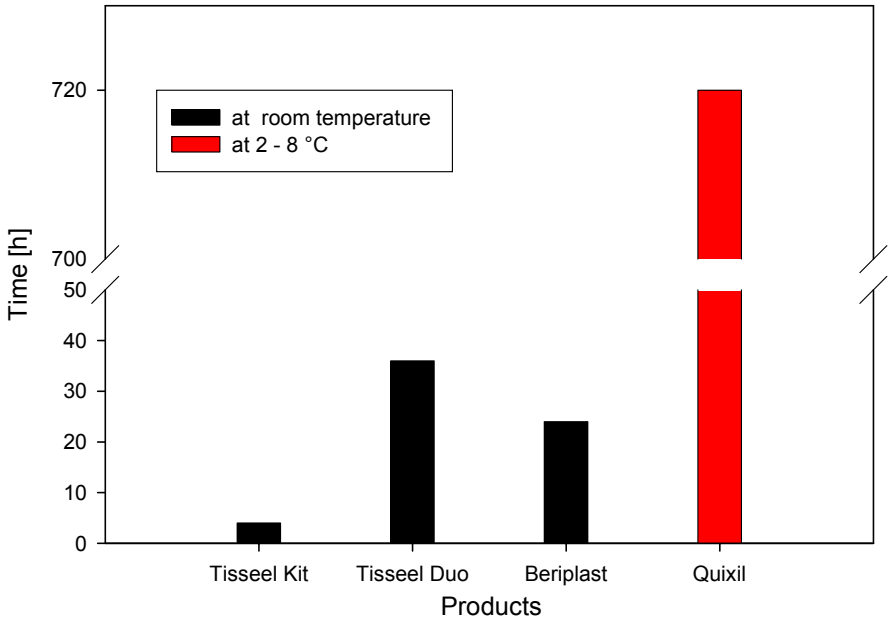

Figure 2: Storage life and conditions after reconstitution/thawing (recommendations of the manufacturers)

6.3 COMPOSITION

Overview
An overview of the chemical compositions of the compared fibrin sealants is given in table 1. The data from table 1 are partially obtained from the product leaflets of the manufacturers and from own analytical data (written in *italics*).

According to the indications of the manufacturers the main active component, the **clottable protein** (including mainly fibrinogen but also fibronectin), is present in the same range of concentration in Tisseel (75-115 mg/ml) and Beriplast (80-90 mg/ml) but in a considerable lower concentration in Quixil (40-60 mg/ml). Because the mechanical robustness of fibrin clots increases with fibrinogen concentration, Quixil is less suitable for sealing and gluing applications. We confirmed this in a preclinical study comparing the sealing efficacy of Tisseel VH versus Quixil in a rabbit partial lung resection model (final report K03/02.1).

The influence of **FXIII** concentration is discussed in chapter 2.4.

Antifibrinolytic agents are used to reduce the enzymatic lysis of the fibrin clot in vivo (catalyzed by plasmin and other proteases) with the aim to stabilize the gluing or sealing or to prevent rebleeding. As antifibrinolytic agent Tisseel and Beriplast contain **aprotinin**, a protease inhibitor of bovine origin, whereas Quixil contains **tranexamic acid (tAMCHA)**, a synthetic antifibrinolytic agent. These antifibrinolytic agents differ in their mechanism of action. Since aprotinin forms an stable 1:1 complex with specific proteases, blocking their catalytic active center, tAMCHA hinder the specific interaction between plasmin(ogen) and fibrin(ogen) through the lysine binding sites of plasmi(nogen). There is a long and positive clinical experience with the use of aprotinin as a component of fibrin sealants and as a stand-alone medicinal product (Trasylol). Due to its bovine origin rare hypersensitivity reactions occur in patients with no prior exposure to aprotinin and anaphylactic reactions are possible in patients who are re-exposed to aprotinin containing products. In the last years concerns

associated with the risk of TSE transmission by products of bovine origin arose, but no case was reported. This risk is very low in the case of aprotinin due to its production process (for which a high degree of elimination of deliberately added TSE agent was demonstrated) and the fact that only cattle from BSE free countries are used as the source in production. The use of tranexamic acid (**tAMCHA**) as an anti fibrinolytic agent in Quixil circumvents these disadvantages but has other drawbacks. Two fatal clinical cases were reported in association with the use of Quixil in neurosurgery from UK. These were associated with the effect of tAMCHA on the nervous system. It was shown that tAMCHA causes convulsions by a γ-aminobutyric acid A receptor antagonistic effect (Schlag MG et al, 2002). According to the Omrix leaflet accompanying the product, Quixil is contra-indicated in "neurosurgery or any surgery where direct contact with the cerebrospinal fluid or dura mater is expected (due to risk of cerebral oedema and seizure)". However, besides the risk of inappropriate uses the consequences of contact with the peripheral nervous system is not convincingly answered. tAMCHA is present in the sealer protein component of Quixil in an extremely high concentration of 95 mg/ml (604 mM). This concentration was chosen to assure the stability of the thawed product at refrigerator temperature in a liquid, ready-to-use form (to prevent gelation). In cell cultures a cytotoxic effect of tAMCHA on lung fibroblast has been found at concentration 9 times lower than in Quixil. Cell viability was reduced to about 50 % and severe morphological changes were observed at concentrations similar to those in Quxil (Final report V03/05). Aprotinin does not have any effect on cell viability and morphology when used in the same concentration as in Tisseel. These finding may allow inferring impaired wound healing after use of Quixil.

Table 1 Composition of competitive fibrin sealants (preliminary results). Legend: Values written *in italics*: analytical results of R&D BioSurgery/Vienna. All the other values are from brochures of the manufacturers. Y - the manufacturer declares the presence but not the concentration.

	Tisseel		**Beriplast**		**Quixil**	
	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin
Total protein	100-130 mg/ml *123 mg/ml*	45-55 mg/ml *43.8 mg/ml*	118 mg/ml *102 mg/ml*	*0.8 mg/ml*	60-80 mg/ml *89 mg/ml*	7.2 mg/ml *7.4 mg/ml*
Clottable Protein	75-115 mg/ml	----------		----------	40-60 mg/ml	------
Fibrinogen	70-110 mg/ml *90 mg/ml*		90 mg/ml *73 mg/ml*		*61 mg/ml*	
Fibronectin	2-9 mg/ml *10 mg/ml*		*4 mg/ml*		*9 mg/ml*	
Thrombin	----------	400-625 U/ml	----------	300 U/ml 500 U/ml	----------	900-1000 U/ml
FXIII	10-50 U/ml (EU) *21.4 U/ml (EU)* *1.0 U/ml (US)*	----------	40-80 U/ml *32.3 U/ml*	----------	*0.4 U/ml*	----------
Plasminogen	40-120 µg/ml		*5.48 µg/ml*		*63.1 µg/ml*	

Table 1 (continuation)

