X-RAY SCATTERING AND DIFFRACTION ENHANCED IMAGING STUDIES OF IN VITRO BREAST TISSUES

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ACADEMIC DISSERTATION

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Helsinki 2006
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Preface

This work is part of an important effort of close collaboration between three institutions: the University of Helsinki (UH), the Helsinki University Central Hospital (HUH) and the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. As a result, the collaboration brought together a broad, very international and interdisciplinary group of people with different backgrounds. My doctoral studentship has been equally shared between the ESRF and the UH. Therefore I have travelled every third month from Helsinki to Grenoble –back and forth– during a large part of my doctoral studies. This has proved to be a great personal experience.

First of all, I would like to express my warmest gratitude to Professor Pekka Suortti, who gave me the opportunity to do science while studying to be a scientist. I would like to thank Professor Ritva Serimaa for her comments on the manuscript. I owe gratitude to Alberto Bravin head of ID17, the Medical beamline at the ESRF, and to Stefan Fiedler. Stefan first and Alberto later were my local supervisors during my stays at the ESRF. Thanks guys, for sharing with me excellent experimental science and good humour, especially during the long night-shifts at the beamline.

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I would like to express special thanks to the ESRF staff, to the directors and to the administrative personnel, which took care of me and my dossier. I am grateful to the extraordinary team of the Medical beamline ID17, especially to Christian Nemoz, Thierry Brochard, Michel Renier and Dominique Dallery. My gratitude to Paola Coan, for her help during the DEI experiments. I would like to thank Irina Snigireva, who helped me with the electron microscope images and to the personnel of ID02, the High Brilliance beamline at the ESRF. I express my gratitude to José Baruchel, head of the X-Ray Imaging Group at ESRF, for supervising my exams. In Helsinki I owe gratitude to Pirjo Tuomi, Anna-Stiina Joensuu and especially to Pekka Pihkala.

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I owe a great deal to my friends in Helsinki and Grenoble. They have been always there for a chat, a coffee, a beer or a dinner or –much better– a nice combination of all these. Thanks folks! I also would like to thank my parents Manuel and Mercedes and my sister Loreto for their absolute and selfless support during all these years despite the distance.

Finally, I would like to express my deepest gratitude to Nina. Her unlimited patience, kind understanding and caring love have been my strongest support and my source of inspiration.

Helsinki, May 2006

I feel gratitude towards the anonymous patients who participated in this research. Their altruistic donation was a moving gesture from persons that, despite the difficulties of their illness, made an effort to help others.

This work is dedicated to them.

Abstract

Small-angle x-ray scattering (SAXS) and diffraction enhanced imaging (DEI) techniques using synchrotron radiation were combined in this work. Human breast samples, surgically excised from patients, were studied in vitro. The samples were formalin fixed and included benign tissues as well as malignant tumours. They were shaped as small disks approximately 1 mm in thickness and about 20 mm in diameter. Histo-pathological examination was performed on the samples after the experiment. In the first phase of the study, SAXS patterns were acquired from small spots (200 × 200 μm) of the samples, following mesh scans. Scans included 50 to 100 measuring points per sample. A single scattering pattern was acquired at every spot. Representative scattering patterns from the tissues were identified and a model for collagen fibril scattering was presented. Preliminary findings of the scattering signatures of fibrillar collagen were observed and recorded. The scattering pattern of collagen was found to be significantly different in malignant tissues compared with healthy ones, indicating structural changes at supra-molecular level of the fibrils. Particularly interesting findings were that, in collagen invaded by cancer cells, the axial period of the fibrils was 0.7% longer than in normal collagen and that the background intensity was found to be 2 to 3 times higher in invaded collagen. These findings were confirmed in consequent experiments.

In another phase of the experiment the SAXS findings were contrasted against DEI images of the samples and their histology. Colour-coded maps of the scattering indicators of collagen degradation were built for some samples. These maps were found to be in an excellent agreement with the morphology and histology of the samples as well as their pathology.

The supra-molecular structure of the tissue samples cannot be solved directly from the intensity distributions of the SAXS patterns. A structural model for fibrillar collagen was constructed using results from electron microscopy and other methods, and this model was used for simulation of the SAXS patterns. The model was refined by a fit to experimental patterns, and a close agreement was achieved.

Classification (INSPEC): A6110F, A7870C, A8760J, A8770E.

Key Words: synchrotron radiation, small-angle x-ray scattering, diffraction enhanced imaging, breast cancer, breast tissues
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List of Original Publications

This thesis consist of an introductory part followed by four publications, referred in the text as Articles I-IV.


Articles I-IV are re-printed with permission from the editors of Physics in Medicine and Biology (http://www.iop.org/journals/pmb) and Spectroscopy –An International Journal (http://www.iospress.nl).
Aim of the Thesis

This work aims to demonstrate that the small-angle x-ray scattering (SAXS) signal carries information about the pathological state of the tissue in human breast cancer tumours in vitro, and that it has a great potential as basis for a diagnostic method. The thesis consists of a summary part and four publications. The publications detail the results of this study and the experiments. The aim of the thesis is presented in the following as a list of the individual goals of the four publications.

I  The main goals were, first, to demonstrate that pathological modifications on the collagen supra-molecular structure are revealed by SAXS acquiring data from in vitro breast samples, and second, the construction of a scattering model for collagen and prove its validity.

II  This study aimed to find a characterization of the tissues of the breast and their pathologies by SAXS.

III  The aim of this publication was to further characterize the breast tissues by SAXS and to find a one-to-one correlation with the histopathological information of the samples. This was done by building scattering colour-coded maps that would match the histology and morphology of the samples. The latter was revealed by diffraction enhanced imaging, DEI.

IV  The aim of the last publication was to describe the supra-molecular structure of fibrillar collagen by a model, which is refined by comparison of the simulated SAXS patterns with experimental results.

Author’s Contribution

The author was responsible for the selection and preparation of the samples at the hospital, together with J. Keyriläinen. He was also responsible for the histo-pathological analysis with the help of the pathologist M.-L. Karjalainen-Lindsberg. The author has been in charge of the sample environment, experiment design and data acquisition during the SAXS beamtime at the ESRF. In the DEI experiments he has been responsible for the experiment design, sample manipulation and imaging. The author has developed the software that was used for SAXS and DEI data analysis. He developed the model for collagen scattering described in Article I. He has carried out the experimental work for Article IV. The author has written the corresponding parts of articles I-IV and took part in the finishing of the manuscripts.
## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>$2\theta$</td>
<td>Scattering Angle</td>
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<td>AB</td>
<td>Analyzer-Based imaging</td>
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<tr>
<td>2D</td>
<td>2-Dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>3-Dimensional</td>
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<tr>
<td>BM</td>
<td>Bending Magnet</td>
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<tr>
<td>CCD</td>
<td>Charge Coupled Device (2D detector)</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>DCIS</td>
<td>Ductal Carcinoma In Situ</td>
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<td>DEI</td>
<td>Diffraction Enhanced Imaging</td>
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<tr>
<td>ESRF</td>
<td>European Synchrotron Radiation Facility</td>
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<tr>
<td>FReLoN</td>
<td>Fast Read-out Low Noise (ESRF’s CCD camera)</td>
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<td>FWHM</td>
<td>Full Width at Half Maximum</td>
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<tr>
<td>HUCH</td>
<td>Helsinki University Central Hospital</td>
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<tr>
<td>ID</td>
<td>Insertion Device (3rd generation SR source)</td>
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<td>ID17</td>
<td>Medical beamline at the ESRF</td>
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<td>ID02</td>
<td>High Brilliance beamline at the ESRF</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>PB</td>
<td>Propagation-Based imaging</td>
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<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>RC</td>
<td>Rocking Curve</td>
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<tr>
<td>SAXS</td>
<td>Small-Angle X-Ray Scattering</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>SR</td>
<td>Synchrotron Radiation</td>
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<td>TOP</td>
<td>Top position of the RC</td>
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<tr>
<td>UH</td>
<td>University of Helsinki</td>
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<tr>
<td>USAXS</td>
<td>Ultra-Small-Angle X-Ray Scattering</td>
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<td>US</td>
<td>Ultrasound</td>
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<td>WAXS</td>
<td>Wide-Angle X-Ray Scattering</td>
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<td>XRII</td>
<td>X-Ray Image Intensifier</td>
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1 Introduction

Numbers show an undeniable fact: In 2004, in Europe, more than 2.8 million of new cancer cases, and more than 1.7 million deaths due to cancer were recorded (Boyle and Ferlay, 2004). As an inevitable consequence, considerable attention and investment are addressed into oncological studies and research not only in Europe but around the world. Fighting cancer is considered by public opinion of great both scientific and human interest.

The cancer of the breast is the first cause of death among women aged 45-65 (Parkin et al., 2001). In Europe it is by far the most common form of cancer among the female population with more than 27% of all cases, and the most common form of cancer death, 17% (Boyle and Ferlay, 2004). It has been demonstrated that an early detection of breast cancer improves the survival chances of the patients (Webster et al., 2005). This finding has led officials of many developed countries to establish screening programs for their female population. All women in the risk age-fork are encouraged to take periodical mammograms. Despite the efforts, many tumours go undetected and the number of deaths has an increasing trend, parallel to the number of new cases.

Cancer pathology is a macroscopical evidence of a molecular disorder in the body. The reasons for these disorders are not well understood and in some cases are associated with oncogenes. Nevertheless, it is known that the biochemistry of the cells is profoundly altered and that such alterations may develop into an overt disease.

