TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

LAURA KOIVUSALO

DEVELOPMENT OF AN ALIGNMENT METHOD FOR SMALL HISTOLOGICAL SAMPLES

Master's thesis

Examiners:
Professor Pentti Järvelä,
D.Sc. (Tech) Niina Ahola
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ABSTRACT

LAURA KOIVUSALO: Development of an alignment method for small histological samples
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Small histological samples, such as prostate biopsies, make up a significant part of histology laboratories' workload due to their demanding handling and large quantities. During tissue sample handling, the small needle biopsies are embedded in paraffin and sectioned onto microscope slides for pathological evaluation. New methods are needed to improve the handling of thin biopsy cores to optimize the quality of patient diagnosis and cost-efficiency of histological sample preparation.

The aim of this thesis was to develop an alignment method for reducing the amount of manual handling steps in tissue processing and ensuring high quality tissue samples for faster and more accurate diagnosis. For this purpose, a device was designed to hold multiple samples simultaneously and in the same plane for maximal tissue representation on single microscope slide. The device was designed so that it can be removed from the paraffin block to allow for easy sectioning of the tissue samples, without affecting the pathological evaluation.

Two different kinds of prototypes were manufactured for an alignment device: dopamine coated silicone elastomer pads and gelatin-siloxane hydrogel pads. Dopamine is the functional molecule of mussel foot proteins responsible for their attachment to virtually any surface, which is why it was selected for coating material for the inert silicone pads. Gelatin-siloxane hydrogel was selected because it was thought to provide both attachment and supportive functions simultaneously.

We evaluated the performance of the prototypes in the histological sample handling process by testing their capability to attach tissue samples from the biopsy needle and keep them in place during sample processing and embedding. Dopamine coated silicone pads were not successful in keeping the tissue samples aligned. Device prototypes made from flexible and tough gelatin-siloxane hydrogel were successful in attaching the small tissue samples onto their surface using capillary force and wet adhesion. The hydrogel pads succeeded in immobilizing the samples during tissue processing and were removable from the resulting paraffin blocks, as designed. However, the hydrogel pads distorted in the tissue processor, limiting their use in small tissue sample alignment. Further development is still needed to find a suitably stiff material or structure to keep the device from bending during tissue processing. Furthermore, the ease of sample attachment should be improved even further to make the alignment device more attractive for use in clinical practice.

TIIVISTELMÄ

LAURA KOIVUSALO: Pienten histologisten näytteiden orientointimenetelmän kehittäminen
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Pienet kudosnäytteet, kuten eturauhasbiopsiat, luovat suuren osan histologisten laboratorioiden työmäärästä, koska niiden käsittely on hankalaa ja näytteiden määrät ovat suuria. Kudosnäytteiden käsittelyssä pienet neulabiopsiat valetaan parafiiniin ja niistä leikataan viipaleita, jotka laitetaan mikroskooppilasille patologin tarkasteltavaksi. Uusia pienten kudosnäytteiden käsittelymenetelmiä tarvitaan potilaiden diagnoosien ja laboratorioiden kustannustehokkuuden optimoimiseksi.

Tämän diplomityön tarkoituksena oli kehittää kudosnäytteiden tasaiseen asettamiseen menetelmä, jolla saadaan vähennettyä manuaalisten työvaiheiden määrää samalla varmistaen potilaiden nopean ja tarkan diagnoosin. Tarkoitusta varten kehitetty laite suunniteltiin siten, että siihen voidaan asettaa useita kudosnäytteitä samanaikaisesti ja ne pysyvät samassa tasossa, jolloin voidaan saavuttaa mahdollisimman suuri kudospinta-ala lopulliselle mikroskooppilasille. Laite suunniteltiin irrotettavaksi parafiiniblokista, jottei se haittaisi kudosleikkeiden ottamista tai leikkeiden patologista tarkastelua.

Diplomityössä valmistettiin laitteesta kahdenlaisia prototyyppejä: dopamiinilla pinnoitettuja silikonimattoja sekä gelatiini-siloksaanihydrogeelistä valmistettuja mattoja. Dopamiini on simpukoiden tarttumisproteiinien toiminnallinen komponentti, joka mahdollistaa niiden tarttumisen lähes mille pinnalle tahansa. Tämän tarttumisominaisuuden takia se valikoituikin silikonimaton pinnoitteeksi. Gelatiini-siloksaanigeeli valittiin prototyyppimateriaaliksi, koska sen ajateltiin pystyvän sekä tartuttamaan näytteet laitteen pintaan että tukevan näytteitä prosessin ajan.

Prototyyppien toimintaa arvioitiin testaamalla niiden kykyä tartuttaa kudosnäytteet biopsianeulasta sekä pitää ne paikoillaan kudosten prosessoinnin aikana. Dopamiinipinnoite ei onnistunut tartuttamaan tai pitämään näytteitä paikoillaan. Sen sijaan joustavasta ja sitkeästä gelatiini-siloksaanihydrogeelistä valmistetut prototyypit toimivat onnistuneesti kudosten kiinnittämisessä. Kiinnitysmekanismiksi arvioitiin kapillaari-ilmiö sekä kosteiden pintojen välinen adheesio. Kudosnäytteet pysyivät geeliprototyypeissä kiinni tehokkaasti kudosprosessoinnin aikana ja laitteen irrottaminen parafiiniblokista onnistui kudosnäytteiden jäädessä paikoilleen. Geelistä valmistetut laitteet kuitenkin vääntyivät prosessoinnin aikana, mikä vähentää niiden käytettävyyttä merkittävästi. Laite tulisi valmistaa jäykemmästä materiaalista, sillä kudosnäytteiden tasossa pysyminen on keskeistä optimaalisen diagnoosin kannalta. Lisäksi näytteiden tarttuvuutta materiaaliin voisi vielä parantaa kliinisen käytettävyyden lisäämiseksi.

PREFACE

This Master of Science thesis was carried out in Fimlab Laboratories and Satakunta Hospital District and in collaboration with Tampere University of Technology. The project was jointly funded by Satakunta Hospital District and Fimlab Laboratories.

I would like to sincerely thank MD Teemu Tolonen and MD Antti Kaipia, whose passion for their work set this thesis in motion, and without whom it would not have been possible. A heartfelt thank you also to Professor Emeritus Pentti Järvelä for giving me the opportunity to work with this project and for supervising my thesis. My deepest gratitude also goes to D.Sc. (Tech) Niina Ahola for supervising the thesis and providing excellent guidance with the work.

I would also like to thank Krista Näsi of the Materials Science Department in TUT and M.Sc. Janne Koivisto of the Department of Electronics and Communications Engineering in TUT for giving me their time and expertise with my material characterization.

Finally, a special thanks to my husband Eero for supporting me and believing in me throughout this whole process.

Tampere 12.5.2015

Laura Koivusalo

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS AND SYMBOLS

APTES	(3-aminopropyl)triethoxysilane
$CuSO_4$	copper sulphate
DI-water	de-ionized water
DOPA	3,4-dihydroxyphenylalanine
EPO	European Patent Office
GA	glutaraldehyde
GPMS	(3-glycidoxypropyl)-trimethoxysilane
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
KOH	potassium hydroxide
PBS	phosphate buffer solution
PDMS	poly(dimethyl siloxane)
PEG	poly(ethylene glycol)
PTFE	poly(tetrafluoroethylene)
SD	standard deviation
TMSPMA	(trimethoxysilyl)propyl methacrylate
Tris	tris(hydroxymethyl)aminomethane
TRUS	transrectal ultrasound
USPTO	United States Patent and Trademark Office

A_0	initial cross-sectional area
E	compression modulus
ε	strain
F	force in newtons
L	length in mm
L_0	initial length
M	concentration, mol/l
Pa	Pascal (also kPa and MPa)
®	registered trademark
σ	stress
θ	contact angle
™	unregistered trademark
v%	percentage by volume
w	mass fraction
w%	percentage by weight

1. INTRODUCTION

Histopathology is the microscopic study of tissues for changes caused by disease. Histological evaluation remains the most important diagnostic tool in use, and it is the only way to diagnose and grade cancers of different tissues. The term biopsy is used for the pathological examination of tissues removed from a patient to discover presence or extent of disease, but it can also refer to the process of taking such a sample or the tissue sample itself. Pathologist determines the state of the tissue by looking at thin slices of the tissue biopsies on a microscope slide.

The processing of all histological samples includes several steps. First, a small piece of the tissue is obtained and fixed in formalin solution. The second step is dehydration, where the water in the tissue is replaced by a series of alcohols, and the alcohols are in turn replaced by molten paraffin. After the tissue has been impregnated with paraffin, the sample is embedded into a paraffin block. Thin slices are then sectioned from the paraffin block using a microtome blade, and the slices are fixed onto microscope slides. Finally, the glass slides are stained for visualization of tissue structures. [1] The tissue handling process is described more thoroughly in Chapter 2.

Prostate cancer is the most common cancer in Finland; in the years 2008–2012, nearly 4600 new cases were diagnosed [2]. The probability of prostate cancer increases with age [3], and due to population ageing, the need for prostate biopsies will only increase in the future. The diagnosis of prostate cancer is always based on histological examination of tissue biopsy together with laboratory tests and clinical examination [3-5]. Urologists determine whether prostate biopsy is required based on digital rectal examination (DRE) of the prostate and concentration of prostate specific antigen (PSA) in blood serum [3]. If prostate cancer is suspected, the urologist takes 10–12 biopsy cores from different parts of the prostate of the patient using a biopsy needle [3]. The biopsy cores are long and thin, which makes them especially difficult to handle in histological laboratories. Because of their demanding nature and the large quantities, prostate biopsies present an important topic for improving histological sample processing.

The topic of this thesis deals with improving current diagnostic practices of small histological samples, of which prostate biopsies are selected as an example. Figure 1 illustrates the steps and operators in handling tissue samples from patients to obtain a diagnosis and relating the diagnosis back to the patient. All the different operators (patient, operating specialist, histology laboratory and pathologist) involved in the process have their own points of view on the diagnostic process. An accurate and speedy diagnosis is, of course, important for the patient, but it is also valuable for the other operators in the

process. For the histology laboratory it is a matter of business, for the specialist physician and the pathologist, a matter of professional ethics. In development of the alignment method for small histological samples, all the different view-points need to be considered for the ultimate goal of optimal patient diagnosis and treatment. The aim of this thesis is to develop an alignment method for small histological samples, which can reduce the workload and costs of tissue processing while simultaneously ensuring high quality samples for optimal patient diagnosis.

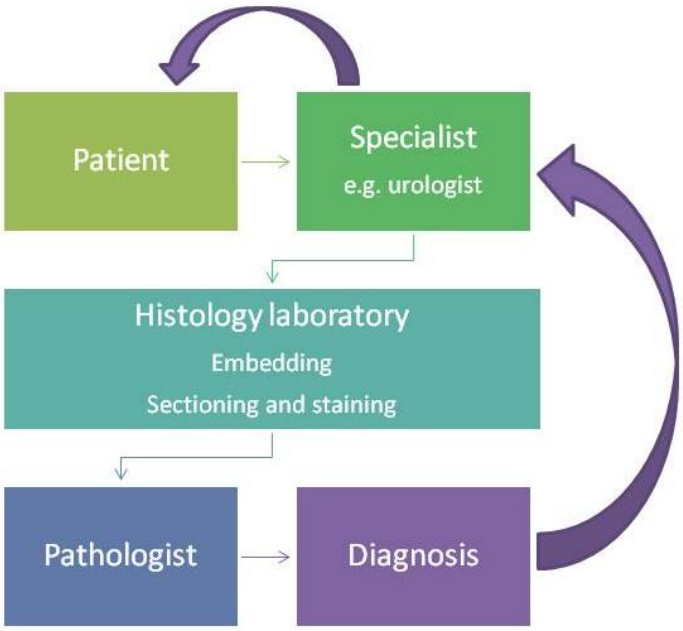

Figure 1. *The process of handling patient's tissue samples for histopathological evaluation and relating the diagnosis back to the patient*

In a broader aspect, the topic of this thesis is connected to obtaining more extensive knowledge from the histological samples. Biobanks are repositories of patient information where tissue samples are stored for research purposes. There are currently four registered biobanks in Finland, but several more are starting their operations in the near future [6]. The paraffin embedded tissue samples stored in the biobanks can be linked to data regarding patients' diagnoses and treatments together with their outcomes. The data stored in biobanks can be used for epidemiological research but also provides a basis for the emerging area of personalized medicine. Personalized medicine is a growing area of medicine, where the treatment is tailored to fit the patients' characteristics and needs [7]. Personalized medicine identifies and targets genetic mutations for development of more effective drugs, especially valuable in the treatment of cancers [7]. Genetic information obtained from the tissue samples stored in biobanks together with the data on diagnoses, treatments and outcomes offers valuable insights on the effects of mutations on the effectiveness of different treatments. The amount of data stored in biobanks is

huge, and the efficient alignment method under development in this thesis aims to concentrate that information for more efficient storage of high quality samples by increasing the amount of tissue material in individual paraffin blocks. For prostate biopsies, this would mean embedding 5–6 cores in the same block. An efficient alignment method would also decrease the amount of slices needed from the blocks, saving more tissue for later use in research.

This thesis is made up of a theoretical part and a practical part. In Chapter 2, the current issues in histological sample processing are outlined and existing solutions to these issues are described. Chapter 3 describes the stages of the actual development process, from initial problem-setting and requirements to the new solutions proposed based on a literature review. Chapter 4 tells about the manufacturing and testing methods of two different prototypes for a tissue sample alignment device. The results of the prototype testing are reported in Chapter 5. Chapter 6 discusses the most important findings and offers new ideas for future development. Chapter 7 presents the conclusions of this thesis work.

The thesis work was done in association with the pathology department of Fimlab Laboratories in Tampere. Fimlab Laboratories is Finland's largest laboratory company. It is owned by the hospital districts of Pirkanmaa, Tavastia Proper and Central Finland and it provides laboratory services for the public health sector of those regions. The pathology department of Fimlab Laboratories in Tampere handles and evaluates approximately 30 000 histological samples annually, of which 1 000 are prostate biopsies. The histological sample handling processes presented in this thesis are based on the practices currently used in Fimlab Laboratories.

2. THEORETICAL BACKGROUND

This chapter deals with the currently existing methods for small histological sample processing. First, the tissue handling process is described thoroughly and the need for its improvement is established. The latter part of the chapter reviews the currently existing methods and products for improving small tissue sample alignment and paraffin embedding.

2.1 Tissue processing

Typical tissue sample processing begins by preparing a small representative sample and placing it in buffered 10 % formalin fixative solution. Formalin fixation will cease all metabolic activity in the tissue by cross-linking proteins, making the tissue slightly more solid [1]. After the tissue sample is fixed, it is put in a plastic tissue cassette. The tissue cassette is a small rectangular container with a lid, with holes to allow fluids to pass to the tissue sample in the tissue processor. The tissue cassette also has a barcode for identifying the sample throughout the process. The cassette lid is closed and the cassette is placed into an automated tissue processor. Inside the tissue processor, the tissue is subjected to a series of alcohols (e.g. 70 % ethanol, absolute ethanol, and isopropanol), which gradually replace the water inside the tissues. Finally, the sample is impregnated with molten paraffin. The duration of the dehydration and impregnation process can vary between 4 to 12 hours, and is usually run overnight [1]. Impregnating tissue samples with paraffin makes them solid in room temperature. The solidified tissue sample is then taken out of the tissue cassette and put on the bottom of a metallic embedding mold. The mold is then filled with molten paraffin, which is allowed to harden leaving the tissue sample embedded into the paraffin block. The paraffin block is then sectioned into thin slices with a microtome blade. These slices are then attached onto microscope slides, which are then stained with tissue dyes for histological evaluation.

For prostate biopsies, the sample preparation process is illustrated in Figure 2. Here, the sample handling starts already in the operating room, when the urologist takes the biopsy with an 18-gauge needle biopsy gun. The biopsy cores are then put into formalin-containing vials, by dipping the needle in the vial and gently shaking it in the liquid. The number of biopsies taken from a patient varies, but current international guidelines suggest a systematic 10–12 biopsy protocol [8]. All biopsy cores may be put into separate containers or multiple samples can be pooled in the same container, depending on the performing urologist’s preference. Submitting cores in individual vials saves the location information of the cores. According to current guidelines, at least the biopsy

cores from the left and right lobes of the prostate must be separated into different containers [4; 8].