	Tisseel		**Beriplast**		**Quixil**	
	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin
Aprotinin	2250-3750 KIU/ml	----------	1000 KIU/ml	---------	-----	------
Tranexamic acid	----------	----------	--------	--------	95 mg/ml (604 mM) *555 /642 mM*	-----
Human albumin	10-20 mg/ml *12 mg/ml*	45-55 mg/ml	10 / l5 mg/ml *16 mg/ml*		*15 mg/ml*	0.6 % w/w
Polysorbate 80 (Tisseel US only)	0.2-0.4 mg/ml					
Triton WR1339 (Tisseel EU only)	0.2-0.4 mg/ml					
Arginine·HCl	----------	----------	12 mg/ml (57 mM)		**Y** *100 mM*	
Glycine	15-35 mg/ml (333 mM)	2.4-3.6 mg/ml			**Y** *100 mM*	
Isoleucine	----------	----------	l2 mg/ml			

Table 1 (continuation)

	Tisseel		**Beriplast**		**Quixil**	
	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin	Sealer Prot.	Thrombin
Na-glutamate · H_2O	----------	----------	9 mg/ml			
Mannitol	----------	----------	-------			2 % (w/w)
NaCl	2-4 mg/ml	8-12 mg/ml	l5 + 8.5 mg/ml[1)] [1)] 8.5 mg/ml from Aprotinin	4.8 mg/ml	6.9-7.1 mg/ml	
Na_3·citrate · 2 H_2O	4-8 mg/ml		5.5 mg/ml	3 mg/ml	2.4–2.9 mg/ml	
Na-acetate	----------	----------	-------			**Y** *18.7 mM*
Na^+						*125 mM*
$CaCl_2$·$2H_2O$	----------	5.9 mg/ml (40 mM)	-------	5.9 mg/ml	**Y**	5.6-6.2 mg/ml *(44 mM)*
Conductivity	*14.72 mS/cm*	*33.93 mS/cm*	*39.40 mS/cm*	*16.91 mS/cm*	*21.60 mS/cm*	*22.33 mS/cm*
	24.32 mS/cm		*28.15 mS/cm*		*22 mS/cm*	
pH	7.0-7.5 *7.34*	6.5-7.3 *6.71*	*7.15*	*6.13*	*6.98*	*6.70*

The high salt concentration in the Beriplast sealer protein component, in conjunction with its arginine content is advantageous in the view of excellent dissolution times at room temperature compared to the Tisseel VH kit presentation (where dissolution at 37°C is recommended). However, this composition leads to non-physiological glass-like transparent "fine clots" and not to physiological white and opaque "coarse clots" obtained with Tisseel.

Thrombin components

The protein compositions of the thrombin components have been analyzed by discontinuous SDS-polyacrylamide gel electrophoresis PAGE (figure 3).

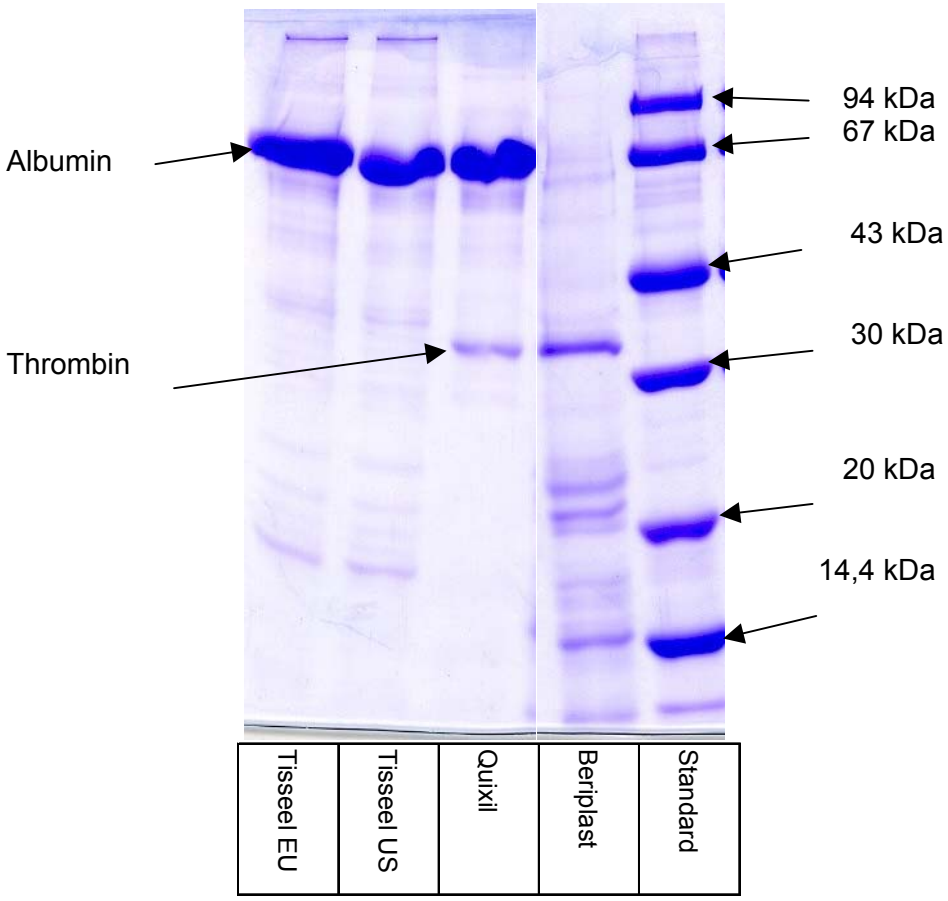

Figure 3 Discontinuous SDS-PAGE (reducing conditions) of thrombin components from competitive fibrin sealants. 8 µg protein/lane were applied.

The electrophoretic analysis has confirmed that the Beriplast thrombin component is free of human albumin. The α-thrombin B-chain is the most prominent band in the electrophoretic pattern of Beriplast. This band is weaker but still visible in the lane of Quixil thrombin component having 6 mg/ml human albumin. Due to the high human albumin concentration (50 mg/ml) of the Tisseel thrombin component, the thrombin band is not visible.
It has to be emphasized that these differences in protein composition of the thrombin components do not have any practical effect on the efficacies of fibrin sealants. More important is the salt and additive concentrations that have to ensure, in conjunction with those of the sealer protein component, a physiologic environment for clot formation.

Sealer Protein components

The protein compositions of the sealer protein components have been analyzed by continuous urea SDS-polyacrylamide gel electrophoresis. The reduced and non reduced lanes show similar patterns for each of the compared fibrin sealants. However, it is remarkable that Beriplast is poor of Fibronectin and that in Quixil, at reduced condition, an additional protein band is visible in the molecular weight range of Fibronectin. It is highly probable that the high molecular weight bands in Quixil and Beriplast represents Fibronectin although this has not been confirmed, e.g. by immunologic methods. The results of densitometric quantification are shown in table 2. The Fibrionectin concentrations of Quixil and Beriplast are calculated from the sum of two bands. Tisseel EU and US contain the highest concentration of fibrinogen and Quixil the lowest. Mechanical strength of fibrin clots and bond strength of tissues glued with fibrin sealants correlates directly with the concentration of fibrin(ogen) Therefore Quixil, having a fibrinogen concentration of only about 60 % of that of Tisseel, shows poor performances in this respect (see 6.6 and 6.7).