The biochemistry of breast cancer can be followed, at least partially, by studying some structural changes of the tissues at the supra-molecular level. Such structures belong to the nanometre scale and can be studied by means of the small-angle x-ray scattering (SAXS) technique. These observations can be further studied and complemented by electron microscopy. On the other hand, morphological changes of the tissues, which are on the sub-millimetre scale, may be depicted with great contrast and resolution by the diffraction enhanced imaging (DEI) technique. The morphological information of the images may be completed with observation of the histological slices of the tissues, which contain key information both about the pathological state of the tissues and their morphology. Histo-pathology contains, therefore, information both in the millimetre and the micrometre scales.

The aim of this thesis is to demonstrate that in breast tumour samples a direct correlation may be established between the microscopic changes (molecular level) and the macroscopical findings (morphological and histopathological level). Such use of combined information may be used as a diagnostic tool for breast cancer.
2 The Breast

2.1 Anatomy

The human breast is an external organ of the body. It mainly consists of the mammary glands, which are embedded in adipose tissue and wrapped in connective tissue. The function of the connective tissue is protective and it gives cohesion to the ensemble of the organ. Collagen is the main component of the connective tissue. Adipose or fatty tissue has a protective function and also serves as nutrients storage for the cells.

The mammary glands are formed by the lobules, where milk is secreted and by the ducts, or tubules, through which the milk flows to the nipple and outside the body. See Figure 1.

![Diagram of the breast]

**Figure 1:** Anatomy of the breast. Mammary glands are embedded in adipose tissue (fat) and wrapped in connective tissue. Glands consist of the lobules and the ducts. During lactation, milk is secreted in the acini.

The distribution of connective and fatty tissue of the breast can be very diverse depending on temporal variables like hormonal cycles, age or diet, but it also depends on genetic factors. Mammographically, breasts rich in collagen are called dense. Mammographs from dense breasts present poorer contrast than normal breasts, due to a decreased transparency of the tissue to the x-rays.
2.2 Histology

A healthy breast includes a variety of tissues. Some of them will be treated in this thesis, namely: adipose, glandular and connective tissues.

- Adipose or fatty tissue is formed mainly by adipocytes, or fatty cells. These cells contain fatty acids, which are used by the cells as source of nutrients and energy. Fatty acids are arranged as triglyceride molecules. The composition of the fatty acids in the triglycerides may vary greatly with diet or age, but they are mainly oleic and palmitic acids (Zhu et al., 1995). In the body adipocytes are rather large cells and they are closely packed.

- The mammary glands consist of a series of bags (acini) were the milk is secreted. The acini are clustered, forming lobules in a branched manner. The conduits from the lobules converge into the ducts. Finally, the ducts reach the exterior of the body through the nipple. The walls of the lobules and the ducts are formed by layers of epithelial cells, and they are wrapped in a layer of connective tissue called the basement membrane. The basement membrane mainly consists of collagen type IV, which is non-fibrillar.

- The connective tissue is mainly made of collagens, but it also has non-negligible quantities of elastine fibers. Its main functions are protection and cohesion of the organs within the body. The nature of collagen is going to be discussed in the next section. Collagen in the breast is mainly fibrillar and it is the principal component of the stroma, or extracellular matrix.

2.3 Pathology

A pathology, or a pathological lesion, is regarded as an abnormality of the healthy tissues\(^1\). The term, neoplasia, or “newly grown” tissue, describes those pathologies that appear as a lump or tumour (Cotran et al., 1989). Neoplasia also refers to the fibrosis and neo-vascularization associated with tumour development. Some breast tumours can be found as palpable masses and others may only be detected mammographically. However, some types of tumour may pass undetected under examination.

\(^1\)There is discussion among experts about the definition of “healthy”. Certain benign lesions are in fact so common that some scientists suggest that they should not be considered pathologies.
According to their pathological nature, the tumours of the breast can be classified as benign or malignant. Malignant lesions are those that are invasive and impose a potential threat to the life of the patient (Cotran et al., 1989). Malignant tumours are said to be a cancer.

**Benign lesions**

*Fibrocystic* changes are the most common alterations of the breast tissues, characterized by an increase of the fibrous stroma and a dilatation of the lobules and the ducts. Certain fibrocystic changes may be accompanied by *epithelial hyperplasia*, an abnormal proliferation of epithelial cells. The presence of atypically grown cells may represent a risk, because the benign neoplasia might develop into a malignancy. Another benign lesion of the breast is the *sclerosis adenosis*, characterized by intraductal fibrosis and proliferation of acini.

**Malignant lesions**

In general, malignant tumours from epithelial origin are referred to as *carcinomas*. There are many different kinds of carcinoma of the breast. The most common breast cancers are the *carcinoma ductale* and the *carcinoma lobulare*, which are situated within the ducts or in the lobules, respectively.

When the tumour is in a late stage of growth, the neo-vascularization does not reach its interior and the cells die. The dead tissue consists of a structureless mixture of the remains of the original tissues and cancer cells, and it is said to be *necrotic*. See Figure 2.

Certain kinds of carcinoma ductale are very well confined in the ducts, delimited by the epithelial wall and wrapped in the intact basement membrane. In this situation there is not direct contact of the cancer cells with the stroma or extracellular matrix. This kind of carcinoma is referred to as carcinoma ductale *in situ*, or DCIS. These lesions have quite good prognosis, with high chances of patient survival after surgical removal of the tumour. In DCIS, the cancer cells are confined very locally (non-invasive), and are very unlikely to spread to other parts of the body where they may produce secondary tumours, *metastasis*. If the cancer cells are in direct contact with the stroma, the lesion is said to be *invasive* and the cancerous cells may use the collagen fibrils as pathways to move throughout the connective tissue. In the lobular carcinoma the cancer cells adopt a very characteristic disposition, aligned one after the other along the collagen strands. These cell formations are known as *indian files*. 

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2 THE BREAST

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4
2.4 Calcifications

Closely associated with many pathologies, it is common to find in the human body depositions of calcium compounds and salts in a very poor crystalline state. They are known in general as calcifications. In the breast, due to their relatively small size, they are called microcalcifications and they are often confined in the ducts and/or in the lobules. Their presence may be indicative of a lesion. Microcalcifications can exist in benign lumps and in healthy breasts, but also be associated to malignant lesions.

![Image of histological section showing cancer cells, necrosis, basement membrane, calcifications, duct, and a scale bar indicating 0, 0.1, and 0.2 mm.]

Figure 2: Histological section of a carcinoma ductale, showing several calcifications and necrosis. The fibrous extracellular matrix is formed mainly of collagen, stained pink. Note the basement membrane around the duct.

The microcalcifications of the breast can be classified in many different ways. Here, two of them are briefly described: a mammographical morphological classification (Ahmed, 1975) and a chemical classification (Frappart et al., 1984). Morphology: Small and almost spherical calcifications are considered to be associated with malignant lesions, whereas relatively large and lobular-shaped microcalcifications are associated with benign lesions. Chemistry: Calcifications may be segregated according to their composition in two types. Type I are microcalcifications composed of weddellite\(^2\), and when they

\(^2\)Weddellite or calcium oxalate dihydrate \(Ca(CO_2)\cdot 2H_2O\).
are exclusive, they are associated with benign lesions. Type II are composed of calcium phosphates like calcium hydroxyapatite\(^3\). They are associated with malignant lesions. Calcium carbonate\(^4\) and tricalcium phosphate\(^5\) are other carbonate salts that may also be found in microcalcifications of the breast.

3 Collagens

Collagens are the principal components of the connective tissues and the extracellular matrix, together with e.g. *elastine* and *proteoglycans*. Collagens are the most abundant proteins in mammals, being about 33% of the proteins in the human body. At least 27 different collagen types, composed of 42 polypeptide chains, belong to the collagen superfamily (Kiely and Grant, 2002). On the basis of their structural features, the different collagen types can be classified into fibrillar and non-fibrillar groups. Each type of collagen has a specific function in the tissues (Ottani et al., 2001). Type I, II and III collagens are examples of fibrillar collagens. Fibrillar collagen represents the biggest part of the total collagen. Every third amino acid is glycine (Gly) and collagen molecule is composed of repeating triplets of Gly-X-Y, where X is often *proline* and Y is often *hydroxyproline*. In collagen molecules three \(\alpha\)-chains are coiled around each other (Jones and Miller, 1991), forming a very long, triple helical molecule with a typical length of about 300 nm and a diameter of 1.5 nm. The non-fibrillar collagen could be divided into several subgroups. A good example is collagen type IV, the main component of the basement membrane, which encloses glandular tissue in the breast (see section 2.2).

In the breast, together with collagen type IV, the most common collagen types are I and III. In soft tissues type III collagen is often found in association with type I, in form of hybrid fibrils (Cameron et al., 2002). In fibrillar collagen molecules are arranged in a particular hierarchical manner forming fibrils and these eventually form fibers (see figure 3). The molecules are packed one against the other in a quasi-hexagonal close-packed array (Hulmes et al., 1995; Wess et al., 1998a).

There are hydrogen bonds within single collagen molecules provided by abundant hydroxyprolines, moreover, different molecules are jointed by covalent lysine or hydroxylysine-derived cross-links (Orgel et al., 2001). Inter-molecular cross-links form a structure in a staggered regular manner provid-

\(^3\) Calcium hydroxyapatite, \(Ca_3(PO_4)_3(OH)\).