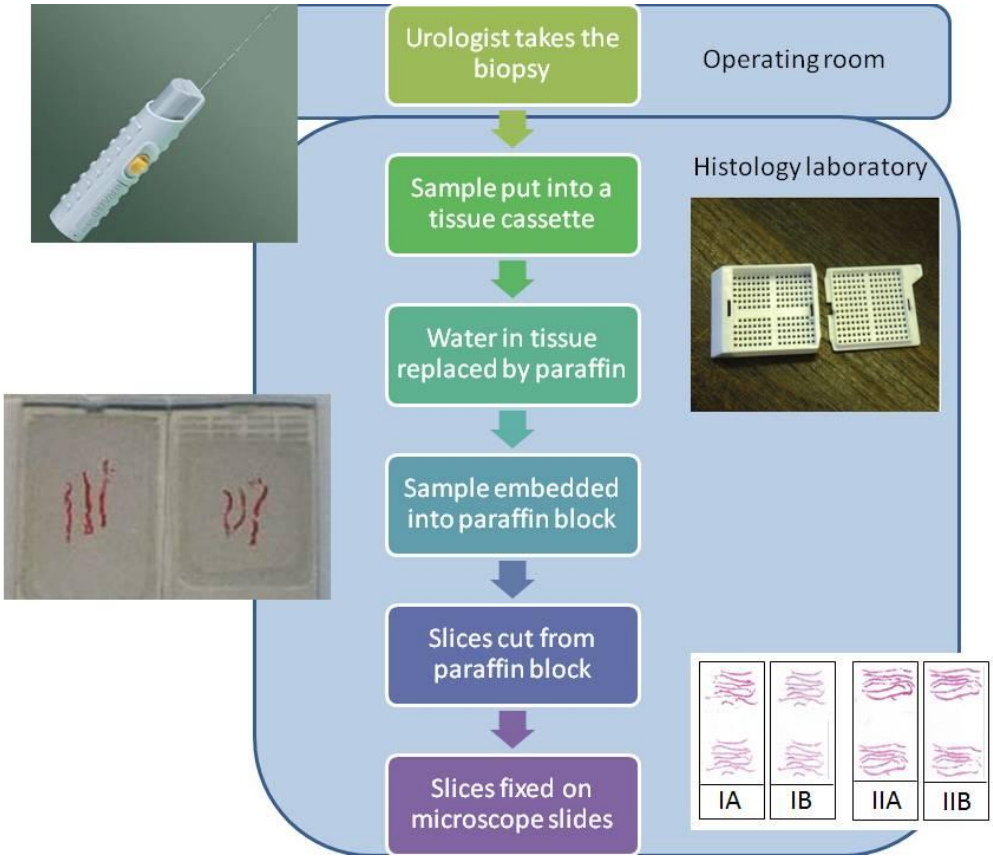

Figure 2. *Prostate biopsy handling process*

The pathology laboratory receives the prostate biopsies in the formalin vials. The biopsy cores are put into a tissue cassette and the cores must be picked out of the containers one by one. Often the biopsy material is fragmented, which renders the work slower and more difficult [9]. Fragmentation of the tissue may also diminish the accuracy of the patient diagnosis [10]. Usually, some pre-embedding method is employed to flatten and straighten the biopsy cores. For example, the tissue samples may be put between two nylon meshes [11] or pieces of paper or sponge [8]. In Fimlab Laboratories, the cores are straightened between two sponges after formalin fixation. The sponges also prevent the biopsy cores from escaping the tissue cassette inside the tissue processor. After the samples are impregnated with paraffin, they must again be picked up from the cassette individually and placed in the embedding mold. While the mold is filled with paraffin, the cores must be held at the bottom of the mold using forceps to make sure that they are properly aligned. It is also possible to use a metal tamper to hold the cores down during embedding [8].

Proper alignment of the prostate biopsy cores is very important for obtaining good quality microscope slides. If one cylindrical core is even 3° out of plane with the cutting blade, it reduces the area available for pathological evaluation by 11.3 % [12]. When

multiple biopsy cores are embedded into the same paraffin block, all of them must be in the same plane to achieve maximal tissue representation for pathological evaluation.

It is customary in Fimlab Laboratories to embed up to six prostate biopsy cores in the same paraffin block if they are submitted in the same biopsy container (pooled bilateral biopsy). Embedding six cores requires skilled technical staff, but does not necessarily compromise the quality of the resulting microscope slides [13]. Embedding multiple biopsies in the same paraffin block would be cheaper, as it requires fewer containers, less reagents and fewer microscope slides and coverslips. It also would save working time from the pathologists, as it is faster and easier to view few microscope slides with more tissue on them than to look at smaller tissue samples on many microscope slides. However, sometimes urologists want more detailed information on the original location of the biopsies, as it may be beneficial for the potential treatment of the patient. If it would be possible to preserve the location information of individual biopsy cores and still submit and embed them together, it would satisfy everyone, pathologists and urologist alike.

2.2 Existing solutions

As already mentioned, most pathology laboratories use some pre-embedding technique to flatten and straighten small tissue samples before paraffin impregnation. Pre-embedding techniques are widely used because they are cheap and simple. Commonly used pre-embedding techniques include enveloping the tissue sample in paper or between two sponges [8] or putting the tissue pieces between nylon meshes before formalin fixation [11]. The main problem with pre-embedding methods is that it does not eliminate the need for manual handling of the individual tissue pieces and may slow down the tissue processing. Using nylon meshes for flattening individual prostate biopsy cores, for example, takes 10 seconds per core [11]. In addition, the pre-embedding methods may cause some small scale deformation on the fragile tissue pieces, which can be seen on the resulting microscope slide.

To maintain the location information of the cores, using ink or dye has been suggested [11]. The cores can be marked before putting them in the formalin vials. Additionally, different color dyes can be used to separate the different locations of the prostate. However, inking of the cores must be done in a way that does not interfere with the pathological evaluation of the samples.

As for devices for alignment of small tissue samples, the existing solutions include both devices currently on the market and devices covered by existing patents in the area. The patent search on the current state of the art was done using both United States Patent and Trademark Office’s (USPTO) uspto.gov-database and European Patent Office’s (EPO) Espacenet-database. The results of the product and patent searches are summarized in the following sections.

2.2.1 Tissue alignment devices by Sakura

A company called Sakura Finetek USA Inc. (Torrance, CA, USA) is the market leader in tissue processing. Sakura manufactures different types of tissue processing equipment and focuses on facilitating automation of tissue sample processing. Sakura's hard plastic tissue cassettes (Tissue-Tek® Uni-Cassettes®) are the current standard in tissue processing in many pathology laboratories, including Fimlab Laboratories. Sakura has also introduced microtome sectionable tissue cassettes on the market: the Tissue-Tek® Paraform® cassettes. The Paraform® cassettes have a rigid external framework similar to the traditional plastic tissue cassettes, but the interior of the cassette consists of mesh-like basket and lid, as shown in Figure 3. [14]

Sakura's Paraform® cassettes are intended to eliminate the need for manual handling in tissue processing. Once put into a Paraform® cassette, the tissue sample remains there throughout the process. Because the Paraform® cassettes are sectionable, they can be embedded into paraffin and sliced with a microtome blade together with the tissue samples. Sakura claims that the Paraform® cassettes can be sectioned with a microtome as easily as the paraffin block itself [14]. However, in a recent study conducted in Tampere University of Applied Sciences, it was observed that the slices of paraffin embedded tissue samples were prone to disintegrate during sectioning when the Paraform® Core Biopsy (device a) in Figure 3) cassette was used [15].

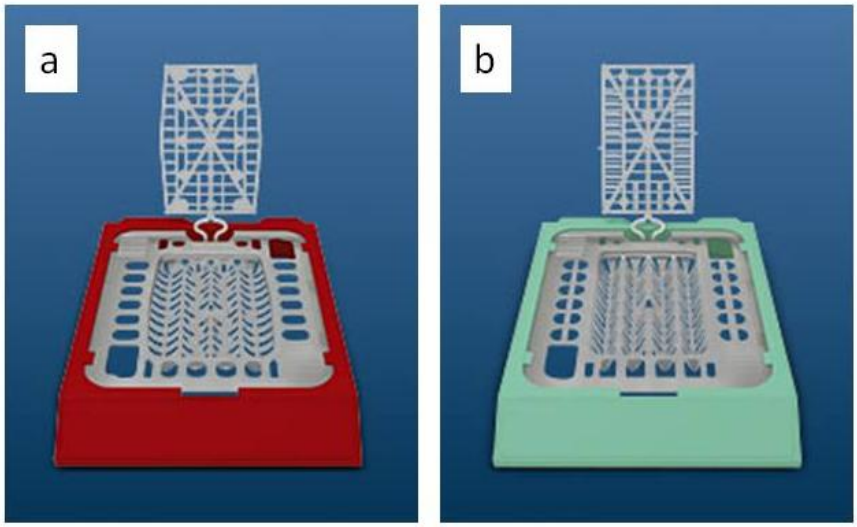

Figure 3. *Sakura's Tissue-Tek® Paraform® tissue embedding cassettes suitable for small sample embedding: a) Core Biopsy Cassette and b) Shaved Biopsy Cassette. Modified from [14]*

There are several different designs of Paraform® cassettes. Paraform® cassettes designed for large or medium sized tissue samples perform quite well, but small tissue samples and biopsies are more difficult to embed [16]. The problem with Sakura's Paraform® cassettes lies in inefficient immobilization of small and thin samples, such

as prostate biopsies [16]. According to Sakura, most suitable Paraform® cassettes for prostate needle core biopsies are the core biopsy cassette and the shaved biopsy cassette (shown in Figure 3) [14]. The samples are intended to be immobilized between the grooves of the bottom of the cassette and the lid. However, the shape of the cassette does not allow for bent or twisted biopsies, and fragmented samples may escape the cassette through the perforations [16]. Furthermore, the biopsy cores are not always easily placed on the bottom of the grooves, which may compromise proper alignment when multiple cores are embedded in the same cassette [16]. Also, the grooves of the cassette can only hold four cores simultaneously, which can be 3–20 mm long and 0.8–1.2 mm in diameter [14].

For a more thorough understanding of Sakura's products, their patents were screened from USPTO's and EPO's patent databases. The earliest patent describing the microtome sectionable cassettes was filed in 1996 [17]. The patent search revealed that there are some provisional aspects to the Paraform® cassettes, which are not utilized in Sakura's current products. Among them is a cassette lid with soft feather-like protrusions to gently immobilize tissue samples to the bottom of the mold [18]. The soft protrusions would help to keep the tissue samples in the bottom of the cassette without piercing them [16]. The reasons for not utilizing the inventions described in the patents may be due to technical or economical challenges, i.e. the described structures may be difficult or expensive to manufacture.

The same inventor, Williamson, has also provided a patent describing a variety of different designs for an alignment device for long and thin samples, which can be placed on the bottom of a tissue cassette. The alignment device is a supportive structure made from the same Paraform® material and it snaps onto the bottom of the Tissue-Tek® Paraform® cassette for medium sized samples as shown in Figure 4. The alignment device has spikes that are intended to hold the biopsies in place. [19] This kind of a device has not yet been commercialized by Sakura. It is possible that the spikes of the alignment device are not successful in immobilizing the samples, or they may pierce the samples and hinder pathological evaluation of the tissue.

Sakura's patent by Williamson et al. from 2014 describes another sample alignment device, which is not currently on the market. The invention describes a microtome sectionable crosslinked gel support structure made of starch and either gelatin or agar. The crosslinking is achieved in both cases with borax (sodium tetraborate). The preference of agar instead of gelatin is noted, because gelatin left on the microscope glass will stain with eosin dye. The patent describes a gel block with a slit for inserting small biopsies (see Figure 5). The immobilization of the biopsies onto the support structure is done by means of mechanical clamping of the two sides of the gel support. However, the patent also describes the possibility to use of an adhesive on the surface of the gel. [20]

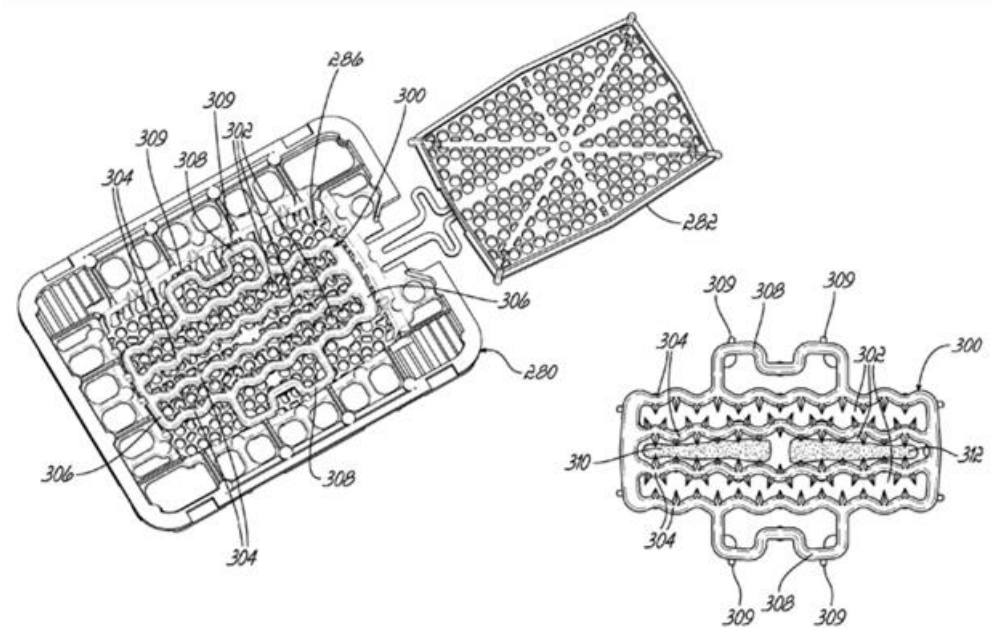

Figure 4. *Tissue alignment device and its placement on the bottom of a Tissue-Tek® Paraform® cassette. Modified from [19]*

For the gel structure, the immobilization of the samples seems much more effective than for the Paraform® cassettes. However, the gel block is quite robust and whether it is actually easily sectionable and able to pass the tissue processing fluids, is not determined. It is also possible that Sakura has not yet utilized the invention commercially because it it not readily compatible with their current line of Paraform® cassette products.

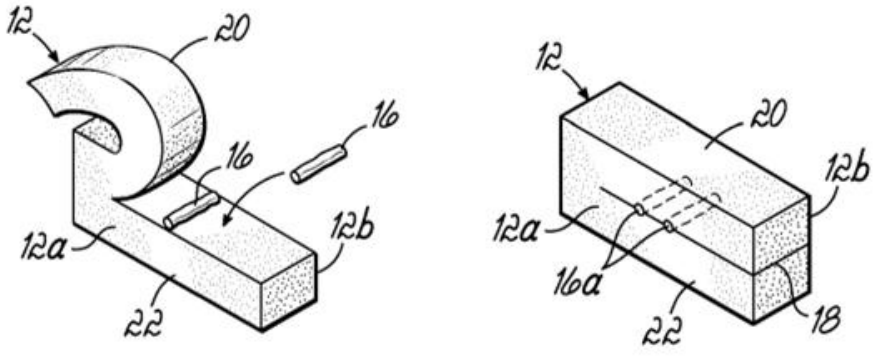

Figure 5. *Sectionable gel support structure for embedding small tissue samples with a slit for inserting the samples. Modified from [20]*

2.2.2 SmartBX™ system

Another currently commercially available solution for processing specifically prostate biopsies is the SmartBX™ system by UC-Care Medical Systems (Yokneam, Israel). UC-Care provides a whole system (Navigo™) for performing TRUS prostate biopsy,

with special focus on maintaining the location information of the cores. This is done by using 3D modeling of the prostate using ultrasound and electromagnetic sensors on the needle guiding probe [21]. The location information of the cores is then saved in the digital 3D model of the prostate [21].

The SmarBX™ tissue collecting device comprises of a biopsy needle gun, needle gun housing, which holds the biopsy needle in place on a sliding table, a cassette holder and a lever, which presses the tip of the needle down on sample sheet [22]. The biopsy is attached onto the sample sheet straight from the biopsy needle. According to UC-Care's patent, the sample sheet is a mesh film of cellulose esters or a film of cellulose filter [23]. The sample sheet is nonsymmetrical to maintain the alignment information of the biopsy cores regarding the biopsy needle (distal vs. proximal ends). The patent also allows the cellulose ester film to be covered with glue or some other adhesive [23].

The SmartBX™ patent also describes a method for tissue sample embedding with the sample sheet and biopsy cores. After tissue impregnation, the sample sheet is embedded in paraffin with the tissue samples toward the bottom of the mold. In the resulting paraffin block, the biopsies are on top of the block and the sample sheet remains embedded in paraffin (see Figure 6). [23]

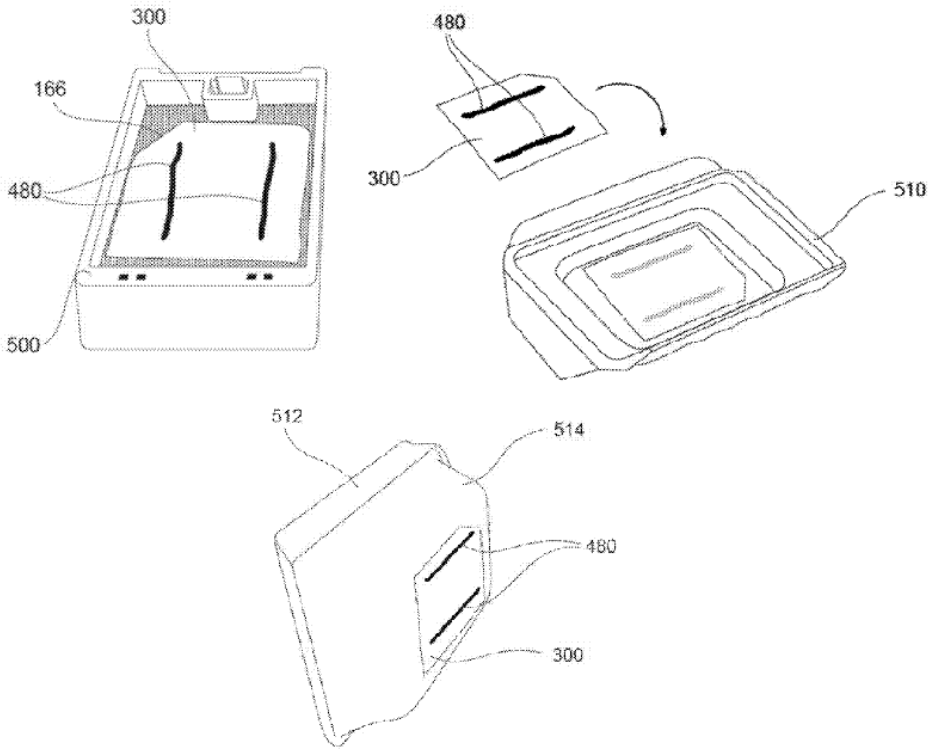

Figure 6. *SmartBX™ system's sample sheet with prostate biopsies is embedded in a paraffin block face down. Modified from [23]*

Cellulose, i.e. paper, is a very good substrate for attaching small tissue biopsies, as the water in the tissues is absorbed into it. It seems questionable whether the sample sheet

actually stays straight during paraffin embedding, but according to users of the SmartBX™ system, the cores stay perfectly straight and level, exactly 100 µm from the surface of the block [24]. The embedding of the samples needs a special mold and associated tamper [22; 24]. The tamper is responsible for the proper alignment of the sample sheet in the paraffin block [22]. Overall, the SmartBX™ seems very promising in prostate biopsy embedding, ensuring sample alignment and reducing the amount of manual handling steps. The SmartBX™ biopsy system is designed to work together with the location information preserving Navigo™ system, but can be also used without it. However, to benefit from the SmartBX™ biopsy core alignment system, procuring the entire biopsy system is required, including the special embedding molds and tampers, and is an expensive investment for pathology laboratories. Also, the SmartBX™ allows only two biopsy cores to be processed on each sample sheet, since the needle gun housing and cassette holder only allow two different positions of the needle tip with respect to the sample sheet [23].