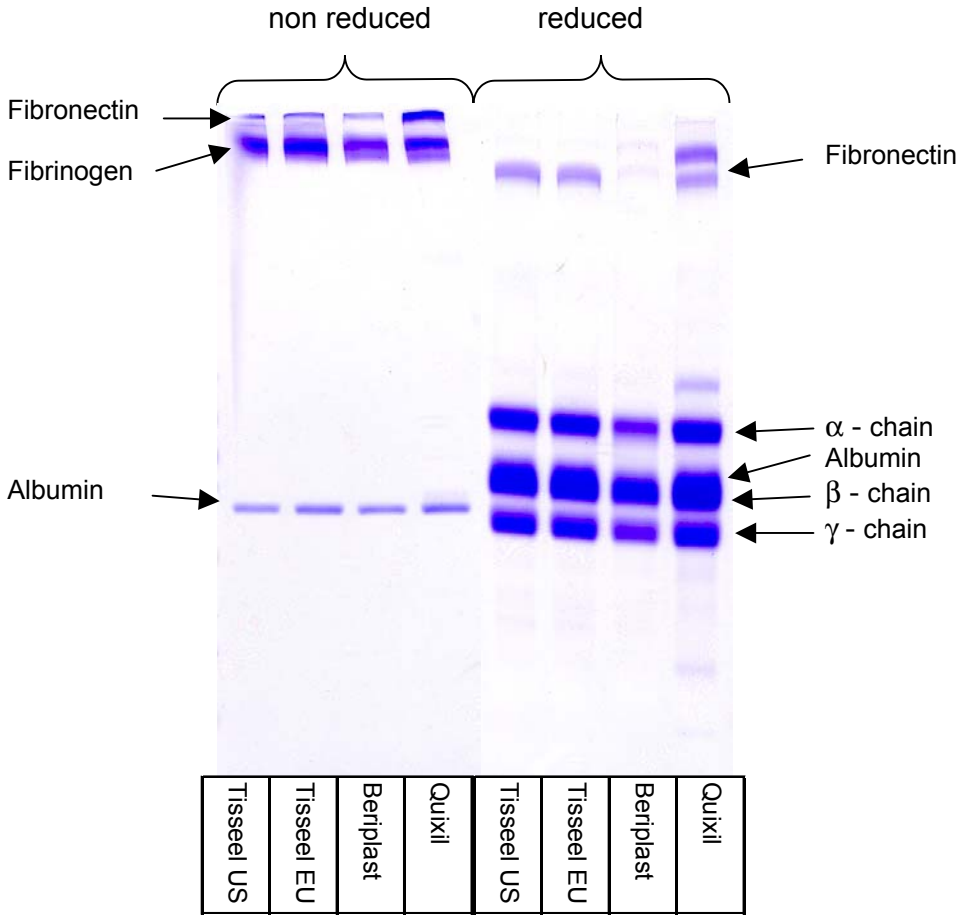

Figure 4 Continuous U-SDS-PAGE (non reducing and reducing conditions) of sealer protein components from competitive fibrin sealants. Protein/lane: 15 ± 4 µg non-reducing conditions; 4 ± 1 µg reducing conditions (Sample preparation 24.09.04 MSz, Electrophoreses 30.09.04 BB)

Table 2 Composition of the sealer protein component of different commercial fibrin sealants. The results of Tissucol are calculated out of 2 determinations and the results of Beriplast and Quixil are calculated out of 3 determinations (Sample preparation and electrophoreses 22.01.2004)

	Tissucol STIM3 EU		Tissucol STIM4 US		Beriplast		Quixil	
	mean	std. dev.	mean	std. dev.	mean	std. dev.	mean	std. dev.
Protein (BCA) [mg/ml]	140,87	6,93	123,44	10,18	101,85	10,18	89,17	10,18
Fibrinogen [mg/ml]	106,56	2,38	89,88	6,09	72,82	4,32	61,47	1,30
Fibronectin [mg/ml]	10,21	2,09	10,37	0,87	3,60	0,52	8,53	1,74
Albumin [mg/ml]	15,64	3,30	12,33	0,89	15,92	2,79	15,16	0,33

6.4 FXIII CONTENT

According to the information provided by the manufacturers FXIII is present in the sealer protein component of Beriplast (40-80 IU/ml) and Tisseel EU (10-50 IU/ml). In Tisseel US and Quixil FXIII is not declared as an active component. In Tisseel US a FXIII content of 1 IU/ml was found and this was 2.5-fold higher compared to the concentration determined in Quixil.
Whereas there is no doubt on the influence of FXIII crosslinking on the mechanical properties of a fibrin clot (e.g. on the clot elasticity and tensile strength), no beneficial effect of FXIII was found for the efficacy of fibrin sealants in vivo. The reason for this is the fact that in gluing and sealing application the weakest point (where rupture first appears) in the system tissue/fibrin clot is the adhesive strength of the clot to the tissue and not the inner strength of the clot. This was the reason why, during the US submission of Tisseel, FDA has approved only a formulation without added FXIII.

Contradictory to Baxter, Aventis promotes Beriplast emphasizing an increased efficacy due to the high FXIII content. However, the data of an Aventis publication (Dickneite G. et al., 2002), after a critical reading, does not support this. For fibrin sealants with the salt composition of Beriplast and different FXIII concentrations, a maximum efficacy was found at a FXIII concentration as low as 3 IU/ml FXIII. The need of FXIII for maximal efficacy could be even lower, as the study has no data point in the range <0,2 - 3 IU/ml FXIII. Further, as can be seen from the kinetics of fibrin α-chain crosslinking (figure 6), due to its salt composition, Beriplast needs higher concentrations of FXIII than Tisseel to attain the same degree of fibrin crosslinking.

Kinetics of fibrin α-chain crosslinking

Some biochemical basics of the conversion of fibrinogen to fibrin and the FXIIIa catalyzed crosslinking of fibrin fibers have been described in the introduction. The following information supports the understanding of the experiments below. Fibrin is composed of three polypeptide chains: the α-, ß- and γ-chain. By the action of FXIIIa in a fast reaction the γ-chain is crosslinked to a γ-chain of an adjacent fibrin molecule resulting a γ-dimer (γ-γ). In a slower reaction the α-chain forms α-oligo- and α-polymers (α_n).
Figure 5 visualizes the electrophoretic pattern of the progress of the FXIIIa catalyzed crosslinking. The quantification of the α-chain crosslinking is shown in figure 6. The kinetics of crosslinking is similar for Tisseel (EU) and Beriplast. Even after 15 min the γ-chains are completely converted into γ-dimer and the fibronectin band disappears completely

(fibronectin is a substrate of factor XIIIa too). For the lots tested, the crosslinking reaction has been slightly faster and the final degree of crosslinking higher for Tisseel (EU) compared to Beriplast (Figure 6). This is a remarkable result since in Beriplast the content of FXIII is, according to the manufacturer, higher (60 IU) compared to the EU-licensed Tisseel (10-50 IU). An explanation of this finding could be the more physiologic composition of the Tisseel clot compared to the Beriplast clot. The physiologic environment might allow a more effective progress of the FXIIIa catalyzed reaction, so that a smaller amount of FXIII is needed to attain the same degree of crosslinking.