\(^4\) Calcium carbonate, calcite or aragonite, \(CaCO_3\).

\(^5\) Beta-tricalcium phosphate \(\beta-Ca_3(PO_4)_2\).
Figure 3: Schematic representation of the hierarchical arrangement of fibrillar collagen in breast tissues. Typical dimensions (taken from Article I) are indicated.

ing a periodical electron density variation along the fibrillar axis. This is the cause for the collagen fibrils to show, under scanning electron microscopy (SEM) inspection, dark and light stripes periodically spaced along their axis (Kadler et al., 1996). In breast fibrillar collagen, the length of this axial period (or d-spacing) is of about 65 nm and the diameter of the fibrils is about 70 nm. These numbers vary greatly by the physical and/or chemical state of the collagen fibrils, like tension or hydration. On the other hand, fibril diameter and d-spacing may be used as representative parameters of the supra-molecular structure of collagen fibrils.

Interestingly, this supra-molecular structure also depends on the pathological state of the tissues where the collagen is located. Studies by Pucci-Minafra et al. (1987) and Kauppila et al. (1998) found aberrant collagen associated with breast tumours. This characteristic is the key for the developments presented in this work. It has been demonstrated in Article I that by studying the supra-molecular structure of the collagen using SAXS it is possible to determine whether the tissue correspond to a benign or to a malignant lesion. This will be discussed thoroughly later in section 8.

Back to the hierarchical structure of the collagen, the fibrils are packed against each other in a similar way as the molecules are packed (Eikentberry et al., 1982a). This packing is also quasi-close-hexagonal and the inter-fibrillar distance is measured by x-ray scattering to be about 100 nm. Resuming, the fibrils form fibers; the fibers form collagen strands and the strands eventually form tissues. Collagen strands are already in the micrometre scale and they can be observed by an optical microscope.
3.1 Collagen Degradation and Cancer Metastasis

As was mentioned in the last section, variations of the supra-molecular structure of collagen in breast tissues may be studied by SAXS and correlated to pathologies in the tissues. These structural changes could either be associated with aberrant structure of collagens found in malignant lesions of the breast (Pucci-Minafra et al., 1987; Kauppila et al., 1998) or be associated with local degradation of the collagen. Such degradation may be produced by the presence of metalloproteinases in the tissues (Uitto et al., 1980). Some metalloproteinases contain zinc (Taylor et al., 2003), whose concentration in the tissues may be measured by x-ray fluorescence (Geraki et al., 2002, 2004). The presence of cells that produce zinc metalloproteinases can be also determined by immunohistological staining with specific antibodies.

The degradation of collagen fibrils in the breast due to the effect of metalloproteinases is linked to cancer invasion and metastasis (Takahashi and Biempica, 1985; Duffy et al., 2000; McCawley and Matrisian, 2000). The exact role of metalloproteinases in the degradation of collagen remain unclear and the biochemistry of the process is very complex (Walker, 2001). Nevertheless, as the results of Article III suggest, the mere presence of cancer cells in the stroma is enough to make the structure of the collagen to be aberrant. In fact, the SAXS signal corresponding to aberrant collagen can be found only from collagen that contains cancer cells among their fibrils (invasive). Healthy collagen is found, for instance, surrounding in-situ islands of carcinoma, which are confined by the intact basement membrane of the ducts. The secretion of metalloproteinases may degrade the collagen, and a breakthrough of cancer cells into the stroma (i.e. extracellular matrix) could occur. Degraded collagen fibrils may be used by cancer cells as pathways to extend tumour invasion.

A typical pathological feature of lobular carcinoma is the presence of Indian files, or single-cell alignments of cancer cells, between the collagen fibrils. Indian files are an example of cell motility along the extracellular matrix, and a sign of invasiveness of the tumour (and possible metastasis).

Moreover, if the collagen fibrils are studied by means of SEM, the structural changes of the fibrils become evident (see Figure 4). The fibrils from a benign region of a tumour appear to be rod-like, with constant diameter and smooth surface. On the other hand, the fibrils from an invaded region of the tumour appear rough and their diameter varies along their axis. In Section 8.2 it will be seen that these observations are coherent with the SAXS signals from the tissues.
Figure 4: Scanning electron microscope images of collagen fibrils. Left, from a healthy spot of a tumour: smooth rod-like fibril. Right, from an invaded spot in the same tumour: rough and uneven looking fibril. Image by the author, obtained at the ESRF.

4 X-Ray Sources

Wilhelm Conrad Röntgen discovered an unknown kind of radiation in 1895. He called it X-Rays and later it was also known as the Röntgen Rays. This particular radiation has a very short wave-length and the propriety to traverse materials allowing to inspect the interior of objects. Very soon the applications of this discovery to the field of the medicine became evident. Röntgen himself performed the first radiographs, imaging the bones inside his wife’s hand. For the discovery of x-rays he was awarded with the first Nobel Prize in Physics in 1901.

As visible light, x-rays are electromagnetic radiation but with shorter wavelength, i.e. higher photon energy than visible light. Thus, x-rays describe a wide range of the electromagnetic spectrum, ranging from energies as low as hundred eV (soft x-rays, close to ultraviolet radiation) to high energies of hundreds keV (hard x-rays, close to gamma rays). These energies correspond to wavelengths from 10 to 0.01 nm, approximately. As a comparison, visible light ranges from 700 nm for the red to 400 nm for the blue.
X-Ray Tubes

From the beginning of the 20th century until the early 1970’s, the production of x-rays has remained more or less unchanged using sealed vacuum tubes. In the 70’s rotating-anode tubes were introduced, however, sealed vacuum tubes are widely in use still now-a-days. Electrons are produced from a filament, which acts as a cathode. They are accelerated by means of an electric field created by a potential of several tens of kilovolts. The electric field guides them into collision against a metallic target, which is the anode. The electrons interact with the material of the anode, producing heat and radiation. Only approximately 1% of the tube power is emmitted as x-rays (Cullity, 1978).

The electrons that are stopped by the nucleus of the atoms in the anode produce the bremsstrahlung (German word for “breaking radiation”), also called white radiation. The maximum energy of bremsstrahlung emission corresponds to the kinetic energy of the electrons. Moreover, the incoming electrons excite the atoms of the tube target and characteristic emission lines appear superimposed on the continuous spectrum of bremsstrahlung.

One of the main limitations to the production of intense x-rays by sealed tubes is the heating of the anode, which has to be cooled to avoid it from melting by an excessive heat load. In the rotating anode x-ray tubes the heat load spreads over a bigger surface, so that the tube power can be an order of magnitude larger than in sealed tubes.

Storage Rings

Synchrotron radiation is produced by centripetal acceleration of relativistic electrons in facilities that are called in a general way synchrotrons. The electrons are created and linearly accelerated in a linac section to an already relativistic speed. Then, they are inserted into a booster ring, where the magnetic field is increased synchronously with mass increase of the electrons, until they achieve the desired energy. Finally they are injected into the storage ring, the actual radiation source. In the storage ring the electrons are kept in an stable orbit by means of magnets. Synchrotron radiation has a very wide spectral range from infrared to hard x-rays (Duke, 2000).

In the so called third generation synchrotrons, there are three kinds of sources, namely: the bending magnets, the undulators and the wigglers. Bending magnets (or BM) constitute the vertexes of the storage ring, where the electrons are bent to follow a circular segment orbit in an uniform dipole filed. Electrons flying through these magnets at relativistic speed emit radiation tangentially to their orbit. Bending magnets were the first sources of
synchrotron radiation (Duke, 2000).

The sections of the electrons’ orbit between the bending magnets are straight lines. In those sections wigglers and undulators can be installed. These sources are called insertion devices (ID) and they are a set of short dipoles with an alternating and periodical polarity. This fast alternation of the dipoles creates an oscillating trajectory of the electrons, which irradiate synchrotron radiation at every bend.

Oscillations in undulators are small, so that radiation from successive source points interfere and the resulting radiation contains only the wavelengths that have constructively overlapped. Radiation from these sources is very well collimated and contains wavelength harmonics.

The oscillations of the electrons in the wiggler are much wider and the interferences are not important. The intensity from $N$ poles is just added up. The vertical opening of the fan beam is about 0.1 mrad and the horizontal opening is typically a few mrad. The spectral distribution is similar to the continuous distribution of bending magnet radiation.

![Figure 5: Schematics of an insertion device (wiggler). Insertion devices are installed in the straight sections of the storage ring.](image)

The experiments described in this work were carried out at two beamlines of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The ESRF is a third generation synchrotron radiation source with an electron energy of 6 GeV. The High Brilliance beamline (ID02) is designed for SAXS and USAXS experiments and uses an undulator as source. The experimental hutch is placed at about 50 meters from the source. The (Bio)medical beamline (ID17) was designed for medical imaging and radiotherapy. The source is a wiggler and the experimental hutch is situated at 150 meters from the source.
5 X-Ray Interactions with Matter

As mentioned in Section 4, x-ray radiation has the ability to penetrate materials. Its short wavelength makes it an excellent probe to study the structure of materials. The scientific use of x-rays is very wide from medicine to chemistry, including physics and biology. Crystallography, fluoroscopy or radiography are just a few examples of the use of some of the properties of x-rays when they traverse the matter. The photon energy $E$ in eV is related to its wavelength $\lambda$ in nm by $E \sim 1200 \text{ eV nm}/\lambda$.