2.2.3 QuickMBed

Although the company QuickMBed Inc. (Bethesda, MD, USA) no longer exists, it provided a product for embedding of needle core biopsies. The QuickMBed product was a silicone pad with flexible base and rows of stems shaped like bowling pins (shown in Figure 7). Different sizes of needle core biopsies could fit between the stems and were held there efficiently [25]. The silicone pad had also holes for tissue processing fluids to penetrate to the samples. Although the silicone pad was marketed for automated embedding by being microtome sectionable [26], silicone is not easily sectioned with a microtome blade.

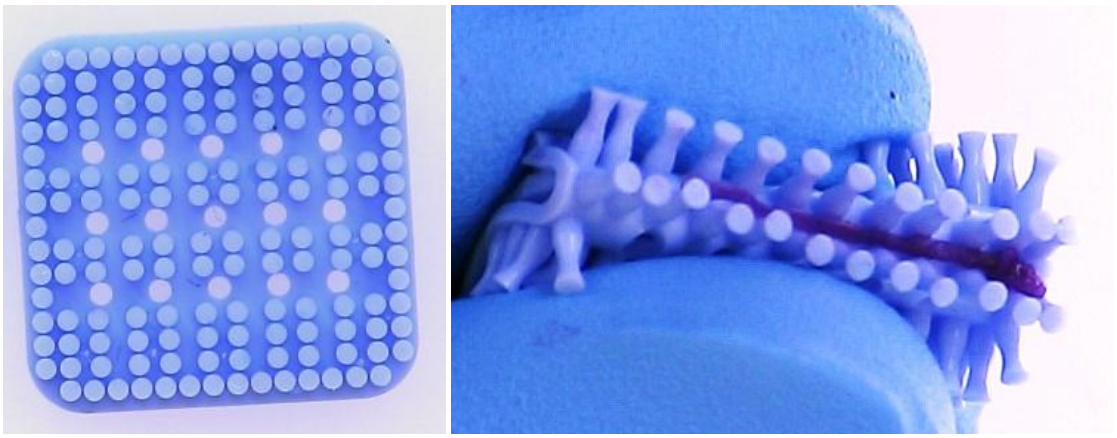

Figure 7. *QuickMBed silicone pad for orienting tissue core samples*

Unfortunately, QuickMBed later abandoned the silicone pad due to patent infringement disputes with Sakura. However, QuickMBed still owns a patent for a tissue sample alignment scaffold made from a hydrogel containing at least one component which is substantially liquid in temperature range 4–37 °C (i.e. water) [27]. The liquid compo-

nent in the scaffold is to be replaced in the impregnation process by paraffin. The patent describes a scaffold, which is flexible enough to allow for it to be bent but sufficiently rigid to hold tissue samples in a particular alignment. The scaffold in the patent is also comprised of a base and supporting stems (like the silicone pad in Figure 7). [27]

Although the shape of the QuickMBed silicone pad was effective in immobilizing needle core samples and keeping them properly aligned [25], manufacturing a similarly shaped structure from a gel material would be technically quite demanding. Also, the patent does not give examples of specific materials, which could be used to manufacture the scaffold, which indicates that the patent describes a concept rather than a product.

3. DEVELOPMENT PROCESS

This chapter describes the steps taken in the development process for the small tissue sample alignment method. The first step was listing different potential ideas for approaching the problem. The requirements regarding usability, processing and structure were used as the basis of the device development. After the suitable approaches were identified, material considerations for an alignment device were listed and specific materials for the device were reviewed based on existing literature.

3.1 Problem-setting

The goal of this work was to develop a tissue sample alignment device for aligning small histological samples, which addresses the issues of current tissue processing; sample processing costs, sample quality and fast and accurate patient diagnosis. To reduce the amount of manual handling steps and material costs in the pathology laboratory, the device should facilitate the embedding of at least 6 small samples simultaneously. The device should ensure high quality samples for pathological evaluation and, thus, improve the accuracy of patient diagnosis and reduce the need for additional biopsies. For optimal patient diagnosis and treatment, the location information of the cores should also be preserved.

After reviewing the currently existing solutions for aligning small tissue samples, possible approaches for developing a new alignment device were outlined. It was noted that all of the currently existing solutions (described in section 2.2) are devices, which are intended to remain in the paraffin block during sectioning. However, differences in the densities of the device and paraffin can harm the samples during sectioning. At least with Sakura's Paraform® cassettes, disintegration of the sample slices has been noted due to the non-uniform composition of the block [15; 27].

To avoid the device material interfering with the slicing of the samples, we chose to develop a tissue sample alignment device, which would be removed after paraffin embedding leaving only the tissue samples in the paraffin block. To be removed from the paraffin block, the device needs to be placed on the bottom of the metallic embedding mold with the tissue samples facing upwards (compare to the setup in Figure 6 on page 10). In the end, the device will be left on the surface of the paraffin block, from where it can be removed.

A key issue in holding tissue samples straight and level is effective sample immobilization. Also, immobilization of the samples from the beginning of the biopsy procedure

can help maintain their location information and also eliminates one additional manual handling step. The immobilization technique must be able to withstand exposure to fixation and impregnation chemicals. On the other hand, the immobilization of the tissue samples has to allow for the device to leave the samples in the paraffin block undisturbed when removed.

3.2 Different approaches

Initially, there were many different ideas for the tissue sample alignment device. Two different approaches for effective immobilization of tissue samples onto the device were considered; mechanical immobilization and surface attachment (i.e. chemical or physical immobilization). The different ideas for the removable tissue sample alignment device are illustrated in the flow charts below. Figure 8 shows the different ideas regarding mechanical immobilization techniques and Figure 9 the chemical and physical surface attachment.

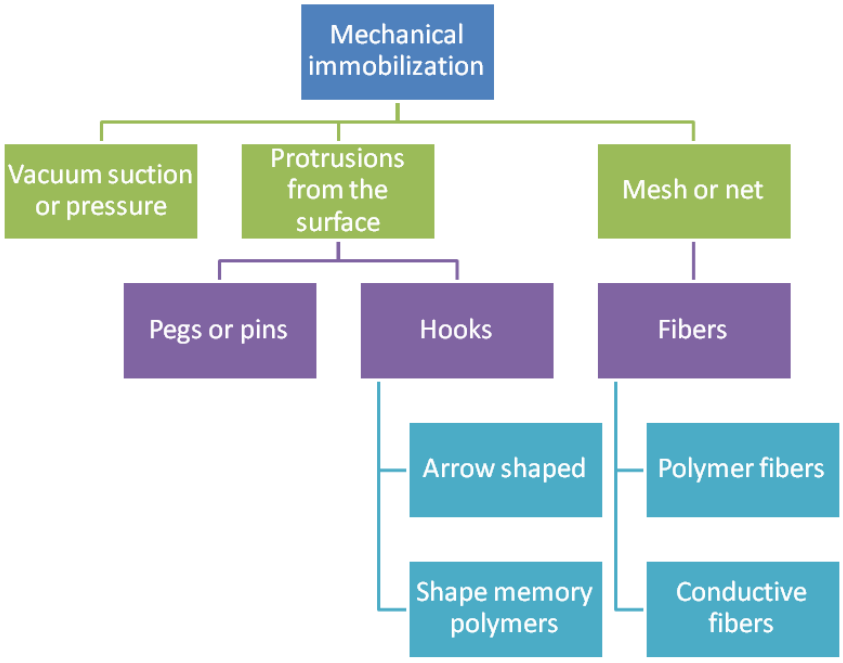

Figure 8. *Ideas for an alignment device based on mechanical immobilization of tissue samples*

The ideas for the mechanical immobilization approaches were mainly inspired by the existing solutions. Sakura's Paraform® cassettes and the QuickMBed pad are examples of immobilization through surface protrusions with different kinds of pegs. However, in a one-sided device, the pegs would need to be hooked to trap the tissue samples. Shape memory polymers were suggested because they can change their shape with changes in temperature. The samples could also be immobilized between fiber meshes, similarly to

the previously described pre-embedding technique. Since both meshes would need to be removed from the paraffin block prior to sectioning, conducting fibers were thought to be removable from the block through heat conduction.

Eventually, all ideas for mechanical immobilization (presented in Figure 8) of the tissue samples were abandoned as unviable. The vacuum suction, for example, was deemed unpractical to carry out and possibly harmful for the fragile tissue material. Most of the ideas were discarded based on lack of available resources. Also, all expensive materials and manufacturing methods had to be discarded because the developed device should be relatively cheap.

Instead of mechanical immobilization, we chose to focus on surface attachment through chemical or physical immobilization. The different suggestions for obtaining surface attachment of the samples are shown in Figure 9. Because the aim is to develop a device, which is able to receive the fresh tissue samples already in the operation room, we have to take into account the wetness of the tissues. This may be a problem in using adhesives, but provides the possibility to make use of capillary action.

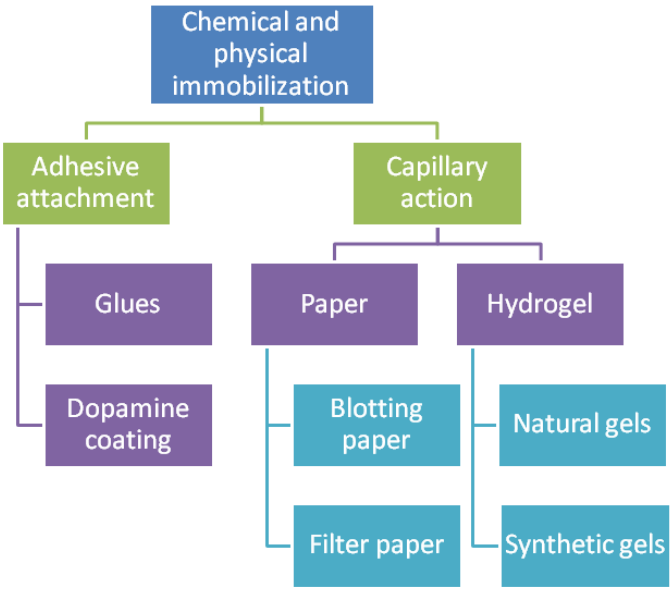

Figure 9. *Ideas for an alignment device based on immobilization of tissue samples using chemical or physical surface attachment*

It has already been established that paper is a good material for attaching tissue samples straight from the biopsy needle (as in the SmartBX™ system). Paper, however, contains the problem of maintaining its composition inside the tissue processor and it may leave traces of cellulose fibers on the tissue samples, which interfere with the pathological evaluation of the samples. The strong suction effect of paper causes the tissue samples to lie flat along the surface of the paper, which may cause them to deform with the surface roughness and pores of the paper. Paper is also not rigid enough, at least on its

own, to keep the tissue samples in plane. As with the SmartBX™ system, a tamper would be needed to hold the paper straight.

Hydrogel materials, three-dimensional polymer networks containing water, were suggested because of their capability of absorbing water into their structure. It was anticipated that the tissue pieces could attach to the gel material by means of capillary action as well as water cohesion between wet tissue samples and water inside the gel. Since hydrogels are a diverse group of materials, it might be possible to find a gel which would be rigid enough on its own to maintain the alignment of the tissue samples.

The idea was to develop a simple mat-like structure, which is able to hold the tissue samples in place throughout the sample handling process and can then be removed from the final paraffin block prior to microtome sectioning. Figure 10 shows a schematic description of existing methods and the suggested methods for tissue sample alignment. As can be seen from the figure, the number of biopsy cores attached to the different alignment devices varies. Sakura's Paraform® cassettes are able to simultaneously hold four biopsy cores in its four grooves and the SmartBX™ system is restricted to attach only two samples on the same sample sheet because of the needle gun housing system [23], although the paper sheet itself could fit more cores. The suggested methods for tissue sample alignment have the advantage of simple design and unrestricted amount of biopsy cores per device. For prostate biopsies, at least 5–6 cores should be attachable to the same device to minimize the number of resulting paraffin blocks to two.

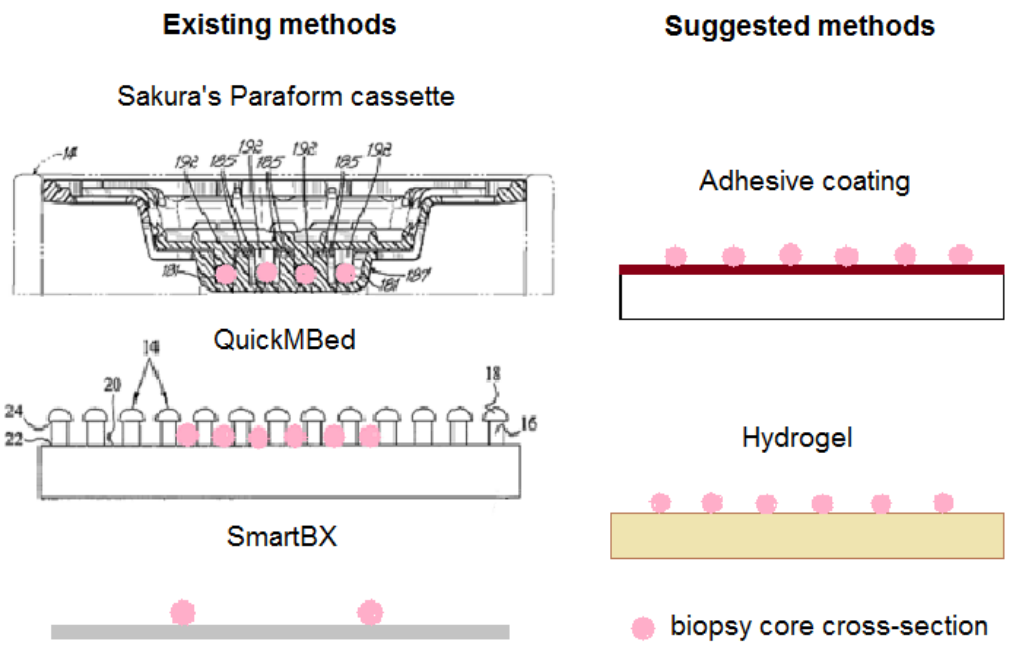

Figure 10. *Illustration of existing tissue sample alignment methods and the suggest methods for biopsy alignment.*

3.3 Requirements for the device

In development of a method for improving the diagnosis of small tissue samples, all the needs of the different operators (patient, operating specialist, histology laboratory and pathologist) in the process must be considered for the optimal result. These needs constitute the requirements the device must fulfill. The patient needs the diagnosis to be fast, reliable and conclusive to ensure proper treatment without the need for repeat biopsies. The specialist physician (e.g. urologist) is in a key role in obtaining the necessary biopsy of high quality and, later, planning the patient treatment based on the diagnosis. For planning the treatment, the physician needs as precise information as possible of the disease, e.g. extent and location of cancer. The histology laboratory wants to process the tissue samples as cost-efficiently as possible with high quality results. The pathologist wants to see maximal tissue representation on the microscope slide for most reliable diagnosis and efficient workflow.

Based on these needs, two goals were set for the development of the tissue sample alignment device. Firstly, the device should reduce manual handling time and material costs. Secondly, the device should ensure the quality of patient diagnosis by maintaining the location information of the tissue samples. More specifically, the device should be able to hold multiple samples simultaneously, preferably 6 for prostate biopsy cores, and the tissue samples should be put on the device already in the operation room.

Multiple sample embedding provides us with certain additional requirements. The first requirement for the alignment device is that it should hold the tissue samples straight and on the same plane for proper sectioning. To fulfill this requirement, the device should be made of a rigid material and keep the tissue samples from bending. Also, the device should hold the tissue samples in place throughout processing. The tissue processor demands that the device material withstands the high temperatures and chemicals used in processing without dissolving or otherwise changing its shape. Details of the tissue processing cycle are listed in Appendix 1. Also, the device should not obstruct the infiltration of the processing chemicals to the tissue. From the quality point of view, the device should not interfere with the histological examination of the tissue, as this could compromise the diagnosis of the patient. The device should not pierce or otherwise damage or deform the tissue samples. Also, there should not be any residues of the device present on the resulting microscope slide, which could affect the pathological evaluation of the sample. For preserving location information of the biopsies, placing the tissue samples onto the device already in the operation room is essential. Because the same biopsy needle is used for obtaining all biopsy cores, the device needs to be sterile to avoid contamination of the biopsy needle between samples. Also, the attachment of the samples should be fast and effective for the urologists to use the device.