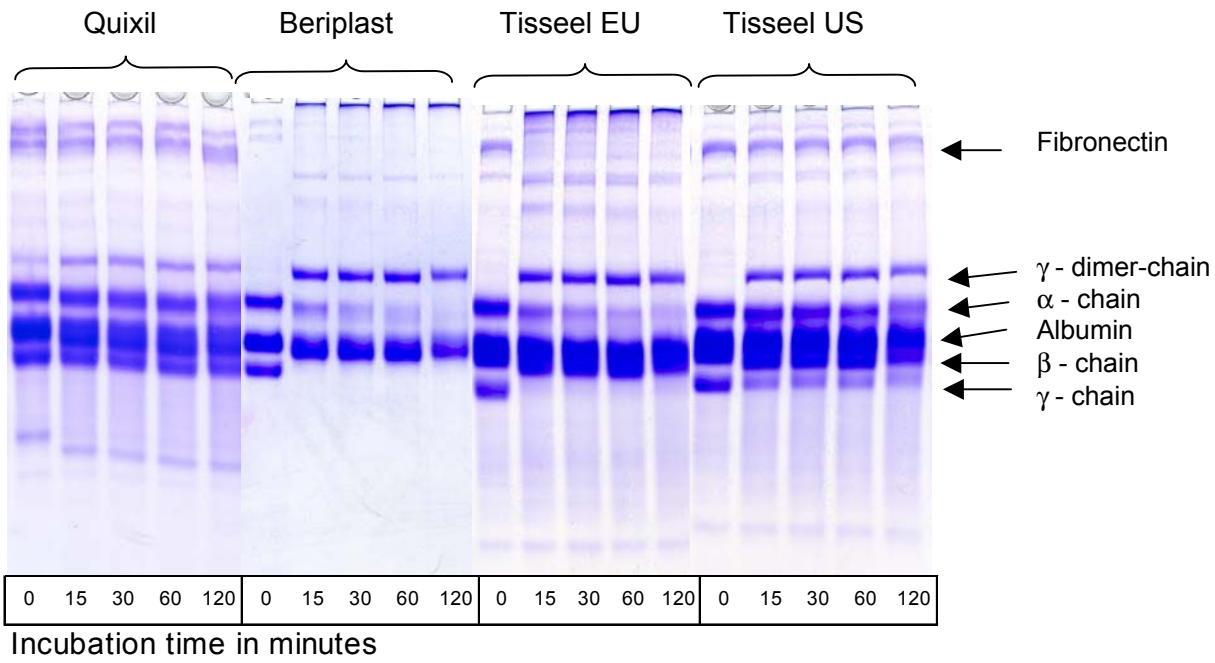

Figure 5 Electrophoretic pattern of the progress of the FXIII catalyzed cross-fibrin clots obtained from different commercial fibrin sealants

In comparison to Tisseel (EU) and Beriplast, the FXIII catalyzed crosslinking was lower for Tisseel (US) and almost completely absent in the Quixil/Crosseal clot. However, Tisseel (US) has shown significantly higher crosslinking than Quixil/Crosseal. In the Tisseel (US) clot, after 15 minutes, the main part of the γ-band was converted to γ-dimer and only traces of the γ-band were detected after 2 hours. On the other hand, in the Quixil/Crosseal clot most of the γ-chain was not crosslinked even after 2 hours (figure 5). The same was observed for α-chain crosslinking. In the case of Tisseel (US) it was increasing continuously to approximately 50 percent crosslinking. For Quixil/Crosseal only less the 10 % α-chain crosslinking was detected after 2 hours (figure 6).

In the view of the fact that only single lots of each fibrin sealant have been analyzed in this investigation, these results, even when they demonstrate a trend, should be considered preliminary and have to be confirmed by the analysis of other batches.

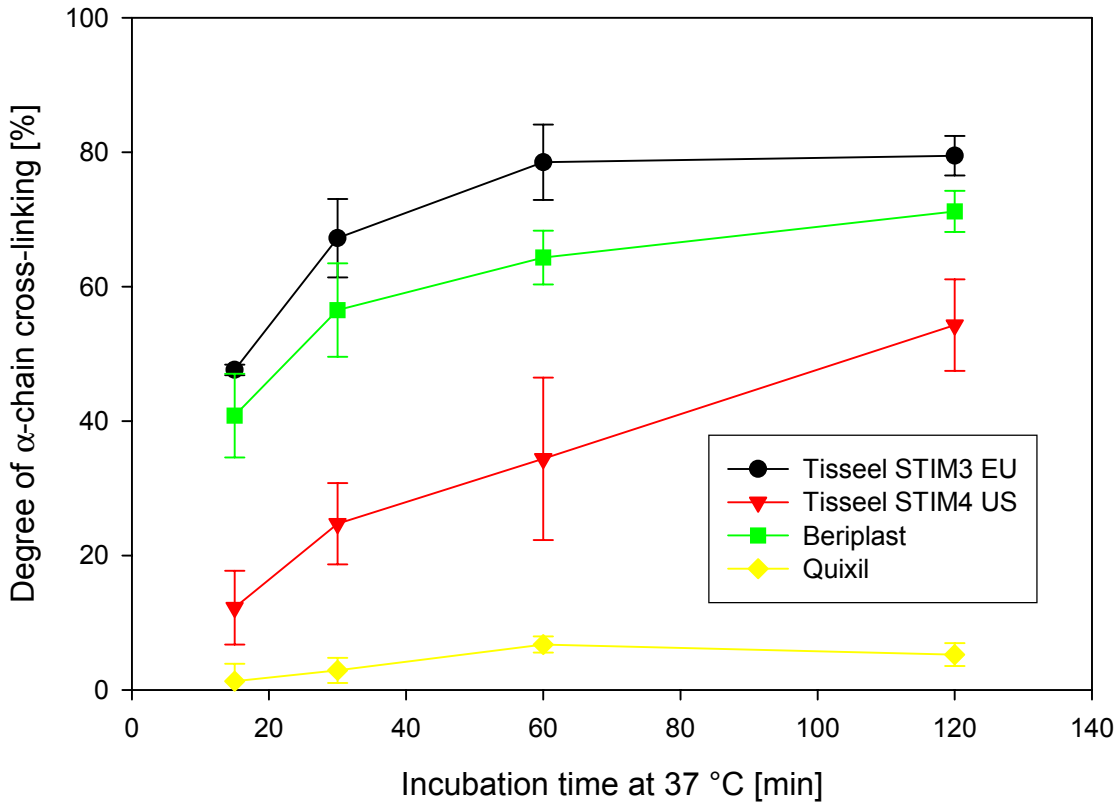

Figure 6 Comparison of Tisseel STIM 3 EU and Tisseel STIM4 US (Baxter) with the competitive products Quixil (Omrix) and Beriplast (Aventis Behring): kinetics of α-chain crosslinking by FXIII. The data were obtained by densitometric analysis of electrophoresis gels shown in figure 5. Means and standard deviations are shown. They are calculated out of four results in the case of Tisseel STIM3 EU and Beriplast. Tisseel STIM4 US and Quixil were carried out in duplicate.