The interactions of x-rays with matter depend on the energy of the photons and on the material itself, its average atomic number $Z$ and its molecular and atomic structure. At relatively low energies, the photons interact basically with the electrons of the atomic shell. For higher energies, some interactions with the nucleus are also possible, as well as photon decay by pair-production. Given the energy used and the composition of the materials studied in this work, the photon interactions to be dealt with will be mainly those with the bound electrons.

In general, when a photon hits an electron its energy gets transferred to the atom and the latter reacts re-emitting a photon and/or an electron. This process is known as scattering and it can be resonant or non-resonant, depending on its behavior with photon energy. Resonance occurs around photon energies near to the electron binding energies. Resonant behavior is element specific, while non-resonant scattering is sensitive to the electron distribution.

When a photon hits an atom, its energy is transferred to an electron of the inner shells (it is absorbed) and the electron is ejected from the atom: a photoelectron. A hole is created in the electronic shell, which is filled by another electron from the higher shells. Then either a photon is produced (with energy equal to the energy difference between the electronic levels), as a radiative process (fluorescence), or a non-radiative rearrangement of the electrons produces the emission of a low energy electron (Auger effect). For a given element the fluorescence radiation is characteristic to that element and the photoelectric cross-section is proportional to $E^{-3}$, where $E$ is the photon energy.

Moreover, the incident photon may scatter elastically or inelastically. In the latter, the energy of the photon is transferred to the atom and an electron is recoiled in Compton scattering process. On the other hand, in elastic scattering the energy of the photon is conserved and interferences between the wavefronts of the scattered photons are possible. The elastic scattering pattern carries information about the structure of the object at atomic and molecular level and this will be used extensively in this work.
Scattering Vector

Let's consider the elastic scattering of a photon of wavelength $\lambda$ from an atom (see Figure 6). The angular distribution of scattering will be the result of the interferences of the wavefronts of the photons scattered from different regions of the electron distribution. If the incoming photon has a wave vector $\mathbf{k}$ and the scattered photon $\mathbf{k}'$, the scattering vector, or wave vector transfer $\mathbf{Q}$, is defined as

$$\mathbf{Q} = \mathbf{k}' - \mathbf{k},$$

(1)

where $|\mathbf{k}| = 2\pi/\lambda$. The interference will be constructive or destructive depending on the phase shift between two different scattered photons. The total phase shift (Figure 6b) is given by

$$\Delta \phi(\mathbf{r}) = \mathbf{k}' \cdot \mathbf{r} - \mathbf{k} \cdot \mathbf{r} = \mathbf{Q} \cdot \mathbf{r}.$$  

(2)

The scattering vector $\mathbf{Q}$ can be calculated from the geometry of the interaction (Figure 6a). Using the law of the cosines, we have

$$|\mathbf{k}'|^2 = |\mathbf{k}|^2 + |\mathbf{Q}|^2 - 2|\mathbf{k}||\mathbf{Q}| \cos \left(\frac{\pi}{2} - \theta\right)$$

and because the scattering is elastic $|\mathbf{k}'| = |\mathbf{k}|$ and $\cos \left(\frac{\pi}{2} - \theta\right) = \sin \theta$,

$$|\mathbf{Q}|^2 = |\mathbf{k}|^2 - |\mathbf{k}|^2 + 2|\mathbf{k}||\mathbf{Q}| \sin \theta$$

Figure 6: Scattering geometry: a) construction of the scattering vector $\mathbf{Q}$ b) the phase shift is given by the difference of optical paths. The thicker segments represent the extra path length. Note that $\mathbf{k} \cdot \mathbf{r} = |\mathbf{k}||\mathbf{r}| \cos \alpha$. 
or

\[ |Q| = 2|k| \sin \theta \]

therefore, since \( |k| = \frac{2\pi}{\lambda} \),

\[ |Q| = \frac{4\pi \sin \theta}{\lambda}. \tag{3} \]

\(|Q|\) is known as the scattering vector length, also represented as \(Q\).

**Form Factor**

The global effect of the interactions between the scattered photons, or the total scattering length of the atom is (Als-Nielsen and McMorrow, 2001)

\[ -r_0 f^0(Q) = -r_0 \int \rho(r) e^{iQ \cdot r} \, dr \tag{4} \]

where \(f^0(Q)\) is known as the atomic form factor, and the minus sign indicates the phaseshift of \(\pi\) upon scattering from negatively charged electrons. The integral in Equation (4) is nothing else but the Fourier transform of the charge distribution of the atom, i.e. its electron density \(\rho(r)\). At \(Q = 0\) (forward scattering) the integral becomes the total number of electrons \(\int \rho(r) \, dr = Z\), the atomic number; therefore \(f^0(0) = Z\). The constant \(r_0\) is the so called Thompson scattering length or the classical electron radius, and its value is \(r_0 = 2.82 \cdot 10^{-6}\) nm.

The form factor can be completed by including the dispersion corrections \(f'\) and \(f''\), which depend very little on \(Q\) but almost exclusively on the photon energy \(E\). These corrections arise from the fact that the electrons are not free, but bound to the atomic nucleus. The form factor is actually a complex number: \(f'\) is the real part of the dispersion correction and it represents the phase shift due to the scattering. The second term \(f''\) is the imaginary part of the dispersion correction and it is the absorption term. The form factor including the corrections is therefore written as

\[ f(Q, E) = f^0(Q) + f'(E) + if''(E). \tag{5} \]

In forward scattering,

\[ f(0, E) = Z + f'(E) + if''(E). \tag{6} \]

A further representation of this formula in terms of its real and imaginary parts may be introduced, and it can be rewritten as

\[ f(0, E) = f_1(E) + if_2(E) \tag{7} \]

where \(f_1 = \Re[f(0, E)] = Z + f'(E)\) and \(f_2 = \Im[f(0, E)] = f''(E)\).
RefRACTIVE INDEX

For visible light, the refractive index $n$ is bigger than unity. For all the wavelengths of the rainbow, and most of the materials, the index of refraction lies somewhere between $n = 1.2$ and $n = 2$. X-rays have, on the other hand, a $n$ smaller than the unity by a tiny amount. The refractive index is in fact a complex number, since it is related to the form factor as shown below. It is usually written as

$$n = 1 - \delta - i\beta. \quad (8)$$

The values $\delta$ and $\beta$ correspond to the dispersion corrections to the form factor,

$$\delta = \frac{r_0 \lambda^2}{2\pi} \sum_j N^j f_1^j \quad (9)$$

and

$$\beta = \frac{r_0 \lambda^2}{2\pi} \sum_j N^j f_2^j, \quad (10)$$

$N^j$ is the number of atoms per unit volume. In terms of the density $\rho^j$, $N^j = \rho^j N_A / A^j$, where $N_A$ is the Avogadro’s number and $A^j$ is the atomic weight in units of g·mol$^{-1}$. The heterogeneity of the material is represented by the sum over $j$.

It was mentioned before that $\delta$ is the phase shift due to scattering and that in the forward direction, for a homogeneous material ($j = 1$), it is

$$\delta = \frac{r_0 \lambda^2}{2\pi} N(f^0(0) + f'(E)), \quad (11)$$

and neglecting the correction $f'$,

$$\delta = \frac{r_0 \lambda^2}{2\pi} N Z \quad (12)$$

$$= \frac{r_0 \lambda^2 \rho N_A Z}{2\pi A}. \quad (13)$$

On the other hand, the value of $\beta$ is related to the linear absorption coefficient $\mu$ as follows,

$$\beta = \frac{r_0 \lambda^2}{2\pi} N f'' \quad (14)$$

$$= \frac{r_0 \lambda^2 \rho N_A}{2\pi} f'' \quad (15)$$

$$= \frac{\mu \lambda}{4\pi} \quad (16)$$
since $\mu = N\sigma_a$, where $\sigma_a$ is the photoelectric cross section, and $\sigma_a = 2r_0\lambda f''$.

Radiography is an absorption based x-ray imaging method. The phase shift is not directly seen in the images, as is the photoelectric absorption. However, at the energies ranging from 10 to 100 keV (this work and general in radiography), and for light elements, the phase shift term of the refractive index $\delta$ offers a higher potential for imaging than the absorption $\beta$, since it is at least one order of magnitude larger.

The ratio

$$\frac{\delta}{\beta} = \frac{Z + f'}{f''} = \frac{f_1}{f_2}$$

(17)

for the element carbon is plotted in Figure 7 and it shows the clear increase of $\delta/\beta$ with increasing energy. Indeed, as can be seen in Equations (13) and (16), $\beta \propto E^{-1}$ and $\delta \propto E^{-2}$. The graph is almost linear in a log-log representation for almost all the energies after the carbon resonance edge at 0.28 keV. For energies in the range 10-60 keV, $\delta$ ranges from $10^3$ to $5 \times 10^4$ times the value of $\beta$. Thus, the amount of phase contrast relative to absorption contrast increases with photon energy, whereas the delivered dose decreases with energy. This allows to perform images with better contrast at lower dose.

![Graph showing $\delta/\beta$ for carbon (Z=6) as defined in Equation (17). Values of $f_1$ and $f_2$ taken from Chantler et al. (2001)](image_url)
5.1 Transmitted Beam

The electric field of a monochromatic plane wave of wavelength $\lambda$ varies with time and space as

$$E(r, t) = \hat{e} E_0 e^{(i k r - \omega t)}$$

(18)

where $|k| = 2\pi/\lambda$, $r$ is the position vector, $\omega$ is the angular frequency $\omega = 2\pi \nu$, and $\hat{e}$ is the unit vector in direction of the electric field. The oscillation of the field is perpendicular to the direction of propagation, so $k \cdot \hat{e} = 0$ and $k \cdot E = 0$. In the propagation direction $z$ the spatial variation of amplitude is $E(z) = E_0 e^{ikz}$, where $kz$ is the phase. When a wavefront traverses an object, it changes its phase and its amplitude (see Figure 8). The amplitude gets attenuated due to the photoelectric absorption and the phase is shifted due to scattering.