For the device to be integrated into the currently existing tissue handling and embedding process, the shape and size of the device need to be designed accordingly. As the metal-

lic paraffin embedding mold is the most restrictive element in the process, it defines the outer dimension for the device. The largest metallic embedding mold is a rectangle with rounded corners with height of 29 mm and width of 24 mm. The embedding mold is approximately 5 mm deep and the device should not be thicker than 2 mm to allow for sufficient filling of the mold with paraffin.

3.4 Material considerations

The suggested solutions for the device were simple flat platform, which provide sufficient support and attachment for the small tissue samples, as depicted in Figure 10 on page 16. In its simplest form it would consist of only one component. The second simplest option is to make the bulk of the device from one material, which provides support, and a coating material for sample attachment. The material for the device should be relatively cheap and easily processed. Additionally, the device should be stiff enough to hold the tissue samples in plane but flexible enough that it withstands bending and can be easily removed from the paraffin block. For the bulk of the device, silicone elastomer was considered because of its easy manufacturing, inertness in the tissue processing fluids and good elastic properties. To provide adhesive properties, we considered different approaches of coating the silicone elastomer.

The first approach was to coat the silicone with some adhesive material. However, using adhesive for attaching the tissue samples proposed some problems. The bonding strength of the adhesive to the tissue samples would have to be weak enough that the samples are left in the paraffin block once the device is removed and the adhesive should remain on the surface of the silicone. The adhesive would also need to survive the tissue processing chemicals. When searching for suitable candidates for surface adhesives, we came across a novel mussel-inspired multi-functional coating material, dopamine.

The second proposed approach to achieving surface attachment of tissue samples was to apply a hydrogel layer on top of the silicone material. It was hypothesized that by using a hydrogel material on the surface of the device, the samples could adhere to the surface by capillary action. Most likely cohesion also plays a role in attachment of the two wet components. There is a wide variety of different hydrogel materials, but the cost of the material and the complexity of its manufacturing method are limiting factors.

Finally, we identified also a material which could provide both structural support and the surface adhesion capabilities, a hybrid gel of gelatin and siloxane. The gelatin-siloxane gel is basically a hydrogel with improved elastic properties. The hybrid gel could allow us to manufacture the simplest possible device, a one-component platform for immobilizing and orienting small tissue samples.

3.4.1 Silicone elastomer

Silicones are polymeric materials comprising of an inorganic Si–O–Si backbone with two functional groups attached to each silicon atom. The most common type of silicone is poly(dimethylsiloxane) (PDMS), which has two methyl groups ($–CH_3$) attached to the silicon atoms of the backbone as shown in Figure 11. Silicone elastomers are three-dimensional crosslinked structures comprising of PDMS molecules, fillers and additives [28]. Silicone elastomers are strong and flexible with relatively constant elastic properties in the temperature range of –40 °C to 180 °C [28]. Silicone elastomers also have high thermal stability, durability and chemical resistance and they are biocompatible. These properties make them appealing in many applications varying from electronics and automotive industry to medical devices [28; 29].

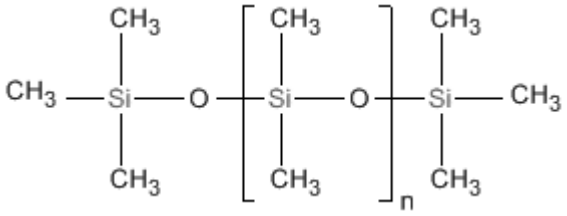

Figure 11. *Structural formula of PDMS*

Silicon elastomers are produced by chemical crosslinking of PDMS chains. The crosslinking can be done via a condensation or an addition reaction in the presence of a catalyst, most often tin or platinum [28]. In many applications, the addition curing reaction is favored over condensation, as there is no shrinkage upon curing [28]. Often laboratory grade commercial silicone elastomer materials are sold as two-component silicone elastomer kits, such as Sylgard® by Dow-Corning (Midland, MI, USA) or Elastosil® by Wacker Silicones (Munich, Germany). The two components are mixed in a suitable ratio and the mixture is cast into a mold and cured. Depending on the additives of the silicone components, the curing reaction may require elevated temperatures or it can occur at room temperature.

Due to its non-polar chemical structure, PDMS is highly hydrophobic, which limits its uses in many medical applications. To overcome the inherent hydrophobicity of PDMS, different methods have been employed to increase the hydrophilicity of silicone surface through oxidation of the surface methyl groups. The most commonly used methods are plasma treatments using ionized gases (oxygen, air or argon) and UV/ozone treatment [30]. In plasma treatments, surface methyl groups in PDMS are replaced by hydrophilic silanol (–SiOH) groups [30; 31]. The plasma treatments are favored for their short treatment times and easy operation, but they require expensive equipment [30]. In addition to plasma processing, PDMS surface has been functionalized through chemical oxidation. Combinations of hydrochloric acid (HCl) and hydrogen peroxide (H_2O_2) [32]

or H_2O_2 and sulfuric acid (H_2SO_4) [33] have been used to oxidize the methyl surface groups of PDMS to silanol groups.

For both plasma treatment and chemical oxidization the increase in hydrophilicity is only temporary. The PDMS surface undergoes hydrophobic recovery, where low molecular weight polymer chains migrate to the surface to minimize surface energy [29-31; 34]. Hydrophobic recovery occurs in a matter of hours after exposure to air [30; 31]. To maintain the hydrophilic property, the silanol groups can be used to covalently graft functional molecules [30]. Silanes, such as (3-aminopropyl)triethoxysilane (APTES), are often used as linker molecules to graft macromolecules, such as proteins, onto PDMS surface silanol groups [30].

Another way to increase PDMS surface hydrophilicity is physical adsorption of polymers and surfactants [31; 32]. For example, a method of introducing hydroxyl groups onto PDMS surface by adsorption of poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) has been suggested [32]. The hydrophobic backbones of the polymers adsorb into the PDMS leaving the pendant hydroxyl groups on the silicone surface [31; 32]. Surface modification of PDMS using physical adsorption is simple, but the resulting surfaces have weak thermal and mechanical stability due to the weak interactions between the coating molecules and the PDMS substrate [31].

Silicone elastomer would be an attractive material for the basis of the tissue sample alignment device, because of its elastic properties and chemical stability. Silicone is cheap and easy to manufacture into wanted shapes. However, its inertness and hydrophobicity make it difficult to provide proper attachment for the tissue samples. To attach the samples onto silicone, we need to provide a surface layer with adhesive function. In the following sections, some promising methods for attaching the samples onto the device surface are reviewed, and the ways to bind them to the silicone bulk material are discussed.

3.4.2 Dopamine coating

Marine mussels are capable of attaching themselves rapidly and strongly on a variety of different material surfaces even in a wet environment. The key element in this strong adhesion property has been attributed to the high concentration of cathecol and amine functionalities in mussel foot proteins containing 3,4-hydroxy-L-phenylalanine (DOPA) and lysine amino acid residues [35]. The small neurotransmitter molecule dopamine (3,4-dihydroxyphenethylamine) contains both cathecol and amine functionalities, and has been recently discovered to act as a universal coating material for virtually any surface, even very hydrophobic materials like poly(tetrafluoroethylene) (PTFE) and PDMS [35; 36]. Dopamine coating on PDMS has been shown to increase surface wettability and cell adhesion and growth on PDMS substrates drastically [37]. In addition to using dopamine as a coating, DOPA or dopamine derived functionalities have been grafted

onto various polymers, such as PEG and poly(acrylic acid), to obtain different adhesives for industrial and, especially, medical use [38-41]. Dopamine has shown great success as a functional component of synthetic tissue adhesive, as it provides strong attachment of wet surfaces without causing inflammatory reactions [41-43].

Dopamine coating can be applied on a material surface in a simple dip coating process. The material to be coated is immersed in an alkaline solution of dopamine in the presence of an oxidant [44]. Most commonly, molecular oxygen (from the ambient air) is used as the oxidant, but also Cu^{2+} ions have been used [45]. In solution concentrations of over 2 mg/ml dopamine is able to spontaneously self-polymerize to form a hydrophilic surface layer [35-37; 44]. The dopamine layer self-assembles on the coated surface through covalent bonding and intermolecular interactions, such as hydrogen bonding, [37; 44] and is very durable [36]. The formation of a polymerized dopamine layer is coupled with a color change from colorless to deep brown [44]. When oxygen is used as the oxidant, a maximum coating thickness of 45–50 nm has been reported, which is reached after 24 hours of immersion [44; 45]. If the coating time is increased, the dopamine coating can become unstable causing it to detach from the substrate [45]. Coating layers exceeding 50 nm can be obtained either by carefully drying the first dopamine layer and immersing the material in fresh dopamine solution or by using Cu^{2+} instead of oxygen for dopamine oxidation [45].

Dopamine coating procedure does not require any complicated instruments or harsh reaction conditions [44], which would enable simple manufacturing of the tissue sample alignment device. One possible disadvantage of the dopamine coating is the brittleness of the coating; bending a dopamine coated PDMS substrate has been shown to cause micron-scale cracks in the coating [37]. Furthermore, the stability of the adhesive has only been documented to be in the temperature range from –40 to 40 °C [46], and the temperature in the tissue processor rises to 100 °C. An additional concern is that the time for adhesive bonding to occur between the surface and the tissue samples may be too long to be useful in this application.

3.4.3 Hydrogel coating

Hydrogels are materials capable of retaining large amounts of water in their structure, consisting of a polymeric network. Hydrogels are inherently hydrophilic, although they can also contain hydrophobic groups in their structure [47]. Because of their high water content, hydrogels possess similar flexibility characteristics to those of the tissue, which makes hydrogels especially interesting in biomedical engineering [47; 48]. Other application areas of hydrogels include agriculture, food industry and drug delivery [48]. Classification of hydrogels can be done in different ways, for example based on their origin (natural vs. synthetic) or crosslinking method (physical vs. chemical) [47; 48].

Natural hydrogels are based on naturally occurring macromolecules, such as polysaccharides or polypeptides. Examples of hydrogels prepared from polysaccharides include cellulose (from wood fibers) [49], agar (from algae) and dextran (from bacteria) [47]. Polypeptides include gelatin (hydrolysis product of collagen from animal tissues) [47] and silk [50]. Synthetic hydrogels encompass a wide variety of different polymers and copolymers. Synthetic polymers have well-defined structures, which makes it easier to tailor their properties than with natural polymers [48]. The most commonly used synthetic hydrogels in industrial production are made of acrylic acid, also known as acrylate, and its salts crosslinked with methylene bisacrylamide [48]. Natural polymers can also be copolymerized with synthetic polymers to obtain a wide range of new hydrogel materials [47].

Hydrogels hold their shape through crosslinking of polymer chains. Crosslinking of hydrogel forming polymers can be achieved via chemical reactions, using ionizing radiation or by creating physical interactions such as entanglements or electrostatic interactions [48]. Natural polymers can also be crosslinked through enzyme catalyzed chemical reactions [49]. Chemically crosslinked hydrogels are more stable than physically crosslinked hydrogels, as physical crosslinking is reversible [47]. Examples of chemical crosslinking could be for example crosslinking gelatin with glutaraldehyde [51] whereas physical crosslinking occurs when gelatin is heated in water and allowed to cool [47]. During heating, the long gelatin molecules disentangle and upon cooling entangle with neighboring molecules [47].

The main disadvantage of hydrogel materials is that they are mechanically quite weak [52]. For the tissue sample alignment device, a certain degree of mechanical stiffness is required for holding the tissue samples in plane. The mechanical properties of hydrogels can be improved by grafting hydrogels onto a support material [48]. In this case we are interested in grafting onto silicone elastomer substrate. Some methods of combining PDMS and hydrogels have been reported in the literature, although it is challenging to attach hydrogels and silicone elastomers due to the discrepancy of their bulk properties [53].

Cha et al. have described a method to polymerize an alginate (an algae-derived polysaccharide) hydrogel onto PDMS surface by first covalently linking alginate molecules onto PDMS surface [53]. The surface-bound alginate molecules participate in the formation of the hydrogel coating and facilitate the permanent attachment of the coating onto PDMS surface. The covalent linking of alginate to PDMS surface was done in three steps, oxidation of PDMS surface, silanization using the silane APTES and conjugation of alginate. The oxidation of PDMS surface was done using the chemical method (H_2O_2 and HCl solution). The alginate linking was shown to improve the bonding of the hydrogel onto the surface significantly when compared to directly crosslinking the gel onto PDMS surface modified in different ways. [53]

Zhang et al. used chemical modification of PDMS surface together with micropillar structures to bond a PEG hydrogel onto PDMS. The micropillars were 66 μm high with diameter of 22 μm spaced 60 μm apart. The chemical modification was done by oxidizing the surface with air plasma and mixture of H_2O_2 and HCl and subsequently treating the surface with a silane linking agent ((trimethoxysilyl)propyl methacrylate, TMSPMA). Figure 12 illustrates the need for both mechanical and chemical bonding of the hydrogel onto PDMS surface. The first row of images shows only covalently linked PEG hydrogel on PDMS surface, the middle row the use of micropillar structures without chemical modification and the third row the use of both chemical and mechanical anchoring. As can be seen from Figure 12, only the hydrogel attached onto PDMS with both chemical and mechanical anchoring withstood the bending without failing. [54]

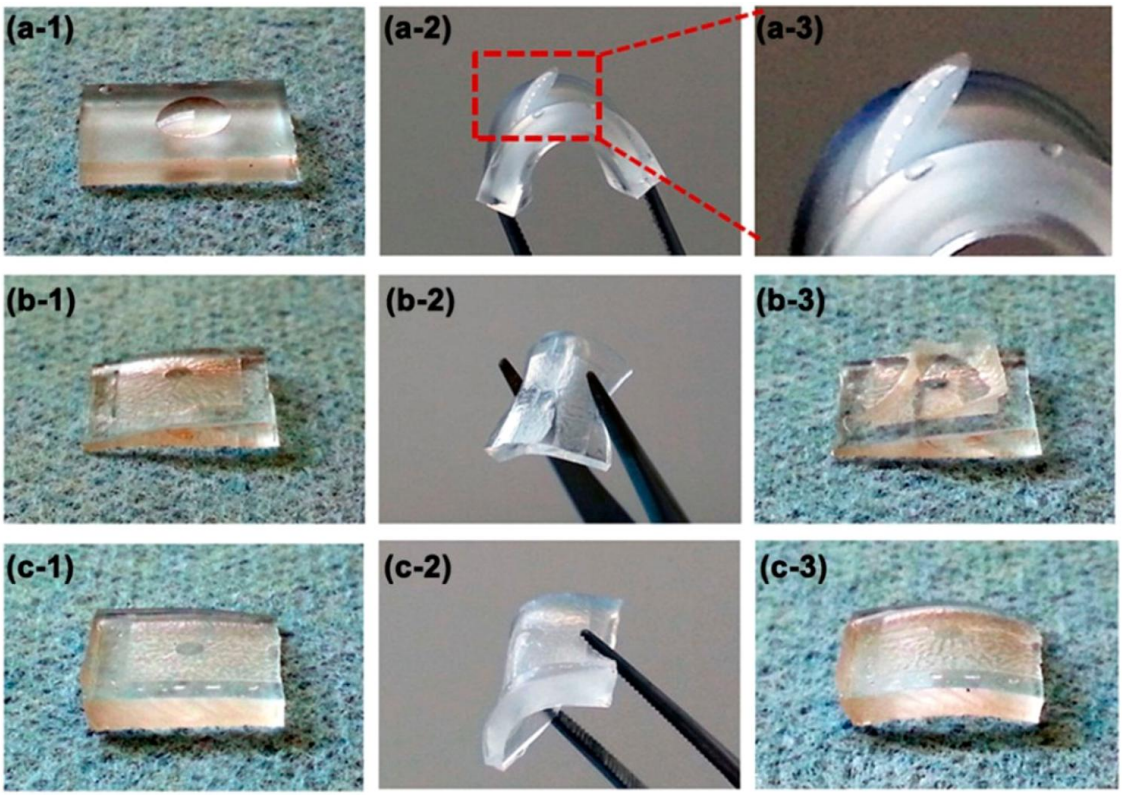

Figure 12. *Attachment of a PEG hydrogel on PDMS surface a) with chemical crosslinking but no mechanical anchoring b) with mechanical anchoring but no chemical crosslinking c) with mechanical anchoring and chemical modification [54]*

Zhang et al. demonstrated that efficient attachment of a hydrogel onto PDMS requires both mechanical and chemical anchoring to the surface [54]. However, the fabrication of micropillar structures with photolithography requires expensive equipment. Another method to produce mechanical anchoring could be to etch the surface of the silicone elastomer using corrosive chemicals. Brook et al. have shown that it is possible to achieve PDMS surface roughness of up to 1 μm using potassium hydroxide (KOH) solution [55]. The KOH surface etching technique could be used similarly to the

micropillars of Zhang et al. to produce mechanical anchoring for hydrogel material. However, the roughness is on a much smaller scale than that obtained with the micropillars. Furthermore, chemical grafting of the hydrogel onto the silicone surface would also be needed.