It is remarkable that Quixil shows a much lower degree of α chain cross linkage in comparison to Tisseel US even though the amount of FXIII (0.4 and 1 IU/ml sealer protein component, respectively) would not suggest such a big difference. Further, results of α chain cross-linking analysis after addition of 20 IU/ml FXIII to both the Quixil and the Tisseel US sealer protein components encourage the previous observation (Figure 7 and 8). The adding of 20 IU/ml FXIII, which is the amount of FXIII contained in Tisseel EU, does not increase the α chain cross-linkage of Tisseel US and Quixil to the same level. Whereas in the case of Tisseel US after FXIII supplementation almost the same level of α chain cross-linking as for Tisseel EU has been attained, the degree of cross-linking for FXIII supplemented Quixil was significant lower. The reasons for this observation could be the non-physiologic composition of the Quixil clot and a partial damage of the fibrinogen molecule in the course of the production process as well. As a consequence of such a damage the resulted fibrin is a less good substrate for FXIIIa catalyzed cross-linking.

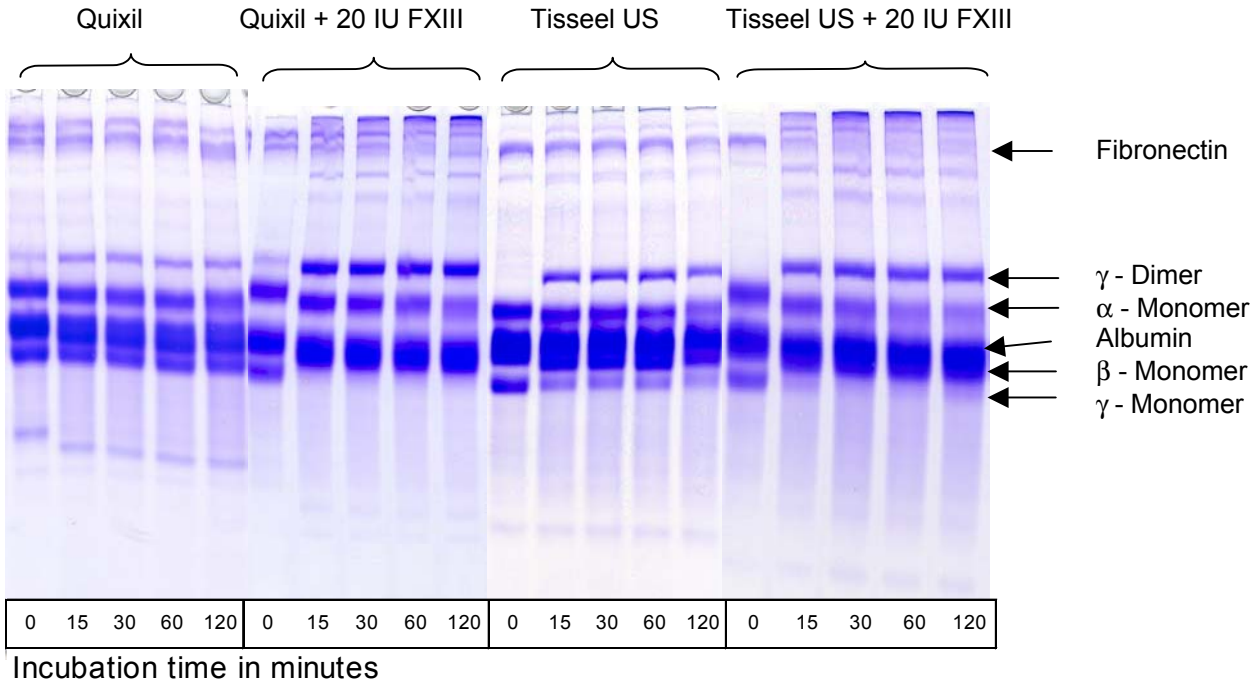

Figure 7 Electrophoretic pattern of the progress of the FXIII catalyzed cross-linking of fibrin clots obtained from different commercial fibrin sealants

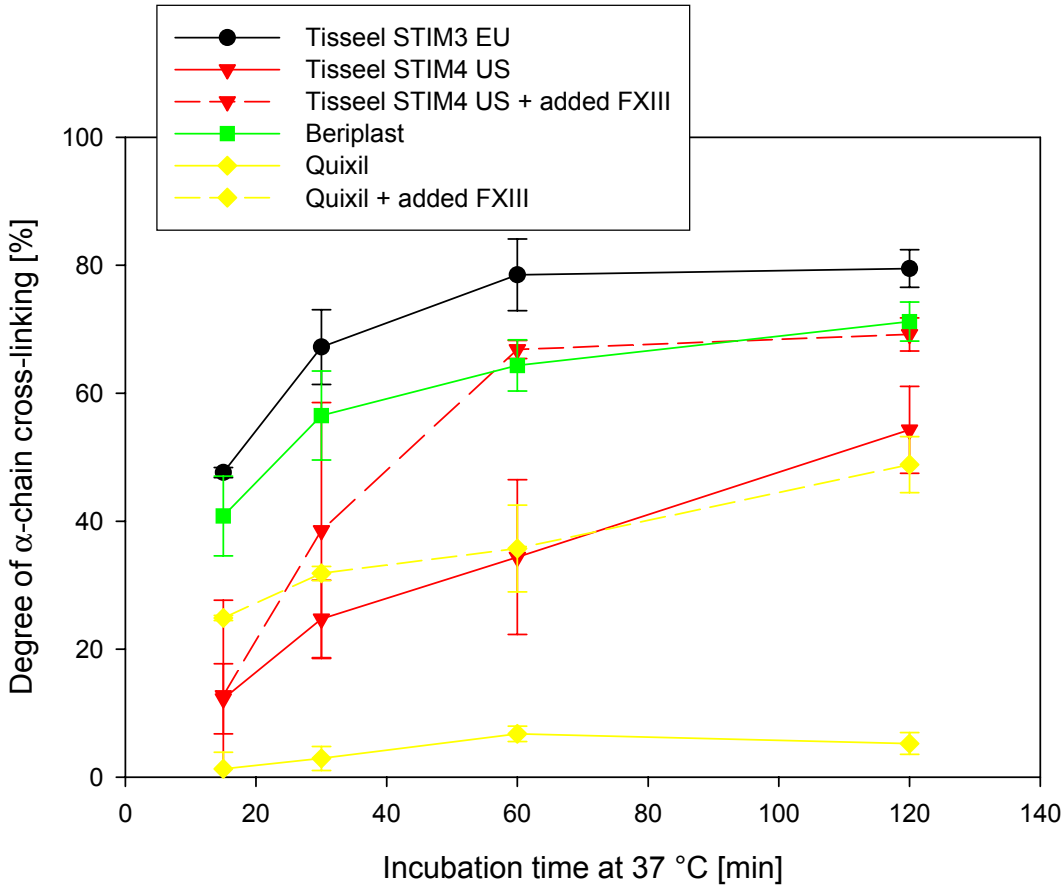

Figure 8 Influence of FXIII addition to fibrin α chain cross-linking of Tisseel US and Quixil. Discription see figure 6. The sealer protein components of Tisseel US and Quixil were supplemented by addition of 20 IU/ml FXIII before analysis.