![Wavefront Deformation](image)

**Figure 8:** Deformation of the wave-front after traversing an object, as amplitude and phase are modified.

In the photoelectric absorption a photon is absorbed by an atom and the excess of energy is transferred to an electron which is ejected from the atom. The atom is therefore ionized. The photo-electrical absorption is quantitatively defined by the linear absorption coefficient $\mu$. The relative attenuation of intensity over an infinitesimal thickness $z$ is

$$- \frac{dI}{I(z)} = \mu dz,$$

(19)

and for a homogeneous object

$$I(z) = I_0 e^{-\mu z},$$

(20)
where $I_0$ is the incident intensity and, from Equation (16),

$$
\mu z = \frac{4\pi}{\lambda} \int_0^z \beta dz.
$$

(21)

It was already mentioned that, between absorption edges, the photoelectric cross section $\sigma_a \propto E^{-3}$, and at the edges there is a jump of approximately one decade, when the photon energy exceeds the electron binding energy.

On the other hand, the phase shift is related to the forward scattering and is given by the expression (Davis et al., 1995; Lewis, 2004)

$$
\phi = \frac{2\pi}{\lambda} \int_0^z \delta dz
$$

(22)

$$
= r_0 \lambda \rho_p
$$

(23)

where $\rho_p$ is the projected electron density in the plane $(x, y)$ at depth $z$.

It is important to note that the measured absorption will be somewhat over-estimated, due to scattering. The angular aperture of the detection system rejects part of scattering so that the observed attenuation is due to the true absorption by the photoelectric effect, quantified by $\mu$, and the scatter rejection. For objects with high scattering cross section, much of the radiation going through them is deviated away from the observed beam. The apparent absorption may be more important than the true absorption for the build-up of the contrast in the image.

### 5.2 Scattered Beam

The photons that are not scattered in the forward direction get distributed along the scattering vector $Q \neq 0$. The actual distribution of the scattered photons depends on the energy, the material and the atomic or molecular structure of the material. In elastic scattering the atomic form factor $f(Q, E)$ is modulated by the arrangement of the atoms and it results in very different scattering patterns depending on this arrangement. On the other hand, Compton or inelastic scattering varies smoothly with $Q$.

The form factor of a molecule or group of atoms is called the molecular form factor and it is defined as the sum of the atomic form factors of every atom multiplied by the phase shift term $\exp(iQ \cdot r_j)$; that is

$$
F_{mol}(Q) = \sum_{r_j} f_j(Q)e^{iQ \cdot r_j}
$$

(24)

where $r_j$ is the position of the atom $j$ in the scattering unit. If the atoms are arranged in a periodic lattice of translation vector $\mathbf{R}_n$, the material is said
to be *crystalline*. The form factor of the unit cell is modulated by a term that corresponds to the interferences due to the lattice translations

\[ F_{\text{crystal}}(\mathbf{Q}) = \sum_{r_j} f_j(Q) e^{iQ \cdot r_j} \sum_{R_n} e^{iQ \cdot R_n} \]  

(25)

Such modulation creates constructive interferences when the scattering vector satisfies \( \mathbf{Q} \cdot \mathbf{R}_n = 2\pi m \), where \( m = 0, 1, 2, \ldots \).

The atoms in a crystal define crystalline planes separated by a distance \( d \), and when the x-ray beam falls on the planes at an angle \( \theta \) it gets scattered and interference phenomena occurs. The only directions where there is constructive interference are the ones that fulfill the so called *Bragg’s Law*

\[ m\lambda = 2d \sin \theta \]  

(26)

where \( m = 0, 1, 2, \ldots \). From the Bragg’s Law it is seen that x-ray photons are scattered to large angles by atoms at short distances, and are scattered to low angles by atoms far apart from each other. The scattering modulated by a lattice is called *diffraction*. The scattering vector \( \mathbf{Q} \) is related to the incident angle by Equation (3)

\[ |\mathbf{Q}| = \frac{4\pi \sin \theta}{\lambda}. \]

The observed intensity \( I(\mathbf{Q}) \) is proportional to the square of the structure factor \( F(\mathbf{Q}) \), and can be written as

\[ I(\mathbf{Q}) \propto F^*(\mathbf{Q})F(\mathbf{Q}) = |F(\mathbf{Q})|^2, \]

where \( F^* \) represents the complex conjugate of \( F \).

Elastic scattering at angles down to approximately 5 degrees is called wide angle x-ray scattering or WAXS. The scattering patterns include information from the interatomic distances and the intra-molecular structure of the material. Crystalline diffraction, or *crystallography*, is used to study the structure of the materials, the crystallography of proteins being an interesting example. Scattering at even lower angles is called small-angle x-ray scattering, or SAXS. Small-angle scattering patterns may be used to study the supra-molecular structures of biological materials. These structures may be weakly ordered, and they can not be solved from the diffraction patterns in the same way as crystalline structures, but still essential information can be extracted from SAXS patterns as discussed in Section 8.

Finally, the photons that are scattered almost in the forward direction (but not quite) are considered to be ultra-small-angle x-ray scattering, USAXS. Only scattering from large particles (in the micron size) is seen at these angles (order of microradians), where the USAXS signal often overlaps with the refracted beam.
5.3 Observation

Because its property to penetrate the materials, x-rays are used as a non-invasive probe to study the interior of objects. The basic general idea is to send a beam of photons through an object and observe what is coming out. Due to different interactions between the x-ray photon and matter there is a variety of signals, which can be analyzed on the basis of the energy loss, angular distribution or interference.

Figure 9 shows the total cross section for carbon (Z=6) as the sum of the contributions of coherent scattering, incoherent scattering and the photoelectric absorption. It is seen that in the energy range that is used in the present work, the cross sections of these scattering processes are comparable.

![Figure 9: Cross sections of the element carbon, C. Shaded area represents the working range of energies in this work. The flat shape of the curve at high energies is due to the pair production: a process beyond the scope of this work. Adapted from Thompson et al. (2001)](image)

The changes in the amplitude and the phase of the x-ray wave were discussed in section 5.1. In the following, the methods for observing these changes are outlined briefly. The observation of the effects of the x-ray interaction with the matter can be divided in two categories: changes in the transmitted beam and observation of the various scattering phenomena. In
the first case, ideally both the amplitude and phase changes should be observed. The observable quantity is the intensity, so that the amplitude change can be recorded directly as a radiograph, where the variations in \( \mu \) are seen as the absorption contrast. On the other hand, the phase changes may be converted to changes of intensity. There are different methods, which are discussed below. These have great potential for imaging applications because the phase contrast is much stronger than the amplitude contrast (c.f. Section 5.1).

The analysis of the phase shifts is divided in propagation-based (PB) and analyzer-based (AB) methods. In the first case the x-ray beam must have a sufficient transverse (lateral) coherence, so that the transmitted waves can interfere. Lateral coherence is defined as \( L_T = \lambda D/x \), where \( \lambda \) is the wavelength, \( D \) is the distance to the source and \( x \) is the size of the source. Independently of the \( \lambda \), good lateral coherence can be achieved by either a small source size or by a long distance from the source. Synchrotron radiation (SR) sources allow high photon flux at long distances, because of their low divergence. Moreover, high brilliance SR allows achieving longitudinal coherence, \( L_L = \lambda^2/2\Delta \lambda \), since very monochromatic beams (i.e. low \( \Delta \lambda/\lambda \)) can be achieved with still good photon flux.

There exist three main techniques (Fitzgerald, 2000) for the observation of the phase-shift \( \phi \):

- In-line holography, PB
- Interferometry, AB and
- Diffractometry, AB.

In-line holography is a PB method. It is the simplest of all and consists of a set-up identical to the conventional radiography, but the detector is situated at longer distance from the sample. In-line holography is sensitive to the Laplacian of the phase, \( \nabla^2 \phi \). The edges of the object, where there are fast variations of the phase, are thus enhanced (see Figure 8).

In x-ray interferometry the incident beam is split in two coherent beams by Laue diffraction. One of the beams traverses the sample and, when the beams recombine, phase maps can be reconstructed from the interference pattern.

Diffractometry is a variation of the in-line holography which includes an analyzer crystal between the sample and the detector. The analyzer is a perfect crystal which acts as an angular slit by using the reflectivity curve of one of its Bragg reflections (Bravin, 2003). The intensity contrast due to in-line holography is modulated by the transmission curve of the crystal. Refracted
photons that suffer an angular deviation wider than the Darwin width of the reflectivity will not go through the analyzer and will not contribute to image formation. Moreover, the scattered photons will be partly rejected, producing an additional attenuation to the beam. The diffraction image is sensitive to the first derivative of the phase $\nabla \phi$.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{There is a variety of signals to be observed for studying materials with x-rays. These signals are a consequence of the different interactions between x-ray photons and the matter. Each of the signals carries a particular information about the material.}
\end{figure}

\section{X-Ray Imaging}

In the following, a short description of the different x-ray imaging methods will be presented and discussed, bearing in mind how they can be applied to breast imaging. In order to simplify, these techniques will be treated as their planar projection version. However, any of the following imaging techniques may be used in the computed tomography (CT) mode, thus the interior of the object could be reconstructed with the whole 3D information.