3.4.4 Gelatin-siloxane hydrogel

For the simplest solution to make a tissue sample alignment device, we studied hydrogel materials, which could provide the mechanical support for the samples to stay in plane and the sample immobilization property. Most hydrogels are weak and fragile, and we required a gel with sufficient toughness to withstand handling and moving between the sample processing steps. For this purpose, a gelatin-siloxane organic-inorganic hybrid hydrogel was identified with the easy manufacture method and cheapness of a natural hydrogel and the good elastic properties similar to silicone.

Gelatin is an inexpensive and easily available material for hydrogel manufacture derived from degradation of collagen from animal tissues. Gels made from gelatin alone are fragile and dissolve readily in aqueous environment, but crosslinking gelatin molecules helps to stabilize the resulting gel. Both physical and chemical crosslinking has been employed. The most common chemical crosslinking agent of gelatin is glutaraldehyde (GA), because high degree of crosslinking can be achieved with even low crosslinker concentrations and it is inexpensive [56]. The use of GA as gelatin crosslinker is avoided in many applications because it is toxic to cells and to the environment and it causes easily irritation on exposure.

One suggested replacement for GA as gelatin crosslinker is (3-glycidoxypropyl)-trimethoxysilane (GPMS). It is a silane molecule containing an epoxy group and three methoxysilane groups. In acidic conditions, the oxirane ring is able to react with amino groups of the gelatin molecule, and the methoxy ($–OCH_3$) groups hydrolyze to form silanol groups. The silanol groups then condense with neighboring silane molecules to form covalent Si–O–Si linkages between the gelatin chains. The reaction mechanism is illustrated in Figure 13. [57] GPMS crosslinking has also been shown to enhance the mechanical strength of the gelatin hydrogel compared to linking with aldehydes [58]. Chemical crosslinking using GPMS reduces the wettability of gelatin, because the silane backbone is hydrophobic, and it simultaneously binds some of the hydrophilic amino and carboxyl acid groups of the gelatin chain [57; 59].

Ren et al. found that the relative amounts of gelatin and GPMS crosslinker have distinct effects on the properties of gelatin-siloxane hybrid gels [59]. They compared gelatin-siloxane gels with different mass fraction (w) of GPMS. With very low ($w = 0.09$) or high amounts ($w = 0.91$) of GPMS compared to gelatin, gelation does not occur. Phase separation of GPMS occurred when w was over 0.5. Increasing the GPMS content in-

creased the shrinkage but reduced the gelation time. Gelation times varied from 120 h for w = 0.33 gel to 24 h for w = 0.75 gel. [59]

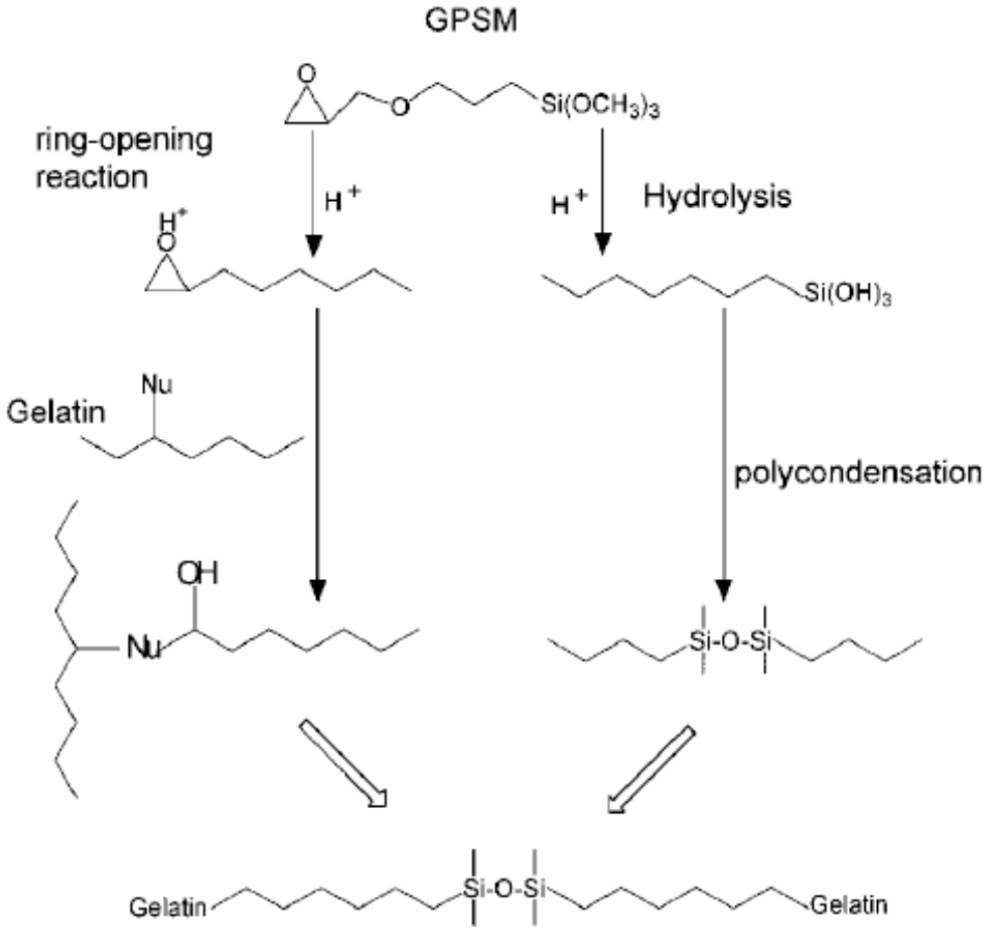

Figure 13. *Chemical crosslinking reaction of (3-glycidoxypropyl)trimethoxysilane (GPMS) crosslinker with gelatin chain side groups. [59]*

In addition to covalently linking gelatin chains using GPMS, the same molecule has been described to form physically linked gelatin-siloxane hydrogels. This physical crosslinking occurs mainly via hydrogen bonding between oxidized silanol groups and amino groups of the gelatin molecule. These gelatin-siloxane gels were made in a simple mixing process at elevated temperature (50 °C) followed by gelation at 37 °C. The physically crosslinked gelatin-siloxane hydrogels showed high failure stresses, up to 3.6 MPa, in compressive tests. [58]

The easy fabrication method of physical gelatin-siloxane hybrid hydrogel makes it an interesting candidate for the tissue sample alignment device. However, the acid catalyzed chemical crosslinking is also a possible means of manufacture as it does not require complicated equipment either.

3.4.5 Summary

As a summary of the materials considered for the manufacture of the tissue sample alignment device, an example from each device type is given in Table 1. The raw materials, number of preparation steps, manufacturing time and estimated costs per device are listed for each method. The costs are estimated for small scale production (500 pieces or less) of the particular device and include only the raw material costs. The costs are estimated based on the price of raw materials obtained from Sigma-Aldrich and other chemical suppliers of Fimlab Laboratories.

Table 1. *Summary of different alignment devices, their raw materials and costs*

Device	Raw materials	Preparation steps	Time to prepare	Cost per device	Sources
PDMS+dopamine	PDMS				
	Dopamine hydrochloride				[37; 45]
	Tris				[37; 45]
	$CuSO_4$	2	2-3 days	0.67 €	[45]
PDMS+hydrogel	PDMS				
	KOH (etching)				[55]
	H_2O_2+H_2SO_4 (oxidation)				[33]
	TMPSMA (linker)				[54]
	PEG diacrylate monomer				[54]
	Irgacure 2959 (initiator)	5	2 days	1.22 €	[54]
Gelatin-siloxane hydrogel	gelatin				
	GMPS		2 days	0.20 €	[58]
	(HCl)[1]	2	(3-5 days)	0.24 €	[57; 59]

[1] chemically crosslinked gelatin-siloxane

The efficient attachment of a hydrogel on the silicone surface would require many preparation steps and different chemicals. Because of the complicated preparation and material costs, the hydrogel coating on PDMS substrate was not selected for the preparation of prototypes for a tissue sample alignment device. Also, the chemical crosslinking method for gelatin-siloxane hydrogels was not used in manufacturing of the prototypes, because of the long preparation times required. Silicone coated with dopamine and physically crosslinked gelatin-siloxane hydrogel were chosen for the prototype manufacture.

4. PROTOTYPES

This chapter describes the preparation and testing methods of prototypes for the tissue sample alignment device. Two kinds of prototypes were prepared: silicone pads with dopamine coating and physically crosslinked gelatin-siloxane hydrogel pads. These materials were selected for the prototypes based on the results of the literature review and because they were easy to manufacture with relatively low cost raw materials.

All of the manufactured prototypes underwent proof of concept testing. In the proof of concept test, the prototypes were evaluated by testing the attachment ability of the tissue sample straight from the biopsy needle onto the device. The devices and the tissue samples were also put through tissue processing to observe the behavior of the prototypes throughout the chemical impregnation process of tissues. The gelatin-siloxane prototypes were also characterized for their material properties: water content, surface wettability and mechanical properties.

4.1 Preparation

The manufactured prototypes represent different structural approaches for the tissue sample alignment device. First, silicone pads coated with dopamine were prepared. Silicone was used as the bulk component of the alignment device to provide support and the dopamine coating was used for its adhesive character. The second approach was to use a hybrid hydrogel made of gelatin and siloxane as the single element of the tissue sample alignment device, which would provide both the structural support as well as the surface adhesion. The preparation methods of the two kinds of prototypes are described below.

4.1.1 Dopamine coating on silicone

The base for the dopamine coated prototypes was prepared from a flexible silicone elastomer, Elastosil® RT 601 (manufactured by Wacker Silicones, Germany, distributor Kevra Oy, Vantaa). The silicone was prepared according to manufacturer's instructions, by weighing the two components of the silicone elastomer in a 9:1 ratio and mixing them thoroughly. The mixture was then poured into metallic embedding molds and allowed to cure for 24 h in room temperature. After curing, the silicone pads were removed from the molds. The curved edges of the pads were removed using a surgical blade (Feather) to improve the setting of the pads on the bottom of the same molds during tissue sample embedding. To facilitate the movement of chemicals inside the tissue processor, punctures were made on the silicone pads using a 1 mm disposable biopsy

punch. Rows of punctures were made on the edges and longitudinally along the center of the device, as illustrated in Figure 14.

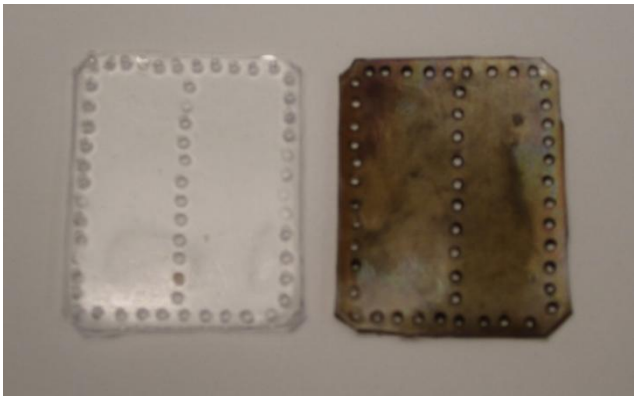

Figure 14. *Silicone pads before and after dopamine coating*

The silicone pads were coated with dopamine to increase their wettability and surface adhesion. Two different dopamine coating procedures found from literature were used, according to Yang and Zhao [37] and Bernsmann et al. [45]. The first one was a simpler method, in which the silicone substrates are immersed in a dopamine-buffer solution. In the latter method, copper sulfate was used as the oxidant to facilitate dopamine self-polymerization. It was found that the latter method provided better deposition of dopamine to the silicone surface.

To provide the alkaline pH in which self-polymerization of dopamine occurs, a buffer solution was used. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane (Tris) was made by dissolving solid Tris (252859, from Sigma Aldrich) in de-ionized (DI) water (Millipore Milli-Q) and adjusting the pH to 8.5 with 1 M HCl-solution. The pH was measured using a calibrated digital pH-meter (Mettler Toledo Seven Compact). The stock solution was then diluted with DI-water to reach the wanted buffer concentration. The same buffer solution was used for both methods but in different concentrations.

In the method according to Yang and Zhao, dopamine hydrochloride (H802, from Sigma Aldrich) was dissolved into 0.01 M Tris buffer solution to achieve dopamine concentration of 2 mg/ml [37]. The solution was initially orange, but turned gradually brownish. The prepared silicone pads were lowered into the dopamine solution attached to pieces of tape and left to react for 24 h in room temperature. The solution was covered with laboratory film (Parafilm®) to avoid evaporation of water. After 24 h, the reaction solution was dark green with black flakes floating on the surface. The silicone pads were removed from the solution, rinsed by dipping in water and left to air-dry in a fume cupboard.

The second coating procedure was done according to Bernsmann et al. by adding copper sulfate pentahydrate ($CuSO_4 \cdot 5\ H_2O$) to 0.05 M Tris buffer solution, to achieve $CuSO_4$

concentration of 0.03 M [45]. Dopamine hydrochloride was dissolved in the $CuSO_4$–Tris solution to achieve a concentration of 2 mg/ml. The silicone pads were again placed into the solution on pieces of tape. The solution was stirred with a magnetic stirrer, covered with laboratory film and left to react for 48 h. When the silicone pads were removed from the solution, 3 of 9 pieces were evenly brown colored, whereas the rest were covered unevenly with black flakes. The coated pieces were rinsed by dipping in water and left to air-dry overnight in a fume cupboard. A successfully dopamine coated silicone pad is shown in Figure 14.

4.1.2 Gelatin-siloxane gel

Physically crosslinked gelatin-siloxane hydrogel prototypes were prepared based on the simple fabrication method described by Xue et al. [58]. The preparation process of gelatin-siloxane gels was slightly altered for the purposes of this thesis. Xue et al. prepared their hydrogels in phosphate buffer solution (PBS) because they aimed to simulate human body conditions [58] but we decided to prepare the gelatin solution in DI-water. Because of limited knowledge of the behavior of these materials, different gelatin and siloxane compositions for the hydrogels were tested. The base gelatin solution used by Xue et al. was 10 w%, but we wanted to also test increasing the gelatin content in the base solution to 15 w% to study whether increasing gelatin content increases the stiffness of the gels. We also wanted to study the effect of different siloxane contents to the properties of the gels. Siloxane concentrations of 5 v%, 10 v% and 15 v% were used. The tested material compositions and their codes are collected in Table 2. These codes are used throughout the rest of this thesis to identify the different gelatin-siloxane gels.

Table 2. *Gelatin-siloxane gel compositions and their abbreviations*

Material code	Gelatin solution concentration (w%)	GPMS content (v%)
10G5S	10	5
10G10S	10	10
10G15S	10	15
15G5S	15	5
15G10S	15	10
15G15S	15	15

The gelatin solution was prepared by weighing proper amounts of gelatin powder from bovine skin (type B, G6650 from Sigma Aldrich) and dissolving it in 50 °C DI-water. The gelatin solution was kept on a hot plate and stirred with a magnetic stirrer for 30 min. (3-glycidoxypropyl)trimethoxysilane, GPMS (440167 from Sigma Aldrich), was added to the gelatin solution to obtain siloxane concentrations of 5 v%, 10 v% and 15 v%. GPMS was measured using a disposable syringe (2 ml or 5 ml from B. Braun). The precursor solutions were then dispensed into molds, covered, and allowed to stand in room temperature for 30 min, after which they were transferred to a heating chamber

at 37 °C for 2 h. For proof of concept testing, the solution was cast into metallic embedding molds. For material characterization tests, cylindrical Teflon (PTFE) molds and plastic Petri-dishes (10 mm in diameter) were used.

Contrary to the report of Xue et al. [58], gelation did not occur during the 2 h in the heating chamber, but the solution in the molds was a viscous fluid when taken out. The covered molds were then transferred to a refrigerator (3 °C) for 48 h where they solidified to form gels. The gels were then removed from the molds carefully using a disposable surgical blade (Feather).

4.2 Testing

The prepared prototypes first underwent proof of concept testing to judge their performance in attaching small tissue samples and holding them in place during tissue processing. The gelatin-siloxane gel prototypes showed promising results in these tests and were further characterized to better understand their material properties. Understanding the material properties and their relationships to the performance of the prototypes can help optimize the material compositions for future development of the tissue sample alignment device. This section describes the testing methods.

4.2.1 Proof of concept

All prepared prototype materials underwent a two-part proof of concept testing to evaluate the performance of the alignment device in tissue handling. Two critical steps of tissue handling process were selected to be tested: the attachment of the tissue samples straight from the biopsy needle and the immobilization of the samples in the tissue processor conditions.

The attachment testing was done with fresh excised tissue submitted to Fimlab Laboratories for pathological sampling. Any tissue was applicable for the usability testing, except very fatty tissue such as breast tissue. A sample from the test tissue was obtained with an 18-gauge needle biopsy gun (Bard Max-Core Disposable Core Biopsy Instrument, Bard peripheral vascular) similar to those used by urologists of Tampere University Hospital to take prostate biopsies. The ease of tissue attachment was evaluated subjectively. Attachment on paper was used as the gold standard for judging attachment of tissue samples. Two or three tissue samples were adhered onto each prototype material. The attachment testing was always conducted by the same person. For the second part of the usability test, the tissue samples and the prototypes were put in tissue cassettes and into formalin fixative, where they remained until they were put in the tissue processor. The samples underwent the normal tissue processing cycle, which is described in Appendix 1.

The tissue sample attachment capability of the dopamine coated silicone pads was also compared to sample attachment capability of uncoated silicone pads. The gelatin-siloxane prototypes were tested on the following day after they were removed from the molds. In the meanwhile, they were kept in closed containers in the refrigerator (3 °C). One prototype from each of the different gelatin-siloxane compositions was tested for attachment of tissue samples and immobilization of the samples in the tissue processor.