6.5 CLOT STRUCTURE (SCANNING ELECTRON MICROSCOPY)

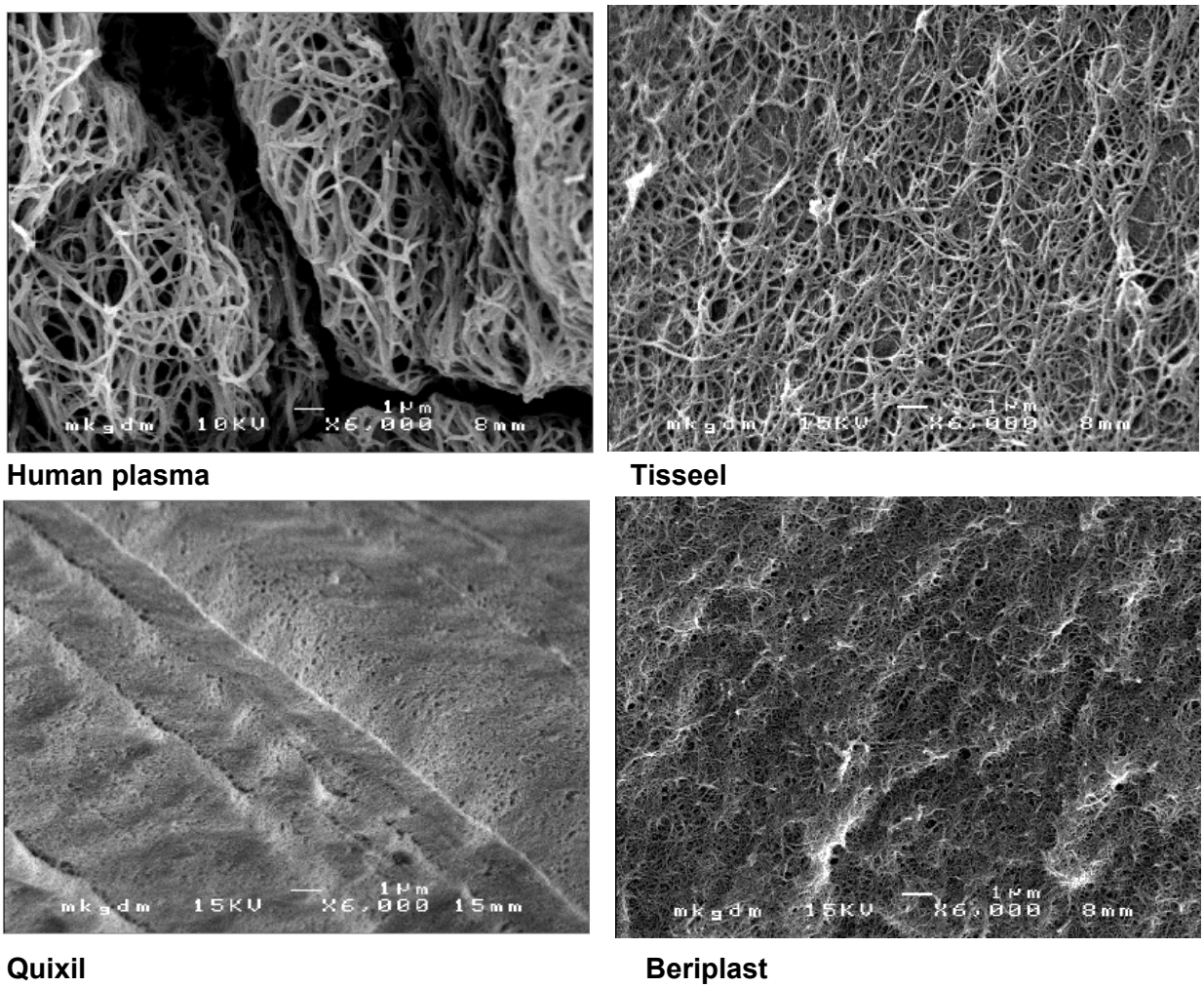

Figure 9 Scanning electron microscopy of fibrin clots obtained from human plasma and various commercial fibrin sealants

As can be seen (figure 9) in scanning electron microscopy (SEM) the fibrin clot obtained directly from human plasma (formed in a "natural", physiologic environment) appears as a three-dimensional network of fibrin fibers. The pores of this network are visible and are an important clot characteristic as they are crucial for the in-growth of cells into the clot during wound healing.
From the commercial fibrin sealants analyzed in this study only Tisseel shows a clot structure similar to clots obtained from blood plasma. The three-dimensional porous network built by fibrin fibers is visible. The network of fibrin fibers appears denser in Tisseel as in the plasma clot. The reason for this is the much (about 20-fold) higher fibrinogen concentration in Tisseel. The higher thrombin concentration, necessary in fibrin sealants for fast clotting, additionally leads to thinner fibers and a denser fibrin network.
Contrary to Tisseel, in SEM Quixil/Crosseal and Beriplast show a compact structure with not any resemblance to a natural fibrin clot.
The clot structures observed by SEM offer an explanation to the fact that in cell culture experiments only Tisseel clots show good cell compatibility.

6.6 CLOT APPEARANCE AND MECHANICAL PROPERTIES

Already in 1947 Ferry and Morrison have described two types of fibrin clots: the white, opaque "coarse clot", obtained at physiological conditions of ionic strength and pH, and the glass-clear, transparent "fine clot", obtained at higher ionic strength and/or higher pH values. Only with Tisseel coarse clots were obtained due to its physiologic salt composition. With Beriplast and Quixil, having compositions optimized for reduced dissolution times or increased storage stability, transparent "fine clots" are obtained (figure 10 and 11).
Aventis is trying to promote the transparent clot appearance of Beriplast with an alleged "high purity". In fact transparency is only the result of different optic properties of the thin fibrin-fibers of the Beriplast clot compared to the thick fibers of a white coarse clot as found in Tisseel. It has to be emphasized that only the Tisseel clot has an appearance similar with clots obtained directly from human plasma, i.e. a natural fibrin clot.

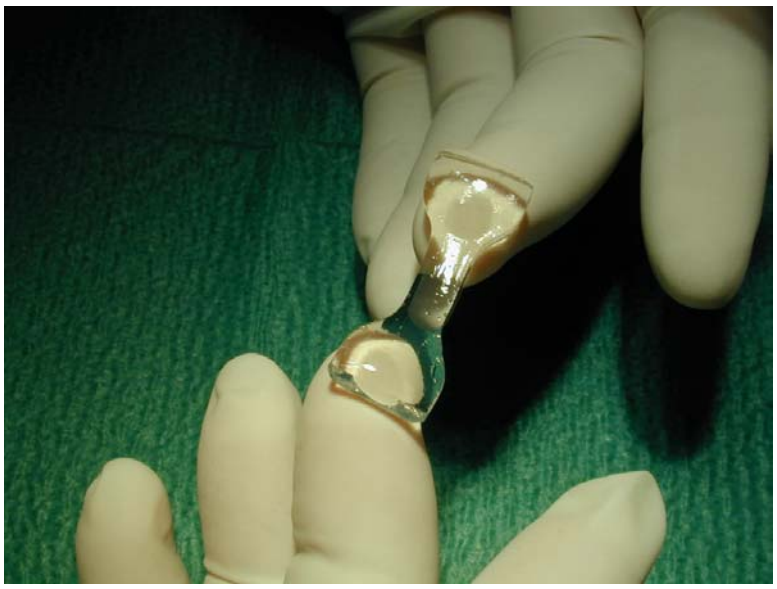

Figure 10
Butterfly-shaped fibrin clot obtained from **Quixil**.
The transparent appearance is characteristic for non-physilological "fine clots".