\subsection{Radiography}

Radiography is the first known application of x-rays after their discovery in 1895 by W.C. Röntgen. It was himself who first applied this new radiation
to non-invasive and in vivo medical examination, performing the first radiograph. Very soon x-rays were well known and widely used as a radiographical tool. New specialized scientific journals, new hospital departments of radiology and a whole new branch of scientific and clinical studies were born very little after, together with an enormous public impact.

To obtain a radiograph, or a radiographical image, a very simple geometry is needed: a source of x-rays illuminates an x-ray sensitive film situated just behind an object. The intensity of x-rays passing through the object is related to the absorption (more absorbing materials let less intensity go through) and to the thickness of the object. This intensity is recorded on the film and a map of intensities, an image, is thus acquired. The film is then developed and the radiograph is ready. For historical reasons, x-ray radiographs are presented as a black and white pictures, where black represents the areas of the object with more transparency to x-rays (i.e. less absorption) and white represents the areas more opaque to x-rays (i.e. more absorption). In photographic argot this is a negative image.

Radiographs of bones in the human body have in general good contrast because bones absorb the photons more than the surrounding soft tissues. Therefore, x-rays are of great help in traumatology, for instance. The situation changes when it is necessary to image soft tissues, where their densities are very similar, and their absorption coefficients very close to each other. Thus, there is not enough contrast in the images to distinguish the boundaries of the tissues. One way to avoid this problem is adding a contrast agent into the body cavities or injecting it into the vascular system. A contrast agent is a substance with higher absorption coefficient (at the desired radiation energy) than the tissues. As a result, the structures of the body to be imaged appear contrasted against the surrounding tissues.

Radiographs are commonly obtained using polychromatic sources (x-ray tubes), but images are blurred by the ”beam-hardening” effect. Besides, the low-energy part of the spectrum emitted by the source does not contribute to the image formation, but it just deposits dose on the object. To some extend, this can be avoided by filtering the radiation. Synchrotron radiography allows the use of almost totally monochromatic radiation, increasing the quality of the image and reducing the dose delivered to the object.

6.2 Phase Contrast Imaging

Obtaining good contrast radiographs of biological materials and soft tissues with similar elemental composition can be difficult, especially at high photon energies. Absorption does not yield enough contrast to differentiate areas of the object with very similar densities and in such cases phase contrast
imaging may play an important role. It is interesting to note that the physical mechanisms that produce phase contrast (refraction and elastic scattering) do not deposit dose in the object, i.e. do not transfer energy to it, in contrary to Compton scattering and photoelectric absorption. Furthermore, as was mentioned in Section 5, absorption contrast scales with $\beta$ and phase contrast scales with $\delta$. The latter varies with increasing energy slower than the former, so $\delta/\beta \gg 1$ for high energies. Phase images will be still of good quality at energies were absorption images loose all contrast, and the doses are low (Lewis, 2004).

6.2.1 X-ray Interferometry

This phase contrast imaging technique has been developed for biological systems by Momose et al. (1999), but the idea was first introduced by Bonse and Hart (1965). It consists of an interferometer built from a single monolithic ingot of silicon which is carved to leave three parallel thin slabs in the x-ray beam paths. The first slab splits the beam (Laue diffraction). Then an object is placed in one of the beams, and the beams are recombined at the last silicon slab. At the recombination point, the beam that went through the sample interferes with the unscattered reference beam. The phase of the reference beam can be changed step-wise by a rotating phase-shifter, and a phase map of the probing beam can be reconstructed from multiple interference images.

Monolithic interferometer crystals are in general relatively small because of the limited size of the silicon ingots. Therefore, the samples can not be bigger than several centimeters, limiting the applications of this technique to mammography.

6.2.2 In-Line Holography

In-line holography exploits the creation of contrast due to the phase shift in the propagation direction of the beam (c.f. Section 5). This imaging method requires a very good transverse coherence of the x-ray beam, so that the shifted waves may interfere with the reference wave of the unaltered beam.

The geometry of planar phase-contrast imaging is basically the same as in planar radiography, with the difference of placing the film (or the detector) at a certain distance from the sample, so that the interferences built up. This distance is given by the coherence length of the beam. The interferences create Fresnel fringes, where intensity varies with the distance of the film from the sample. These fringes depict the density contours in the object, otherwise invisible by just absorption contrast.
Phase contrast is widely used with well collimated and monochromatic radiation from synchrotron sources. This technique has been successfully applied to weakly absorbing materials (Cloetens et al., 1999), and to imaging of tissue samples (Arfelli et al., 1998). It is interesting to note that in-line holography can be performed using polychromatic x-ray sources, since phase-contrast depends on the lateral coherence of the x-ray beam and this is achievable by reducing the size of the source.

Its relatively simple set-up and excellent results at low dose make this imaging method very attractive for mammographical examination.

6.2.3 Diffractometry or Analyzer-Based (AB) Imaging

Analyzer-based imaging is a general description for all the imaging systems that exploit the angular-slit effect of a perfect crystal (analyzer) situated between the object and the detector. The object is illuminated with well collimated monochromatic x-rays, and the analyzer crystal can be rocked about an axis perpendicular to the plane of diffraction. The Darwin width of the reflectivity curve of the crystal acts as a very narrow angular slit, of the order of microradians. X-rays will get absorbed, scattered, diffracted and refracted by the object and the analyzer can be used to trace the phase-shift due to these interactions. The main drawback of the method is that it is sensitive to the phase-shift only in the direction of the diffraction plane.

The diffraction enhanced imaging (DEI) is an example of AB imaging method, developed by Chapman et al. (1997). It has been extensively studied during the last years (Bravin, 2003), with great success in soft tissue imaging (Zhong et al., 2002; Kiss et al., 2003; Lewis et al., 2003) and especially in mammography. Breast imaging is an example where DEI can greatly improve the visualization of tumoral masses, microcalcifications and collagen strands (Pisano et al., 2000; Hasnah et al., 2002; Kiss et al., 2004; Keyriläinen et al., 2005; Fiedler et al., 2004; Hasnah et al., 2005). Especially interesting is that DEI synchrotron based mammography has substantially lower-dose compared with conventional mammography (Bravin et al., 2002). Moreover, the DEI technique can be used to build integrated density maps of the object with very little noise (Wernick et al., 2006). Furthermore, DEI has been compared (Fiedler et al., 2003) and combined (Coan et al., 2005) with propagation phase-contrast, or in-line holography.

The DEI reconstruction consists of a pixel-by-pixel algorithm that retrieves the refraction and the apparent absorption from two images of the object, recorded when the analyzer crystal is tuned to reflect the middle-slope position of the rocking curve. In DEI terminology, the refraction image is a map of the deviations of the beam from the incident direction due to
variations of the refractive index \( n \) within the sample. On the other hand, apparent absorption is a map of the attenuation of x-rays through the sample due to combined photoelectric absorption and scatter rejection.

### 6.3 Scattering Based Imaging

In mammography, the scattering from the tissues (either elastic or Compton) is a nuisance because it blurs the images, therefore decreasing the image quality. However, elastic scattering contains very useful information about the structure of the tissues (Evans et al., 1991). The basic idea for recording the scattering signal is to place a detector slightly off the axis of the incident beam, so it will count the photons scattered at that fixed angle. If the angular aperture of the detector is \( \Omega \), the differential cross section \( d\sigma/d\Omega \) is measured (Leclair and Johns, 2002; Johns et al., 2002). The scattering signal can be also picked-up by a 2D detector, using a pencil beam as probe in CT mode (Harding et al., 1987; Westmore et al., 1996). In general, biological materials have not long range order, but they often have it in the short range, which is the key for their possible characterization by elastic scattering. On the other hand, Compton scattering can be also used for tissue characterization, as was demonstrated by Kleuker et al. (1998) in a CT application.

The elastic scattering signal in the WAXS region can be used as a tissue characterization tool, as was demonstrated in early studies by Evans et al. (1991) and later by Speller and Royle (1992). But it is the SAXS signal that apparently has better potential as a diagnostic tool, since it gives information about the structure of the tissues that can be directly associated with their pathologies. This is demonstrated by Lewis et al. (2000) and in Article I and Article II. A preliminary attempt to build a camera to use SAXS as breast cancer diagnostic tool has been published by Round et al. (2005).

The gap between studying the SAXS signal as such, and using it as a tissue imaging signal is still wide. Nevertheless, it has been demonstrated in Article III that the structural information from SAXS patterns and the morphological information from DEI refraction images are complementary and there is a direct correspondence tissue-pathology-SAXS. The other approach to the scattering based imaging is the use of a DEI set-up as a Bonse-Hart camera, which is able to pick-up the USAXS and SAXS signals.

Moreover, a new imaging technique that uses USAXS as a contrast mechanism was introduced by Levine and Long (2004). This technique images an object with a broad beam in a Bonse-Hart camera using a 2D detector.
7 Breast Cancer Diagnosis

Many different diagnostic and screening techniques are used in the every-day hospital oncology (Basilion, 2001). Some techniques are more routinely used than others, depending on the particular cases. Mammography is probably the most common of them, due to its extensive use in the screening programs and its good spatial resolution.