4.2.2 Water content

Hydrogel characterization is often difficult, because their mechanical and physical properties can change with time as they interact with the surrounding environment, in this case mainly through water evaporation. As water evaporates from the hydrogels, they may change their shape and lose elasticity. The water content of the gels was also thought to affect the tissue attachment capability of the prototypes. The change in water content was studied with storage time. The knowledge of the rate of water evaporation of the gel pads during storage is also relevant from the point of view of determining device shelf life.

Five test pieces of each gelatin-siloxane material were prepared in metallic embedding molds as described in Chapter 4.1.2. The gelatin-siloxane prototypes were stored in a refrigerator (3 °C) for the first 24 h (because pads manufactured at the same time were also used in tissue attachment testing) and subsequently in closed containers in room temperature. After 7 days of storage, 10G5S and 15G5S gels became moldy, and they were discarded. Because of the contamination, the overall water content of the 10G5S and 15G5S gels could not be measured from the same samples, but new samples were made from those materials.

The gels were weighed using an analytical scale (Mettler AE 204, Mettler Toledo). The weights were recorded immediately after the pieces were removed from the molds and at time points 1 day, 2 days, 5 days and 7 days after removal from the molds. The overall water content of the materials was determined by weighing the samples after they were completely dry and comparing their wet and dry weights. The gels had dried completely after approximately 2 weeks.

4.2.3 Surface wettability

Contact angle, θ, is used as a quantitative measure in determining the surface wettability of a material. The contact angle is defined as the angle formed by a liquid at the three-phase boundary where liquid, gas and solid intersect, as shown in Figure 15. The measurement is based on optical recording of an image of the droplet and applying suitable curve fitting algorithms to the image. Large contact angles (θ>90°) indicate hydrophobic surfaces, meaning that the wetting of the surface is unfavorable causing the liquid

droplet to minimize its contact with the surface. Small contact angles (θ<90°) indicate high wettability and are observed when the liquid droplet spreads on the surface. [60]

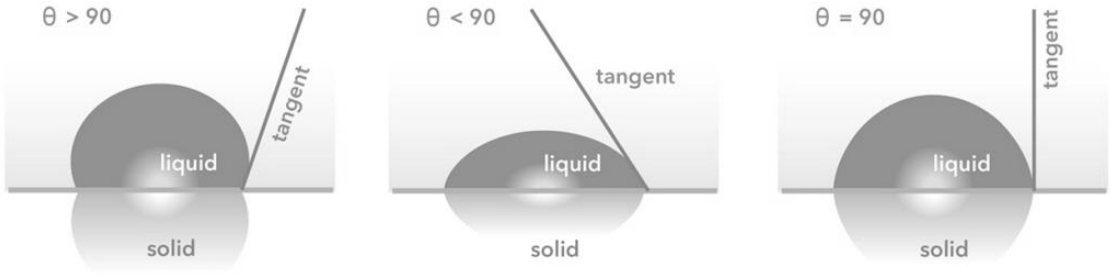

Figure 15. *Contact angle, θ, values and corresponding droplet shapes [60]*

The gelatin-siloxane materials with different gelatin and siloxane compositions were mold cast in Petri-dishes as described in section 4.1.2. The contact angles of the materials were measured 3 days after mold casting. In the meanwhile, the material samples were kept refrigerated and covered. As a reference, contact angles for the silicone elastomer (Elastosil® RT 601) were measured. All contact angle measurements were conducted on the same occasion, except for 15G15S, because the surface of the original sample was visibly irregular, which lead to notable irregularities in the shape of the droplets. A new contact angle measurement sample was prepared from 15G15S material, and measurements were performed in the same way as for the other materials.

The surface wettability of the gelatin-siloxane gels was determined using the static contact angle method with water as the measurement liquid. Contact angles were measured using KSV Cam 200 Optical Tensiometer (Biolin Scientific, Sweden) with an automatic multidispenser syringe. The measurements were carried out in constant temperature of 23 ± 2°C and humidity of 50 ± 5 %. Long rectangular test pieces were cut form each material and put on microscope glass slides for support. The contact angles were measured using 2 µl distilled water droplets, dispensed 5 mm apart. For each material, 10 measurements were made and their average was calculated. Unsuccessful measurements, for example due to malfunction of the automatic dispenser, were excluded from the calculation of the mean.

4.2.4 Compression testing

The test pieces were prepared on the previous day before compression testing. The test pieces were cast into Teflon molds with cylinder shaped cavities with removable caps. Approximately 1 ml of gelatin-siloxane solution was dispensed into each mold cavity. The mold cavities were 12.3 mm in diameter, made to fit a 5 ml syringe piston (BD). The caps of the mold cavities were removed right before each piece was measured and the test piece was gently pushed from the mold cavity using the syringe piston. The outer dimensions of the test pieces were determined using a digital slide gauge.

The compression testing of the gelatin-siloxane gels were done using Bose BioDynamic ElectroForce 5100 compression testing machine with a 225 N load sensor in room temperature. Three parallel samples for each material were tested. Compression speed of 5 mm/min was used for all samples. The test pieces were compressed 80 % of their original height. The recording rate for the measurements was 4 scan points per second.

The stress vs. strain curves for all the samples were plotted using Matlab R2013b (version 8.2.0.701, MathWorks Inc.) from the load and displacement data obtained from the compression testing software. The measured diameter of the cylindrical test pieces was used to calculate the initial surface area (A_0) of the loading contact. The stress (σ) was calculated using the formula $\sigma = \frac{F}{A_0}$, and the strain (ε) with formula $\varepsilon = \frac{\Delta L}{L_0}$, where ΔL and L_0 denote the change in height and initial height of the test piece, respectively. Failure stress was determined as the maximum stress applied to the sample before fracture occurred, obtained from the maximum value of the stress-strain graph. Failure strain was the corresponding strain, also obtained from the stress-strain graph. The compression modulus (E) was determined as the slope of the linear portion of the stress-strain graph, defined as the part up to $\varepsilon = 0.2$ from each graph.

5. RESULTS

This chapter discusses the observations and measurements made during the preparation and testing of the two kinds of prototypes. The results are also compared with values found from literature. The validity and reliability of the findings are also discussed in relation to the aims of the thesis.

5.1 Observations

For the preparation of the dopamine coated silicone prototypes, two different methods were used for the dopamine deposition. In the first method, no additional oxidative agents were used, whereas the second method included $CuSO_4$ as an oxidant to catalyze dopamine film formation [45]. After dopamine coating with the first method, the silicone pieces and the reaction solution surface were covered with black flakes, which were easily removed either during rinsing or afterwards by gently wiping the surface. These black flakes were most likely aggregates of polymerized dopamine nanoparticles [37; 61]. Overall, the silicone pads appeared clear and transparent as before coating, although slight discoloration could be observed on the edges of the pads, suggesting a deposition of a very thin dopamine film. However, with the $CuSO_4$ assisted coating, a more noticeable dark brown color was observed on the surface of the silicone pads (as shown in Figure 14 on page 28). Although it is reported that thicker coating layers can be achieved using $CuSO_4$ [45], the color difference of the silicone pads coated with the two methods should not be caused by a difference in thickness. Dopamine deposition with the $CuSO_4$ method is slower; $CuSO_4$ oxidation should result in a similar coating thickness in 48 h as with the first method in 24 h [45]. It was concluded that with the first method, the oxidation of dopamine was not effective enough to result in a visible coating. To improve oxidation in the first method, oxygen concentration in the reaction solution could be increased by means of aeration [45].

During the preparation of the gelatin-siloxane prototypes, observations differed from those reported in the literature. Firstly, the physically crosslinked gelatin-siloxane gels prepared by Xue et al. were reported to form gels already during the 2 hours inside the 37 °C heating chamber [58], whereas our gels solidified only upon cooling in 3 °C. According to Xue et al., the physical crosslinking mechanism of the gelatin-siloxane gels is based on hydrolysis of the methoxy groups of GPMS to silanol groups and subsequent hydrogen bonding between the silanol groups and amino groups along the gelatin chains [58]. Gelling upon cooling, which we observed, may suggest that physical crosslinking was based on entanglements of the gelatin molecules. The differences observed in the

gelation behavior of the gelatin-siloxane materials could be because of DI-water was used as the solvent instead of PBS. It is possible that the ionic components of the phosphate buffer cause hydrolysis of the GPMS molecules, functioning similarly to the proton (H^+) from acidic solution for the chemical crosslinking reaction depicted in Figure 13 on page 25. Also, it was later noted, that Xue et al. used type A gelatin (obtained from acid cured tissue) whereas we used type B (lime cured). If the type of gelatin affects the pH of the reaction solution, this may affect also the gelation of the material.

Secondly, the gelatin-siloxane gels prepared by Xue et al. were white and opaque, suggesting phase separation of the siloxane component [58]. Initially, all of our gelatin-siloxane pads were transparent and slightly yellowish. After manufacturing, the gelatin-siloxane gels were kept in closed containers to observe their behavior during storage. After 2 days of storage, the 10G15S and 15G15S gels lost some of their transparency and became slightly translucent. The 10G10S and 10G15S materials turned milky white after 6 days out of the molds, which was most likely due to phase separation of the siloxane component [59]. As they became milky in appearance, they also lost some of their elasticity and became more fragile and rigid. Also the 15G15S gels showed increased rigidity at the end of the week-long storage period. Although significant phase separation of the siloxane component was observed in only few samples of 10G10S and 10G15S materials, it may also be responsible for the slight loss of transparence of the 15G15S gels [59]. The delayed phase separation of the siloxane indicates that the siloxane component can move inside the gel material and condense to form siloxane-rich areas. The phase separation was first observed in the materials with the highest GPMS content. For the materials with lower gelatin concentration, the phase separation was more pronounced. Possibly, the reason is that the less concentrated gelatin network allows more movement of the siloxane molecules. When the gelatin-siloxane gels were kept in the fume cupboard, the 10G5S and 15G5S gels became moldy after 1 week of storage and were discarded. No mold growth was seen on the other gel materials, which would indicate that the materials with the lowest siloxane content were most susceptible to contamination.

5.2 Proof of concept testing

For proof of concept, the tissue sample attachment capability of the prototypes and the ability of the prototypes to keep the tissue samples in place during tissue processing were evaluated. The tissue samples were attached to the prototype pads straight from the biopsy needle, to imitate the intended use of the alignment device in an operating room. It was noticed, that the sample notch of the needle was difficult to place evenly onto the surface of the device, because the biopsy needle is long and flexible. Often the central part of the sample attached to the surface but the ends of the core did not properly detach from the needle. The attachment of the tissue cores required a certain technique of placing the needle tip on the pad and rotating it slightly while pressing the tip down.

Some method of applying pressure evenly on the needle tip would be helpful in attaching the samples on to the device, as is used with the SmartBX™ prostate biopsy system [22].

The dopamine coated silicone pads did not perform well in the tissue attachment test. The tissue samples showed no particular affinity to the surface of the pads and the tissue did not adhere to the prototypes any better than to the plain silicone, which was used for comparison. The tissue cores required several attempts before they detached from the biopsy needle. The tip of the biopsy needle had to be pressed down and dragged along the surface of the pads for the cores to detach. The gelatin-siloxane hydrogel pads showed better results. All of the gelatin-siloxane pads were able to attach the tissue samples from the biopsy needle with relative ease. Because the gel pads were quite soft, the needle tip came well in contact with the surface. The different gelatin-siloxane compositions showed only slight differences in tissue attachment ability. The material judged to have the least attachment capability was the 15G15S gel pad. 10G15S and 15G5S materials were judged to attach tissue samples most easily. However, none of the materials matched the attachment capability of paper, which was considered as the gold standard for easy attachment.

When put through the tissue processor, all tissue samples detached from the dopamine coated silicone pads. When a sponge was used together with the dopamine coated pads, the samples stuck to the sponge rather than to the prototype. After tissue processing, the tissue cores were stained dark whereas the surface showed lighter areas where the samples had been attached. It would seem that the tissue samples were attached to the dopamine coating, but the coating did not survive the tissue processor conditions. The temperature of the processor rises to 100 °C, which may have been too high for the dopamine layer causing it to degrade [46].

For gelatin-siloxane prototypes, all of the tissue samples remained immobilized on the prototypes during tissue processing. The gelatin-siloxane pads after tissue processing are shown in Figure 16. As seen in the figure, the prototypes had not retained their shape, but had become bent and wavy. The prototypes had also shrunk notably during the process. The shrinkage of the pads was most likely caused by the removal of water from the gels. The gels also became rigid in the processor, most likely because water was replaced by paraffin.

One gelatin-siloxane prototype was also embedded in paraffin with the samples to see whether the device could be removed from the block as intended. Because the prototype was bent and shrunken, it did not stay on the surface of the paraffin block, but became slightly embedded in the block. After scraping the excess paraffin off the top, the prototype was removed from the block with the tissue samples successfully left embedded in paraffin, as shown in Figure 17.

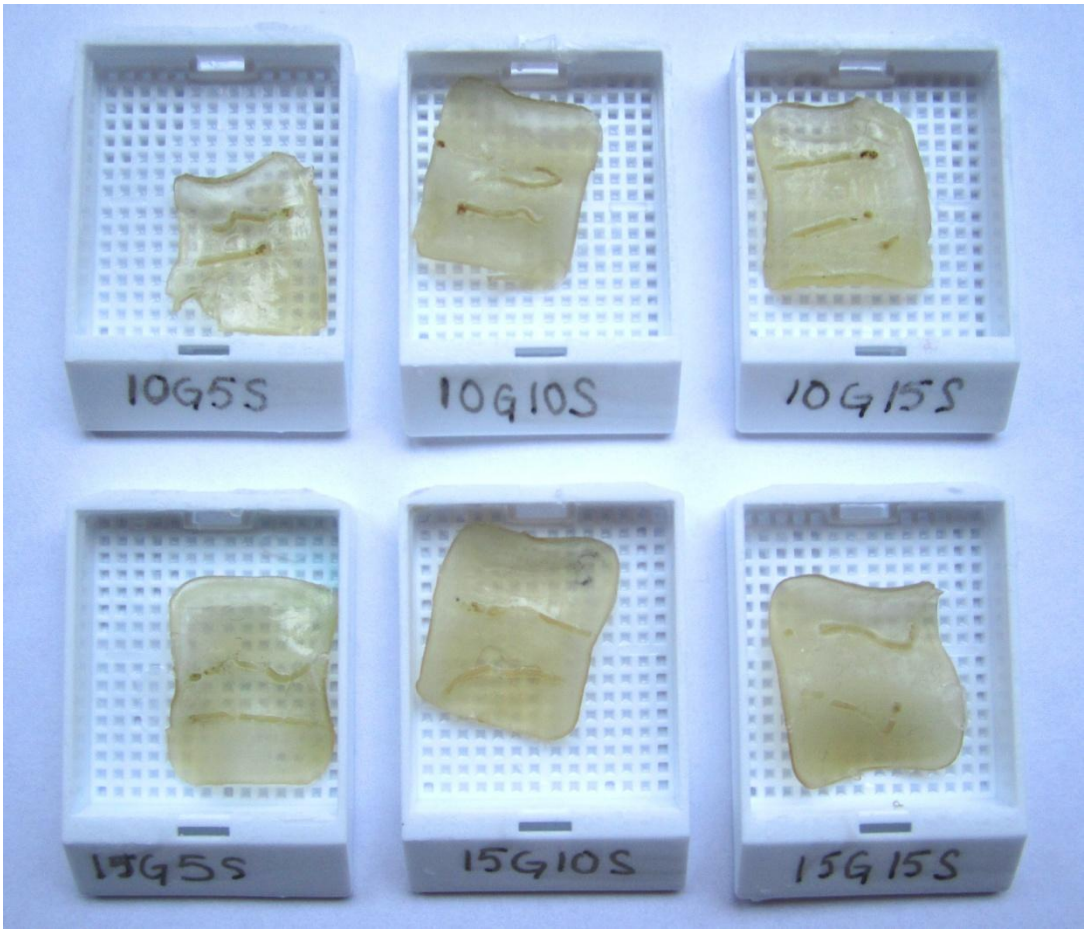

Figure 16. *Gelatin-siloxane prototypes with tissue samples after tissue processing*

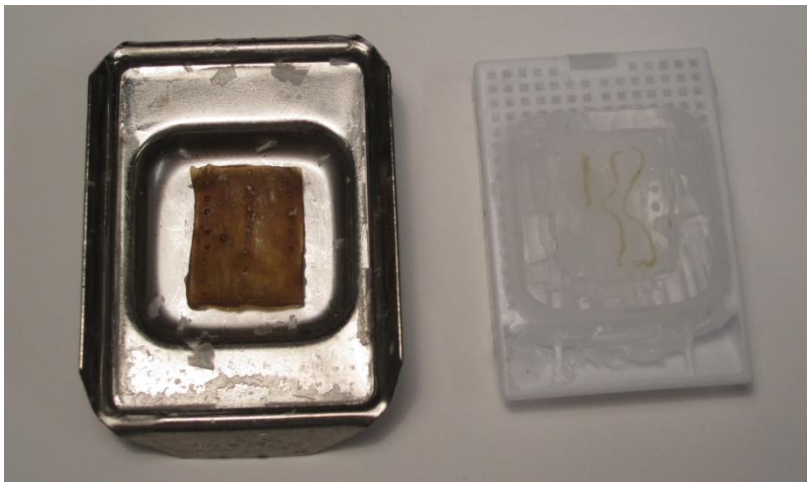

Figure 17. *Gelatin-siloxane pad successfully removed from the paraffin block with tissue samples still embedded*

5.3 Water content

The gelatin-siloxane gel prototypes were stored in closed containers and their changes in weight were observed to obtain the evaporation rate and water content of the gels. The gels were stored for one week, because after that time the 10G5S and 15G5S pads became moldy and had to be discarded. Few of the test pieces broke during the measurements, because they had become rigid and fragile, and were discarded. The broken test pieces were of the higher siloxane content materials, 10G10S, 10G15S and 15G15S, and their fragility may also relate to the observed phase separation, as previously discussed. The 10G5S and 15G5S materials remained transparent and flexible for the longest, up until they had to be discarded due to contamination.