Clots obtained from Beriplast have a similar appearance.

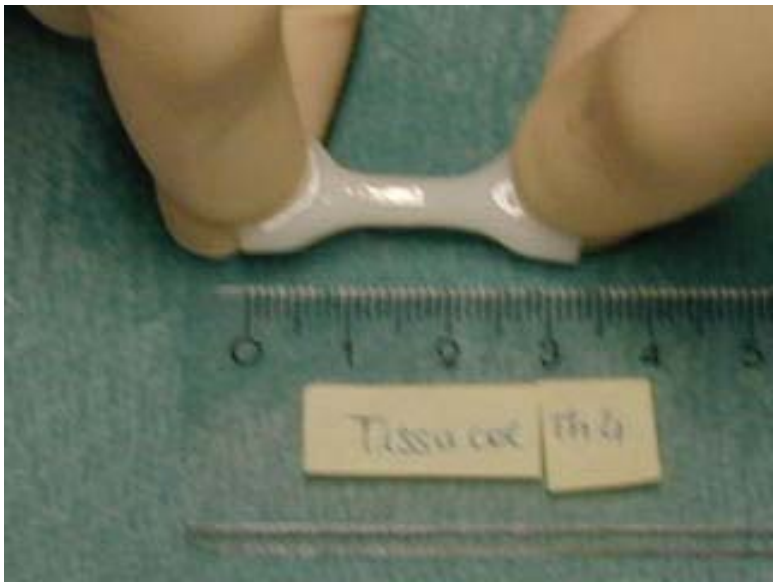

Figure 11
Butterfly-shaped fibrin clot obtained from **Tisseel**.
The opaque appearance is characteristic for physilological "coarse clots".

Inner tensile strength and clot elongation at rupture of fibrin clots

The inner tensile strength and the elongation at rupture are mechanical characteristics of fibrin clots that have been largely used in comparative studies of fibrin sealants. The factors influencing these properties have been outlined in the introduction. Because the adhesive strength between the fibrin clot and the tissue is the decisive parameter for fibrin sealant efficacy, the clinical relevance of these intrinsic clot properties is controversial. Due to their

widespread use in comparative studies of fibrin sealants they have been included in this investigation.
Experimentally, after casting a fibrin clot into a mold, after an incubation time of 30 min at 37°C in a moist chamber, a butterfly-shaped fibrin clot is obtained (figure 10 and 11). This clot is stretched with a constant velocity (5 mm/min) until rupture. The maximum clot elongation (elongation at rupture) in mm and the maximal force applied (N) are recorded (figure 12)

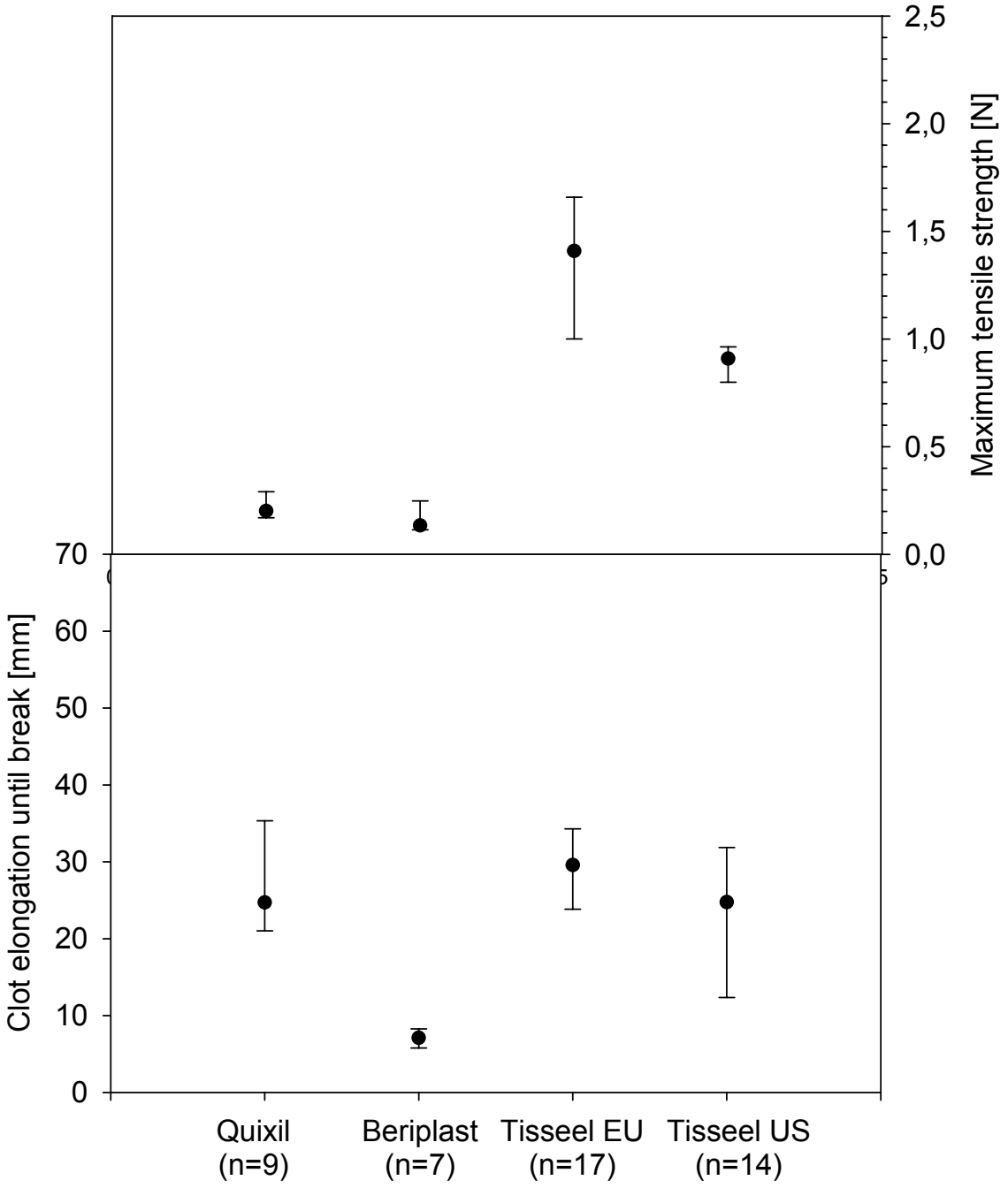

Figure 12 Elongation at rupture and inner tensile strength of fibrin clots obtained with competitive fibrin sealants. After casting a fibrin clot into a mold the clot is stretched with a constant velocity (5 mm/min) until rupture. The maximum clot elongation (in mm) and the maximal force applied (in N) are recorded. Medians, 25 and 75 percentiles are depicted.