Ultrasound imaging (US) is widely used to check non-mammographical lumps, for instance those found after a breast self-examination. US has the advantage of being dose-free, contrary to the relatively high doses of x-ray mammography. However, its spatial resolution is poorer. The use of US is favoured in the case of young patients (c.f. Section 2) where mammographical examination is not recommended: first because the delivered dose and the risk of radiation induced neoplasia, and second because of the low mammographical contrast in dense breast tissue. Benign lesions of the breast as cysts (common in young patients) are more clearly identified by means of the US technique (Sickles, 2000).

Besides the ultrasound techniques, and depending on the patient and/or the situation, a large arsenal of techniques are available to the clinician: magnetic resonance imaging (MRI), x-ray computed tomography (CT), positron-emission tomography (PET), thermal imaging, electrical conductivity, diaphanography (trasillumination), and so forth. PET technique is sensitive to the metabolism of the cancer and, despite its poor spatial resolution, is very useful to trace metastatic tumours, otherwise unseen.

The great advantage of MRI and ultrasound compared with CT and mammography is the absence of dose deposition during examination (Jung, 1998). A great disadvantage is the poor spatial resolution, of just couple of millimetres, compared with few tens of micron resolution for the mammography (Keyriläinen, 2004). A common advantage of all these methods is their non-invasiveness.

7.1 Clinical Mammography

In clinical mammography practice, distinction between screening mammography and diagnostic mammography must be made. The former refers to the images\textsuperscript{6} which are performed systematically to large groups of patients in screening programs. The latter refers to the additional mammographies taken when is required further examination of non-conclusive findings during the screening (Keyriläinen, 2004).

\textsuperscript{6}Usually four images are taken in a routine mammographical examination: one cranio-caudal and one mediolateral-oblique views per breast.
Contrast in mammography arises from differences on the linear absorption coefficient of the different tissues and their thickness. The breast consists of soft tissue with very small density differences. These differences are enough to create a good contrast in breast where the fat contents is high. On the other hand, contrast is poor in dense (i.e. fibrous) breast. In these cases some types of tumours, as carcinoma lobulare, may go unseen, particularly when there are no other signs of their presence, like microcalcifications. These tumours have usually no palpable masses and only mammographical inspection may be able to spot them.

There exist several techniques to improve the image quality of the mammograms. Two of them are of common use in clinical practice. One is the inclusion of a scatter-rejection grid between the breast and the mammography screen-film (Nykänen and Siltanen, 2003). Scattered x-rays (mainly SAXS and USAXS but also Compton) from the tissues create a “halo” of intensity that is spread over the image. This produces blurring and a loss of contrast and resolution in the image. The grid eliminates part of the scattered radiation improving the image quality. The other technique to improve the resolution in mammographs is the magnification. The radiation for mammographical cameras is produced by an x-ray tube (rotating anode), which produces a divergent polychromatic beam. The magnification is achieved by increasing the distance between the breast and the film, so that the size of the image is bigger than the imaged object. The magnification of certain areas of the breast allows to inspect closely suspicious features found in previous examinations.

Despite the effort and the improvement of the techniques, many tumours are not spotted and there are significantly frequent cases of false positive findings and, more unfortunate, false negative diagnosis of breast cancer.

8 Small-Angle X-Ray Scattering Imaging and Histo-Pathology

In the precedent sections, it has been seen that scattered x-rays are a nuisance for conventional radiography but, on the other hand, elastically scattered photons contain useful information about the structure of the tissues at the molecular level. Structural information from the tissues can be retrieved collecting and quantifying the scattered radiation in a proper manner. In this section it will be demonstrated that a direct correlation can be made between the structural information retrieved by SAXS from human breast tissues and their histo-pathological and morphological properties. These properties cor-
respond to biophysical and biochemical processes at different length scales, and they need to be studied in a different manner.

### 8.1 Small-angle X-ray Scattering

The photons elastically scattered from the supra-molecular structures of the tissues are in the range of the SAXS and USAXS. The general formulation for studying SAXS patterns has been developed for a statistically isotropic system of particles, which do not have long-range order (Glatter and Kratky, 1982). With certain assumptions and structure modeling this treatment has been extended to studies of densely packed objects (as found in body tissues in general), which may exhibit anisotropic alignment and/or certain long-range order, like collagen fibrils in connective tissue (Hulmes et al., 1995).

As discussed in Section 5, the elastic scattering amplitude $F(Q)$ is the Fourier transform of the electron density of the object. However, the observed quantity is the intensity,

$$ I(Q) \propto |F(Q)|^2 = F(Q)^* F(Q) \quad (27) $$

where $F^*$ represents the complex conjugate of $F$. In terms of the electron density $\rho$, the intensity scattered by a system of particles can be written as

$$ I(Q) = \int \tilde{\rho}^2(r) \exp(-i \cdot r \cdot Q) d^3r, \quad (28) $$

where

$$ \tilde{\rho}^2(r) = \int \rho(r_1) \rho(r_1 - r) d^3r_1. \quad (29) $$

The measured intensity is the Fourier transform of the quantity $\tilde{\rho}^2(r)$, called the auto-correlation function. It is not possible to determine the actual electron density $\rho(r)$ uniquely from the scattering patterns, because the phase of $F(Q)$ is not known. Some kind of a priori information about the scatterers (like shape or size) is needed in order to obtain a complete picture of the object. Scanning electron microscopy (SEM) may provide the necessary information.

The interference function $G(Q)$ counts for interferences between closely packed objects. Thus, the final intensity is

$$ I_i(Q) = I(Q)G(Q). \quad (30) $$

The spherically averaged interference function $G(Q)$ is related to the radial distribution function $g(r)$ through Fourier transform (Guinier, 1963).
Besides the structural information given by the overall SAXS pattern, there are two important limiting cases described by the Porod’s law (Porod, 1951) and the Guinier’s approximation (Guinier, 1963).

Guinier’s equation is an approximation to the scattering distribution at very small angles (|\(Q\) = \(Q\) small), or in the so called central peak. The intensity is defined as (Glatter, 2002)

\[
I(Q) = (\Delta \rho)^2 V^2 \exp(-Q^2 R_g^2/3) \tag{31}
\]

where \(\Delta \rho = \rho - \rho_0\) is the electron density difference of the scatterer \(\rho\) and the medium \(\rho_0\), and \(V\) is the volume of the scatterer. The factor \((\Delta \rho)^2 V^2\) is known as the total scattering length, and \(R_g\) is the radius of gyration, defined as

\[
R_g^2 = \frac{\int_V \rho(r_i)r_i^2dV_i}{\int_V \rho(r_i)dV_i} \tag{32}
\]

where \(r_i\) is the electron position with respect to the origin, and \(\rho(r_i)\) is the electron density. The scattering pattern shape at small \(Q\) is given by \(R_g\).

Porod’s law is another approximation to the shape of the scattering curve, but this time at large values of \(Q\). In other words, it is an approximation to the asymptotic behavior of the scattering pattern. It states that

\[
I_\infty \propto S/Q^4 \tag{33}
\]

where \(S\) is the total surface of the particle per unit mass. The subscript \(\infty\) refers to asymptotic behavior. Porod’s approximation in this form is valid for 3D particles, but it can be applied to heterogeneous and closely packed particle systems, because the inter-particle interference has little effect at high angles, i.e. its interference function is \(\sim 1\) when \(rQ \gg 1\), where \(r\) is the size of the particles. This region of the scattering vector is known as the Porod’s regime (c.f. Article I).

Two further considerations about Porod’s approximation are worth noting. First, in the Porod’s regime the scattered intensity is directly proportional to the total surface of the particle per unit mass \(S\). This will be very important for the understanding of the degradation of fibrillar collagen, as it will be seen later. Secondly, for particles that are not 3-dimensional (i.e. with one or more dimensions being very small, like long rods or platelets), the intensity in the asymptotic region falls with a factor different from \(Q^{-4}\): for long rods, the factor is \(Q^{-1}\), for disk-like particles \(Q^{-2}\). On the other hand, scatterers with fractal dimension present an intensity fall between \(Q^{-1}\) and \(Q^{-3}\) if they are mass fractals, and between \(Q^{-3}\) and \(Q^{-4}\) if they are surface fractals.
The scattering vector was defined in Section 5, Equation (3) as

$$|Q| = Q = 4\pi \sin \theta / \lambda.$$ 

It is seen from Bragg’s law that a real space distance \(d\) and the length of the scattering vector \(Q\) are related by

$$d = \frac{2\pi}{Q} = \frac{1}{s},$$  

(34)

where an alternative definition of the scattering vector \(|s| = s = 2\sin \theta / \lambda\) is also used.

### 8.1.1 Scattering from Tissues

Scattering patterns from different breast tissues are given in Article II. Scattering patterns from breast samples fall into three basic categories depending on the kind of tissue: adipose, connective and necrotic. Often the patterns are combinations of some of these three basic patterns, weighted by the tissue composition. Scattering patterns from adipose tissue are of relatively low intensity, in clear contrast with the scattering patterns from necrotic tissue, which are couple of orders of magnitude more intense. The scattering pattern from collagen-rich tissues include the typical characteristics of the fibrillar scattering, described in the next subsection.

Moreover, Article II shows the possibility to characterize the tissues of the breast by SAXS, stressing its potential ability to differentiate benign from malignant tissues. In Article III this ability is compared with the histology and the morphology of the tumours, and shown to have potential use for diagnostics.