The measured weight change during the 7 days of storage is plotted with time in Figure 18. The initial weights of the gels varied between 0.36 and 1.01 g. The graph of weight change of the gels shows that water evaporation occurs fairly linearly for all of the gels. The evaporation rate was slightly slower during the first day, when the gels were stored in a refrigerator (where they were kept for attachment testing), and increased when they were moved to room temperature. The evaporation rates for the different gel compositions were approximately equal, except for 10G5S, which showed faster weight loss than the other materials.

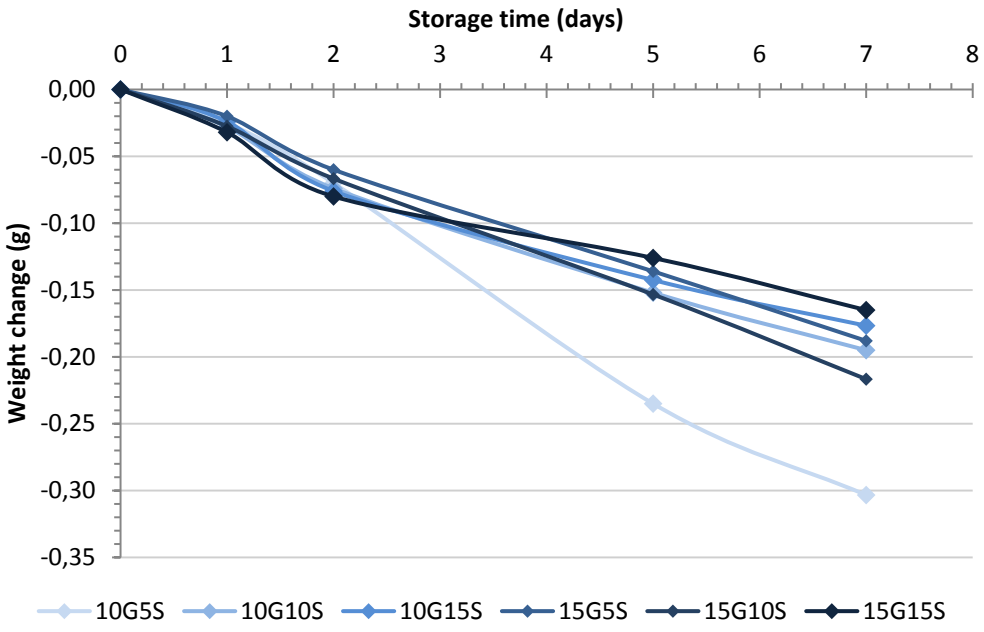

Figure 18. *Weight loss of gelatin-siloxane gels during one week of storage*

The overall water content of the gelatin-siloxane materials was determined after they had dried completely. The mean and standard deviation (SD) of the water contents of the gelatin-siloxane materials are shown in Table 3 as percentages of the initial weight.

The results show that the 10G gels contained more water than the 15G materials. This is intuitively true, as less concentrated solution contains more solvent. The high water content of the 10G5S gel may be the reason for the rapid water evaporation during storage. Overall, the water contents of the gelatin-siloxane materials are quite low compared to similar values found in the literature. Xue et al. measured water contents of their 10 % gelatin and 5 %, 10 % and 15 % siloxane gels to be 85.7 %, 85.5 % and 83.1 % respectively [58]. They also kept their gels in a refrigerator after preparation, but only for 24 h. As we kept the pieces refrigerated for 48 h before removal from the molds, there has probably already occurred some water evaporation before the initial weight was recorded.

Table 3. *Water content of the gelatin-siloxane gels*

Material	Water content (w%)	
	mean	SD
10G5S	75.13	1.50
10G10S	68.92	3.51
10G15S	66.78	5.75
15G5S	66.41	1.57
15G10S	61.05	11.96
15G15S	56.42	4.18

The results also show that increasing the amount of siloxane in the gels reduced their water content. Xue et al. observed a similar trend for their gelatin-siloxane gels [58]. The reason is most likely the hydrophobic character of the siloxane, which does not allow the large amounts of water binding into the structure.

5.4 Surface wettability

Static water contact angles were used as a quantitative measure of the surface wettability of the different gelatin-siloxane materials. Also, the water contact angle of silicone was measured as a reference. The means and standard deviations of the water contact angles, θ, are shown in Table 4 and plotted in Figure 19.

Table 4. *Static water contact angles of silicone and the gelatin-siloxane materials*

Material	θ, °	
	mean	SD
silicone	111.47	6.59
10G5S	75.97	14.96
10G10S	82.91	7.11
10G15S	90.74	3.98
15G5S	92.55	14.19
15G10S	93.16	9.95
15G15S	91.21	8.68

The contact angle measured for silicone (111.47 °) was consistent with literature values (water contact angle for native PDMS is 104–113 ° [30]). Although the water contact angles for the gelatin-siloxane materials were lower than for silicone, they were overall higher than expected for these hydrogels, which were considered to be hydrophilic. Only the contact angles for 10G5S and 10G10S are lower than 90 °, which is considered the limit for hydrophilic surface character. No literature values were found for the physically crosslinked gelatin-siloxane gels, but static water contact angle for dry chemically crosslinked gelatin-siloxane film was reported to be 72.2 ± 2 ° [57].

The 10G gels had slightly lower water contact angle values than the 15G gels. However, as can be seen from Figure 19, there is some overlap in the error bars of the measurements implying that the differences in the contact angles of the various gelatin-siloxane compositions are not very significant. Possible reason for the slight differences in water contact angle may lie in the water content of the materials (see Table 3 on page 39), as the 10G materials have more water in their structure. For the 10G materials, the contact angle values seem to be increasing with increasing siloxane content. It was expected that increasing siloxane content would increase the hydrophobic surface character of the materials, as the introduction of GPMS has been shown to increase the hydrophobic character of gelatin [57]. However, this trend was not observed for the 15G materials.

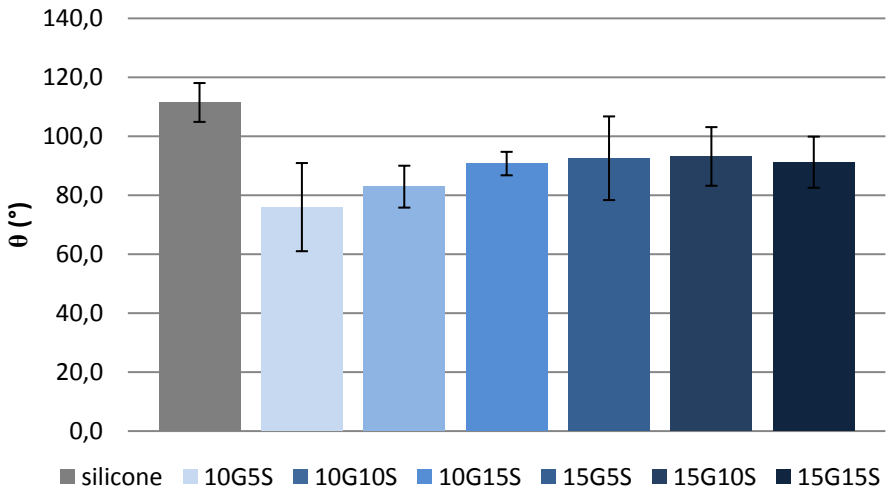

Figure 19. *Static water contact angles of silicone and gelatin-siloxane hydrogels of different compositions. Error bars show the standard deviation.*

The large variations in the measurements suggest uneven surface character of the studied materials. The uneven surface character could be explained through water evaporation from the surface of the samples. Although the Petri-dishes where the samples were cast were covered, the lids of the Petri-dishes were not air tight and they had quite a large surface area for water evaporation. Also, the mixing of the two components in the gels may not be entirely uniform, and siloxane-rich areas on the surface of the material could lead to localized increase in hydrophobic character. Siloxane-rich areas might also

result from phase separation, although it was not visually observed from the tested samples.

It was also observed, that the water droplets were slightly absorbed to the materials when they were on the surface for a while. The water contact angle does not tell everything of the surface wettability of the materials, whereas swelling tests could tell more about the rate of water absorption of the gels. However, swelling tests were not performed within the scope of this thesis.

5.5 Compression testing

Before compression testing, the outer dimensions of each test piece were measured with a slide gauge. The measurements were rounded to the nearest decimal, because the softness of the gels made exact measurement of the dimensions difficult. The diameter of all the test pieces was approximately 12.3 mm but the height of the samples varied from 8.7 to 10.2 mm.

During the compression tests, all of the different gelatin-siloxane samples demonstrated a very pronounced barreling effect upon loading, where the sides of the cylindrical test piece bulged outwards from between the compression plates. The following fracture occurred in a peculiar manner, as the edge of the piece was peeled off from the cylinder almost in a single piece, as can be seen from Figure 20. This may be because the test pieces bulged outside the compression plates, causing the fracture to originate along the edge of the plates. Only two samples (one 10G15S and one 15G5S test piece) fractured without the separate edge fragment.

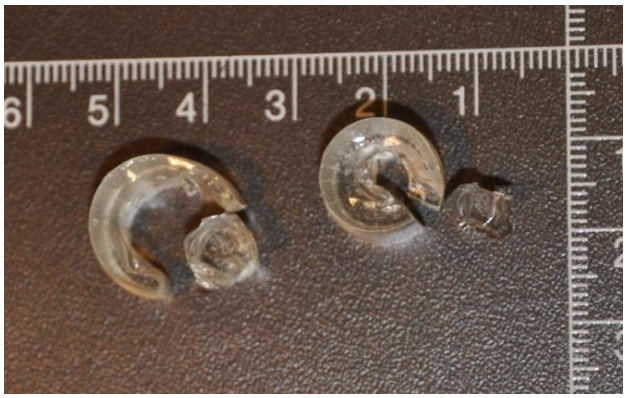

Figure 20. *Two gelatin-siloxane test pieces after fracture. Left: 15G10G Right: 15G15S.*

One test sample of 15G10S and two samples of 15G15S material showed no fracture during the 80 % compression test. However, after the test ended and the crossheads remained at 80 % compression, fracturing of the samples occurred along the outer edges in a similar manner to the other test pieces. After opening the crossheads, a cylinder

from the center of the sample remained intact. These are shown in Figure 20. The center cylinder of material 15G10S was larger in diameter than the 15G15S sample's cylinder. If we assume that the pieces fractured along the edges of the compression plates, this suggests that a larger portion of the 15G15S test piece was squeezed from between the compression plates, which would indicate that 15G15S was more elastic than 15G10S.

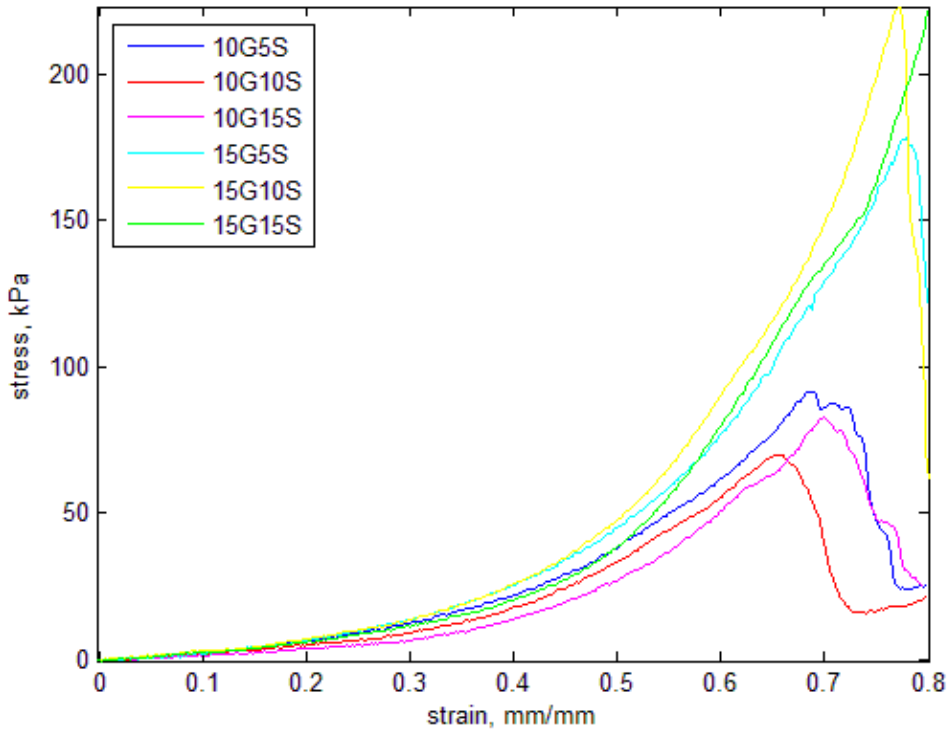

Figure 21. *A typical stress-strain curve for each gelatin-siloxane composition*

One representative stress-strain graph for each gelatin-siloxane material is shown in Figure 21. From the graphs, it can be seen that the 15G gels withstood much higher stresses than the 10G gels. Also, fracturing occurred at higher strains for the 15G materials, with no fracture occurring for two of the three test pieces of 15G15S. The shapes of the stress-strain curves for the gel materials are similar to those measured by Xue et al. [58]. At first, the gels deformed easily under low stresses, then increasing resistance to deformation and finally fractured.

Failure stress, failure strain and compression modulus (E), of the gelatin-siloxane gels are collected in Table 5. The table shows the values obtained for each sample and means and standard deviations for the three samples of each material. For the samples, which did not undergo fracture, no values were obtained for failure stress and strain. The samples, which did not fracture along the cylinder edge are indicated with an asterisk (*) after the sample number in the table. The mean compression modulus for each of the materials is also plotted in Figure 22 and the failure stresses and strains are shown in Figure 23.

Table 5. *Mechanical properties of gelatin-siloxane gels*

Material	Sample	Failure stress (kPa)	Failure strain (mm/mm)	Compression modulus (kPa)
10G5S	1	84.16	0.657	37.04
	2	91.56	0.685	31.42
	3	113.70	0.714	24.96
	mean ± SD	**96.47 ± 15.37**	**0.685 ± 0.03**	**31.14 ± 6.05**
10G10S	1	70.02	0.651	25.52
	2	74.56	0.655	25.19
	3	72.80	0.667	19.11
	mean ± SD	**72.46 ± 2.29**	**0.658 ± 0.01**	**23.27 ± 3.61**
10G15S	1*	112.02	0.735	19.10
	2	82.90	0.699	18.83
	3	85.67	0.762	16.34
	mean ± SD	**93.53 ± 16.07**	**0.732 ± 0.03**	**18.09 ± 1.52**
15G5S	1*	198.36	0.700	53.24
	2	178.42	0.778	33.58
	3	169.16	0.706	45.08
	mean ± SD	**181.98 ± 14.92**	**0.728 ± 0.04**	**43.97 ± 9.88**
15G10S	1	169.24	0.755	37.36
	2	–	–	35.57
	3	219.52	0.771	33.19
	mean ± SD	**194.38 ± 35.52**	**0.763 ± 0.01**	**35.37 ± 2.09**
15G15S	1	194.10	0.770	28.84
	2	–	–	38.62
	3	–	–	30.50
	mean ± SD	**194.10**	**0.770**	**32.65 ± 5.23**

It can be seen from Table 5 that the values for failure stress have quite large variation between the different test samples. Although the test pieces which did not fracture along their edges had higher values for failure stress and E compared to other samples of the same material, this did not seem to cause any significant increase in variation for 10G15S and 15G5S compared to the rest of the gel compositions. Overall, the variation in the measured values for failure stress is quite high. To reduce the variation, more parallel sample of the same material should be tested.

The mechanical properties of the gelatin-siloxane gels measured were very different from those reported in literature for the similarly prepared physically crosslinked gelatin-siloxane gels. Although the composition of our 10G5S, 10G10S and 10G15S gels were the same as those characterized by Xue et al., they observed significantly higher failure stress values for their gels [58]. The failure stresses measured by Xue et al. were 2.23 MPa for the 5 % siloxane gel, 3.56 MPa for the 10 % siloxane gel and 3.19 MPa for the 15 % siloxane gel [58]. The corresponding values measured for our gels were 0.10 MPa for 10G5S, 0.07 MPa for 10G10S and 0.09 MPa for 10G15S. Furthermore,

Xue et al. observed that 10 % GPMS content provided the strongest gels [58], whereas in our measurements, the fracture of 10G10S occurred at the lowest stress and strain values. Another significant difference between the compression measurements is in the compression modulus values. We measured 10–30 times higher compression moduli for the 10G materials than those measured by Xue et al. [58], indicating that our gels were more rigid.

One possible explanation for the differences in the mechanical properties may be that the molecular weight of the gelatin was different than that used by Xue et al. The molecular weight is quantified by the bloom number, which is based on viscosity measurements [62]. The gelatin we used had a bloom number of approximately 75 [63]. Xue et al. do not disclose the specific product number of their gelatin, only that it was type A porcine skin gelatin from Sigma-Aldrich. A quick product search revealed that the bloom numbers of the possible gelatin products are 90–110, 175 and 300, which would indicate that they have used higher molecular weight gelatin for their gels. The higher the molecular weight of gelatin, the more they form entanglements in the gel structure [62]. It has also been shown that increase in molecular weight of gelatin is coupled with an increase in strength [62].