6.7 GLUING PROPERTIES

The gluing properties of the tested fibrin sealants have been evaluated by measuring the adhesive strength of two prepared rat skin strips glued to each other under standardized conditions. The results are shown in figure 13. For the tested fibrin sealant lots the best and similar results were obtained with Beriplast and Tisseel EU. For rat skin strips glued with Quixil approximately only half the force compared to Beriplast and Tisseel EU was necessary to disrupt the gluing. Tisseel US showed to confer an intermediate adhesive strength, but the mean variation of the values was unusual high during the measurements.

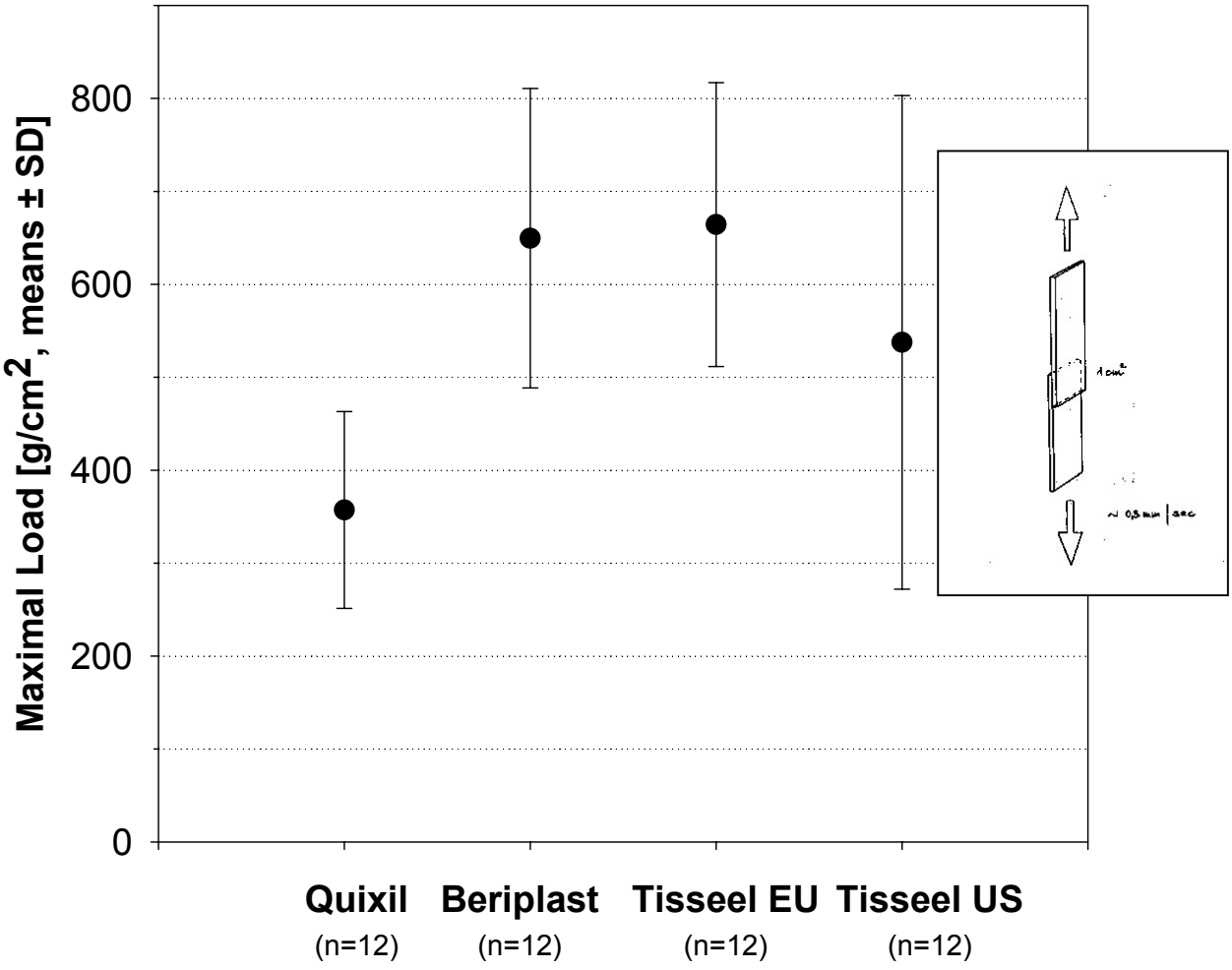

Figure 13 Adhesive strengths of fibrin sealants (rat skin model).
Two rat skin strips (3x1 cm) were glued together overlapping over 1 cm^2. After 30 min the strips were torn apart (insert) with 0.3 mm/sec and the maximal force applied was recorded.

7 RAW DATA

7.1 LABORATORY BOOK

Margarete Szekely, Book 2, Pages 31-34, 38, 39, 45-51, 54-65, 70, 72-75, 79, 86, 106, 146, 164, 165
Barbara Breicha, Book 5, Pages 44,45

7.2 ATTACHMENT

U/SDS Electrophoresis of Tisseel US and EU, Beriplast and Quixil. 2 pages, MSz

Immunol. Bestimmung von Plasminogen, 1 page

Determination of protein content (BCA), 5 pages MSz

Determination of FXIII Content, 2 pages MSz

Determination of acetate content, 2 pages MSz

Discontinuous SDS Electrophoresis (Laemmli), 2 pages MSz

Continuous urea/SDS electrophoresis (Densitometer printout)
Gele: reduced and non reduced samples, Tissucol STIM3 EU, Tissucol STIM4 US, Beriplast, Quixil, 21.01.04, 2 pages
Densitometer printouts: reduced and non reduced samples, Tissucol STIM3 EU, Tissucol STIM4 US, Beriplast, Quixil, 20 pages

Fibrin α-chain cross-linking
Gele: Tissucol STIM3 EU, Beriplast, Quixil, 21.10.03, 3 pages
Densitometer printouts: Beriplast, 12 pages
Tisseel STIM3 EU, 13 pages

Gele: Beriplast, Quixil, 27.10.03, 2 pages
Densitometer printouts: Quixil, 12 pages
Beriplast, 11 pages

Gele: Tissucol STIM3 EU, Tissucol STIM4 US, 19.11.03, 2 pages
Densitometer printouts: Tisseel STIM4 US, 12 pages
Tisseel STIM3 EU, 12 pages

Gele: Tissucol STIM4 US + 20 IE/ml FXIII, Quixil + 20 IE/ml FXIII, 04. und 10.12.03, 4 pages
Densitometer printouts: Tissucol STIM4 US + 20 IE/ml FXIII, 22 pages
Quixil + 20 IE/ml FXIII, 22 pages

Amino acid determination (QC), 28 pages

Na^+ and Ca^{2+} determination (QC), 2 pages

Inner tensile strength and clot elasticity

Rat skin adhesive strength, (Department Dr. Auer) 2 pages