Other factors affecting the measurements may have been the different measurement parameters. The diameter of the compression test samples was 5 mm for Xue et al., whereas we tested samples with a diameter of 12.3 mm. The large diameter of our samples caused squeezing of the test pieces from between the crossheads which may have given rise to fracturing along the edges of the compression plates. Compression rate was also different between the measurements; Xue et al. measured the mechanical properties at a compression rate of 1 mm/min whereas we used 5 mm/min. It is possible that the faster compression does not allow the movement of the polymer chains as well as the slower rate, resulting in a more brittle behavior of the material.

Besides comparing our results to those found in the literature, we can compare the effect of different gelatin and siloxane contents on the mechanical properties of the materials. The bar graph in Figure 22 indicates a distinctive trend for the compression modulus of the different gelatin-siloxane materials. The 15G materials have higher E values compared to the 10G gels with respective siloxane contents. Inversely, increasing the amount of GPMS lowers the value for E. The higher the E value, the more the material resists deformation, i.e. the more rigid it is. Increasing the gelatin content seems to increase the rigidity and increasing the GPMS content decreases it. In other words, increasing the siloxane content increases the elastic character of the gels. Figure 22 shows that the gel with the highest gelatin and siloxane contents (15G15S) has approximately the same value for E as the material with lowest gelatin and siloxane contents (10G5S).

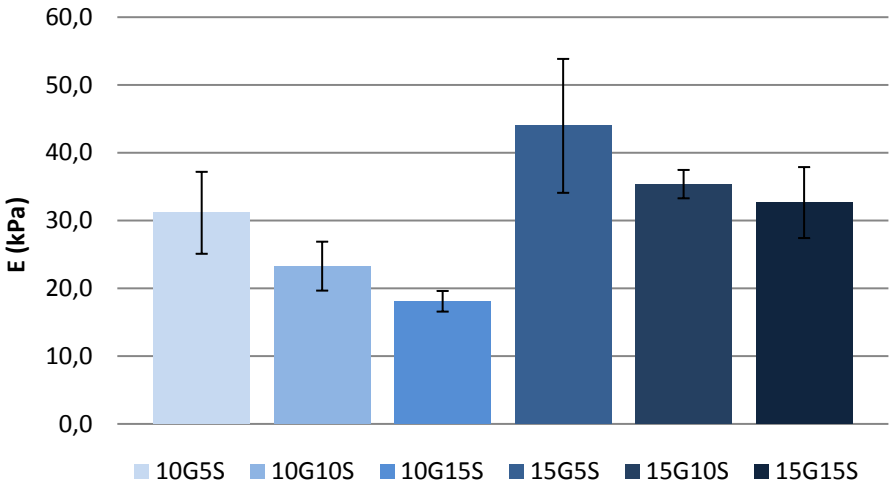

Figure 22. *Compression modulus (E) of the gelatin siloxane materials, error bars show the standard deviation*

The failure stresses and strains of the gelatin-siloxane gels are shown in Figure 23. The results show that the 15G gels have significantly higher failure stress values than the 10G gels. No error bar is shown for the 15G15S material, as only one of the three test pieces experienced fracturing during measurement. The obtained mean failure strain and stress values for 15G10S and 15G15S gels did not entirely reflect the strength of the materials, as some of the test pieces did not show fracture during the measurement and, thus, no numerical results were obtained for them. These materials were, in fact, stronger than the plotted mean values suggest.

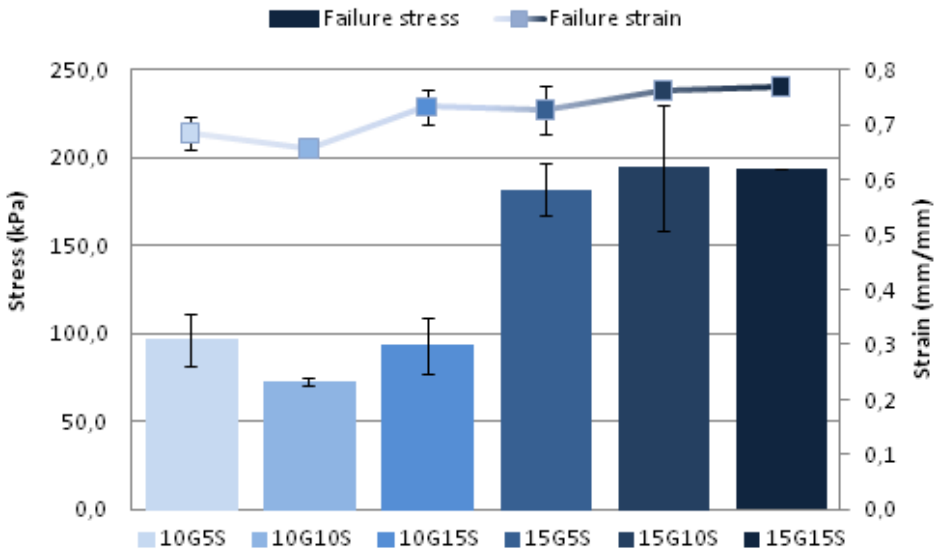

Figure 23. *Fracture strengths of the gelatin-siloxane materials, error bars show the standard deviation.*

Overall, the gelatin-siloxane gels were quite tough. They were able to withstand compression of 65–80 %. The materials made from 15 % gelatin solution were tougher than the 10 % gelatin materials. The compression modulus of the materials seems to be dependent on the gelatin and siloxane contents. With higher gelatin content, the modulus is higher and with increasing siloxane content the modulus decreases. The fracture along the edges of the test pieces may have affected the results, especially the values of failure stress and strain. To overcome this limitation, either smaller diameter samples or larger diameter compression plates would need to be used. For more significant differences in the results, more than three pieces of each material would need to be tested.

6. DISCUSSION

This section summarizes the results of the developed alignment method for small histological samples. The most important results of the device prototype testing are discussed here. Based on the results, future development areas are identified and possible approaches for research are proposed.

6.1 Results

Although current literature has praised dopamine for its potential as a tissue adhesive, it did not appear effective in this application. The dopamine coated silicone prototypes were not successful in attaching the tissue samples from the biopsy needle and did not keep the tissue samples in place during tissue processing. Based on observations of the dopamine coated prototypes after tissue processing, it was concluded that tissue samples did attach to the dopamine coating but the coating did not withstand the high temperatures of the tissue processor, but detached from the silicone substrate. It would seem that the tissue attachment did not occur fast enough to be useful in this application.

The physically crosslinked gelatin-siloxane hydrogels performed significantly better than the dopamine coated prototypes. The hydrogels were capable of attaching the tissue samples and the tissue samples remained in place throughout the entire tissue processing. The method of attachment was attributed to both capillary function and water adhesion between the wet gels and wet tissue samples. The physical attachment method was efficient in immobilizing the tissue samples while simultaneously allowing the device to be removed from the paraffin block without removing the samples. The attachment of the tissue samples from the biopsy needle was successful, but not as easy as attachment onto paper. To be useful in practice, the attachment of the tissue samples should be more efficient and easy.

Although the evaluation of the tissue sample attachment capability of the prototypes was only subjective, the prototypes judged to perform the best were 10G15S and 15G5S. The gel material which showed the least attachment capability was 15G15S. The water content measurements of the different gelatin-siloxane gels suggest that the reason for poor attachment of the 15G15S gels might be its low water content, only 56.4 ± 4 %. The water contents for 10G15S and 15G5S gels were very close to each other, 66.8 ± 6 % and 66.4 ± 2 % respectively. It is interesting that although the water content of 10G5S gels was the highest (75.1 ± 2 %), they did not attach the tissue samples most easily. On the other hand, the gels may have already lost some water due to

evaporation, as they were stored overnight in the refrigerator before the tissue attachment testing was done. For more evidence on the dependence of the water content to the attachment behavior, further studies should be conducted. In the attachment test, only one pad from each material was tested, which does not take into account the possible variation between different gel pieces. As shown from the large standard deviations in the measurements, the variation between different individual pads was quite large.

Surface wettability was initially considered to provide knowledge on the attachment capability of the hydrogels but the results of the water contact angle measurement were inconclusive, showing no significant differences between the results. Swelling test could be performed to find out the more on the capability and rate of water absorption for the gel materials.

Although the gelatin-siloxane gels were efficient in immobilizing the samples, they were not stiff enough to hold their shape and the prototypes became distorted during tissue processing. Even slight distortion of the alignment device is not acceptable in small tissue sample embedding, as this will prevent the whole tissue sample from being visualized on the same microscope slide. Also, the prototype was not easily peeled away from the solidified paraffin block as it became rigid during the paraffin impregnation and did not stay on top of the paraffin block during embedding. Furthermore, shrinking of the pads during processing may cause curling of the tissue samples, which could also make their pathological evaluation more difficult.

In the compression tests, the compression modulus values of the studied gels showed that increasing the gelatin content of the materials increased the rigidity of the gels, whereas increasing the siloxane content decreased it. The most rigid gelatin-siloxane material was the 15G5S gel with E value of 44.0 ± 10 kPa. However, the rigidity, as measured by the compression modulus, did not reflect on the performance of the prototype pads during tissue processing. It was observed, that the 15G5S gel pad was equally distorted as the rest of the prototypes. Furthermore, it seemed that the gels with higher siloxane content were stiffer and did not bend during processing as much as the lower gelatin content gels. Thus, it can be concluded that rigidity of the material does not imply stiffness of the gel pads since stiffness is a property of shape and not an inherent material property.

The strength of the gelatin-siloxane gels was evaluated based on the failure stress and strain. The 15G gels were mechanically stronger than the 10G gels, as they were able to withstand significantly higher stresses before fracturing. For the measured 80 % compression, one test piece of 15G10S and two pieces of 15G15S gels survived without fracturing. However, the observed fracture method of the gelatin-siloxane test pieces was unusual, along the edges of the test pieces. This fracture method, which was observed for nearly all of the tested gels, was thought to be caused by edges of the compression plates, as the test pieces were squeezed out from between them during the

measurement. To provide more reliable measurement of the fracture strength of the materials, the test piece cylinders should have smaller diameter. Also, the compression testing should be carried on to higher strains to observe a fracture for all of the materials. The mechanical properties of the gelatin-siloxane gels were quite good, although similar values to those found in the literature were not achieved in this study. It was determined that the most likely cause of the differences in the mechanical properties to the literature values were due to the use of a lower molecular weight gelatin.

6.2 Future development possibilities

The developed alignment method was very promising, since it was capable of attaching the tissue samples straight from the biopsy needle and keep them in place until the samples were embedded in paraffin. However, there is still room for improvements. The most important area for future development is making the alignment device stiffer to hold its shape better inside the tissue processor. The device should also retain some of its flexibility after the tissue processor to be more easily removed from the paraffin block. Secondly, making the attachment of the tissue samples easier would increase its usability in the biopsy procedure. Additionally, the observed contamination issue should also be addressed.

As already mentioned, the gelatin we used for preparation of the gelatin-siloxane gels had, in fact, quite a low molecular weight. By using a higher molecular weight gelatin, stronger gels could be achieved [62]. Chemical crosslinking could also provide stronger and more rigid materials. The chemical crosslinking of gelatin and siloxane can be achieved through a mild acid catalyzed reaction, as described previously in section 3.4.4. Although the chemically crosslinked gelatin-siloxane gels were left outside the scope of this thesis because they required quite long preparation times [59], their manufacturing is also inexpensive, as listed in Table 1 on page 26. Chemical crosslinking of gelatin using other crosslinking agents, such as aldehydes, could also be possible. In this application, the cytotoxicity of glutaraldehyde might not be harmful, as we are dealing with excised tissues.

In addition to crosslinking, other methods of achieving improved mechanical properties in hydrogels have been developed. Double network gels combine the properties of the two network forming polymers to achieve a stiff yet ductile behavior [64]. Double network gels consist of two interpenetrating networks of crosslinked polymers, one with high crosslinking density and the other loosely crosslinked inside the first [64; 65]. Different combinations of network forming polymers can be used, but with a combination of poly(2-acrylamido2-methylpropanesulfonic acid) and poly(acrylamide) double network hydrogels can be achieved, which are able to withstand stresses in the order of tens of MPa while still containing 90 % water [64].

Another method for increasing the stiffness of the tissue sample alignment devices would be to use some form of reinforcement. Nanocomposite gels are reinforced by inorganic clay nanoparticles, which act as the crosslinkers. These clay-nanocomposite gels are also highly stretchable [65]. In addition to nanoparticle reinforcement, reinforcement with fibers could increase the planar stiffness of the gel pads. For example, fiber-reinforcement using cellulose nanofibers would provide a relatively simple method for achieving increased stiffness for hydrogels [66]. Nanofibrillated cellulose has been studied in reinforcement of gelatin [67] and PEG hydrogels [66]. The use of cellulose in attaching and aligning tissue samples has been avoided, because the cellulose fibers can interfere with the pathological evaluation of the tissues. However, encasing the cellulose fibers inside a hydrogel matrix may prevent them from being embedded in the resulting paraffin block while providing improved attachment ability.

In addition to providing fiber-reinforcement the cellulose fibers could also improve the water absorption capability of the gels [66]. Increased water absorption capability could make the attachment of tissue samples onto the device surface easier. Another method of facilitating the attachment of the tissue samples from the biopsy needle would be to implement a method for even pressure of the needle tip onto the device, as used with the SmartBX™ biopsy system [22].

One point of development with the hydrogel alignment devices is the contamination of the gelatin-siloxane gels. This susceptibility to microbial growth is an inherent property of natural polymers. Although the growth of microbes on the gel pads would most likely be prevented simply by sterilization of the devices, also preservatives could be used. For example, methyl paraben has been suggested as a preservative in Sakura’s patent for the gel alignment structure [20]. Decreasing the susceptibility to contamination could also be done by replacing the natural protein gelatin by some synthetic polymer, which is not degradable by enzyme catalysis.

7. CONCLUSIONS

The aim of this thesis was to develop an improved method for aligning small histological samples to improve the quality and speed of patient diagnosis while taking also into account the cost-efficiency of histological laboratories. Prostate biopsies were used as a specific case, because of the high epidemiological frequency of prostate cancer, large sample sizes and their demanding manual processing in histological laboratories. After reviewing currently existing solutions for aligning small tissue samples, a simple pad-like device was suggested. The suggested device was intended keep the small tissue samples in place and on the same plane throughout the tissue handling process, from the operating room to the resulting paraffin block. For reducing the amount of manual handling steps and handling time, a device was designed, which could fit at least 6 small tissue cores simultaneously. The device was also designed to be removed from the resulting paraffin block leaving only the tissue samples embedded.

Two different approaches were used for making device prototypes for the small tissue sample alignment device. Both prototypes were simple to manufacture with relatively low cost raw materials. The first prototype was a silicone elastomer pad coated with dopamine. Silicone was selected as the base for the first device prototype because of its suitable mechanical properties, inertness to chemicals and ease of fabrication. Dopamine coating has been shown to increase adhesion to surfaces in wet environment, and it can be applied even onto inert silicone surface in a simple dip coating process. The second approach was to prepare a physically crosslinked hydrogel pad made out of gelatin and siloxane. Adding siloxane (GPMS) to gelatin was intended to provide flexibility and toughness and the hydrogel material was thought to have both adhesion capability and supporting function required of the alignment device. Both kinds of device prototypes underwent proof of concept testing. Also, hydrogels with different amounts of gelatin and siloxane were prepared and characterized to determine the effects of the compositions on the properties of the gels.

The results of the proof of concept study showed that the dopamine attachment approach was not successful in attaching and immobilizing the tissue samples on the silicone elastomer pads during tissue processing. The attachment of the tissue samples was not easy enough to be useful in operating room conditions. Furthermore, the dopamine coating was not stable on the silicone surface in the tissue processor conditions, resulting in detaching of the tissue samples. The gelatin-siloxane hydrogel performed much better in the proof of concept testing. The tissue samples attached onto the pads straight from the biopsy needle and were effectively immobilized throughout tissue processing.

Moreover, the gelatin-siloxane pad was successfully detached from the paraffin leaving the tissue samples untouched in the block. However, the gelatin-siloxane gels with different component ratios did not stay flat but bended and shrank during tissue processing.

The method of attaching the tissue samples onto the hydrogel devices was based on both capillary forces and adhesion between the wet gels and tissue samples. The attachment and immobilization of the tissue samples was effective and strong, and most importantly, reversible. Also the resilience of the gels helped in achieving good contact between the biopsy needle tip and the surface of the device. However, further development is still needed to find a suitably stiff material or structure to keep the device from bending during tissue processing, as proper alignment of the small histological samples does not allow distortions of the device. Furthermore, the attachment of the tissue samples should be improved even further to make the alignment method more attractive for use in clinical practice.

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APPENDIX 1: THE TISSUE PROCESSING CYCLE

1. Formalin fixation, 20 min ramp to 50 °C and hold for 10 min
2. Flushing and rinsing with 70 % ethanol, 2 times 2 min
3. 100 % ethanol impregnation, 24 min ramp to 65 °C, hold for 1 min
4. Isopropanol impregnation, 25 min ramp to 68 °C, hold for 35 min
5. Evaporation, heating to 100 °C then ramp to 60 °C, hold for 1.5 min
6. Wax impregnation, with 10 min ramp to 70, hold for 16 min and 28 min ramp to 65, simultaneously lowering the pressure from 500 mBar to 100 mBar.