### 8.1.2 Scattering from Fibrils

Densely packed cylindrical fibrils constitute an anisotropic system, where two directions of scattering are clearly defined: the meridional direction, parallel to the fibrils’ axis and the equatorial direction, perpendicular to the fibrils’ axis (Wess et al., 1998b). In the the meridional direction, photons are mainly scattered by the electron distribution that varies along the axis. In the equatorial direction, the scattering pattern contains information about the fibrils’ size and their packing.

Connective tissue basically consists of collagen fibrils (see Sections 2 and 3) arranged in a close packing and in a very hierarchical manner. The elastic scattering from collagen fibrils ranges from WAXS region, which contains information about the intra- and inter-molecular structure, to SAXS and USAXS,
Figure 11: Scattering patterns from some breast tissues belonging to the same tumour. The intensity of scattering from adipose tissue (fat) is well below the intensity from collagenous tissues. Collagen peaks are clearly visible in the healthy collagen pattern, while they are almost non-existing in the invaded collagen. Fifth and tenth orders of the collagen peaks are indicated. Another interesting difference between healthy and invaded collagen patterns is the background intensity, higher in the latter. The double peak from adipose tissue at \( s \approx 0.22 \text{ nm}^{-1} \) arises from the packing of triglycerides. Note that the scattering vector is \( s = Q/2\pi \).

which contain information about supra-molecular and fibrillar structures (see Article I). The scattering from collagen fibrils is well understood (Eikenberry et al., 1982a,b) and it can be modeled with great accuracy in the SAXS region (see Article IV).

In the meridional direction there are scattering features that consist of several ring-like sharp peaks, called the collagen peaks. They are the different orders of the same Bragg diffraction peak, associated with the periodical axial electron density of the fibrils, or the \( d \)-spacing (see Section 3). These peaks can be modeled from a periodical function representing the electron density.

In the equatorial direction, scattering radiation bears information about the fibril size. The scattering looks like a series of concentric broad rings. Their shape can be modeled by a Bessel function of first order, c.f. Article I,
therefore they are dubbed Bessel peaks. Modulation intensity related to the inter-fibrillar distances is also observed, which can be represented by an appropriate interference function $G(Q)$, or just simply by a single interference peak.

Scattering from fibrils is anisotropic. In connective tissue the collagen fibrils are arranged more or less parallel to each other forming fibers, which have a lesser degree of ordering. Finally, the collagen strands may present any orientation, depending on their location within the tissues. An exception is collagen in tendons, where the ordering at all scales is preserved. Furthermore, the connective tissue may be layered and the collagen fibers randomly oriented in the layers. In a loose connective tissue there is no preferred orientation and the collagen peaks appear as isotropic rings. The orientation distribution can be included in the model, which is used for simulation of the SAXS pattern (see Article IV).

8.1.3 Structural Parameters

The structural changes of collagen fibrils are compatible with the model of collagen degradation where the fibrils break and became “loose” and “hairy”. The hierarchical structure of collagen-rich tissues suffers a disruption in the presence of specific degrading proteins (collagenases), associated to certain pathologies, for example cancer. These structural changes could be followed up by observing certain parameters which can be extracted from the scattering patterns.

It has been deduced from the scattering patterns (see Article II and Article III) that the fibrils are thinner and they are closer to each other in malignant neoplasias than in healthy tissues. These differences can be followed up by studying the position of the Bessel peaks and the structure peak, both found in the equatorial direction of scattering.

Moreover, the $d$-spacing of the fibrils is slightly larger in malignant tissues than in benign ones. Larger $d$-spacing results on a shift of the collagen peaks to smaller $Q$ values, since $d = 2\pi / Q$. Finally, another observation compatible with the degradation of the fibrils is the increase of the background intensity in malignant tissues. The scattered intensity is proportional to the total area per mass unit of the objects. Therefore, “hairy”-degraded fibrils will scatter more intensity than the healthy ones. Other parameters can be selected to study structural aspects of fibrillar collagen, but it seems that the ones with better potential to do a systematic study are the background intensity and the $d$-spacing (c.f. Article II).

A description of the study of the scattering signals from pathological and healthy breast tissues is presented in the following.
8.2 Experiment Description

Some aspects of the experimental procedures and techniques are briefly exposed here. The experiments were designed to obtain small-angle x-ray scattering patterns of breast tissues, including several malignant and benign tumours. Several samples were selected and prepared for the data acquisition. The data was treated and interpreted using a software developed by the author. The preliminary results of this experiment are described in Article I, and an association between aberrant collagen and SAXS signatures can be established. These encouraging results led to extension of the study and many other samples were investigated, including the acquisition of USAXS scattering patterns using a Bonse-Hart camera. Results from the USAXS measurements indicated that the scattering pattern presented long tails and they gave a hint for the introduction of a new reconstruction algorithm (Fernández et al., 2006). These studies culminated with the publication of Article II, where a breast tissue characterization by SAXS is presented.

The investigation proceeded along a parallel line with the inclusion of diffraction enhanced images (DEI) of some of the samples and the reproduction of the USAXS patterns by means of the DEI set-up. The images were used to match the morphological features with the histo-pathology of the samples and the SAXS parameters. The result of this matching was excellent and the results where published in Article III.

All the experiments were carried out on two beamlines at the storage ring at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France: the High Brilliance beamline, ID02, and the Medical Beamline, ID17.

8.2.1 The Samples

Sample selection, fixation and manipulation is worth a few comments, since the tissue structures may be altered by these processes. A total of 28 samples were used in this work. They were selected from specimens of tumours surgically excised from patients diagnosed with either breast cancer or bearing benign tumours (c.f. Section 2).

The samples were provided by the Helsinki University Central Hospital, and transported to France for examination. It is important to note here that consent from the patients was granted for the use of the specimens in scientific research, together with the permission of the Hospital’s Ethical Committee and the French Ministry of Health.

The specimens were immediately frozen in liquid nitrogen after excision. The samples were cut in disks of 1 mm thick and 20 mm in diameter. They were let thaw and immediately submerged in formalin (a saline solution con-
taining 10% volume of formaldehyde). After the samples were fixed, they were placed between thin Kapton (DuPont de Nemours, France) windows and mounted in an aluminium frame.

The fixation of the samples is something that raised concern, since the process of fixation dehydrates the tissues, modifying the structure of collagen, which is very sensitive to hydration. This problem is discussed in Article III and it was concluded that the changes due to dehydration of collagen do not wash-out the structural changes produced by pathological processes. Therefore, fixed samples could be safely used for this study.

In order to obtain a priori information about the histology of the samples, a second thin disk was cut just next to the first one. This second disk was immediately processed and sliced for histological examination under the microscope. An experienced pathologist directed this examination. The information obtained was used as a guideline for the scattering experiment, allowing to select for examination potentially interesting regions of the samples.

After the experiments, the samples were prepared for histological examination. The histo-pathology of the samples is of key importance in this work, since the scattering patterns must be correlated with each tissue. Despite that the samples were 1 mm thick and the histological slices of just about 5 μm, the latter represented quite well the composition of the tissues that were probed by the beam.

8.3 Scattering Measurements

The scattering measurements were carried out at the High Brilliance beamline, ID02, at ESRF. The source of radiation of this beamline is an undulator, which produces a high brilliance, low divergence, synchrotron beam. The beam is collimated by means of slits, focused with x-ray mirrors and monochromatized with perfect silicon crystals (Narayanan et al., 2001).

8.3.1 Pin-Hole Camera

The pin-hole camera for SAXS experiments is situated at 55m from the undulator source at ID02. The camera consists of a slit set, a flux monitor (pin-diode), a beamstop with a second built-in flux monitor (pin-diode) and a detector, in this order along the beam direction (see Figure 12). The beam is focused at approximately the detector position, which can be translated inside of an evacuated tube, so the distance sample-detector can be set in a range from 1.5 to 10 metres. For this experiment, the monochromator was set to deliver a beam with a wavelength of ~1 Å or 0.1 nm, equivalent
Figure 12: Scattering set-up (ID02). Inset: flux geometry and coordinates at the sample position for SAXS measurements. The red arrow represents incoming monochromatic radiation. The sample can be translated in the xz-plane. Adapted from Article III.

to the photon energy of 12 keV. The size of the beam was approximately 200×200 μm. The sample was scanned through the beam with stepper motors in a mesh of about 100 measuring positions. From every position a full scattering pattern was recorded. The detector was a 1024×1024 pixel FReLoN CCD camera, coupled to an x-ray image intensifier, XRII. For most of the experimental data set, the distance sample-detector was 10 m, but many scattering patterns were recorded at 3 m from the sample. For these distances and the given energy, the scattering vector ranges from $Q \approx 0.025$ nm$^{-1}$ to $Q \approx 2$ nm$^{-1}$.

An extra problem to deal with was the radiation damage of the tissues. In order to get a proper exposure time, a sequence of irradiations and acquisition of scattering patterns was performed. The structures of the tissues were followed up, and the maximum irradiation time for the tissues — before any measurable structure damage — was established to be of about 1 minute. This is discussed in Article I. The radiation damage was found not to be an important issue after all, since a complete scattering pattern was obtained with integration times of 20 to 50 milliseconds. The photon flux at the sample position in ID02 is of the order of $8 \cdot 10^{12}$ photons/s at 100 mA of ring current.
The scattering patterns were normalized to the transmitted beam intensity using the two pin-diodes as beam monitors (See Figure 12). The flux is measured before and after the sample. If the sample has a constant thickness, the ratio between these two intensities can be used as a intensity normalization factor. The value measured with the beam monitor on the beamstop has a small angular aperture and therefore does not include the intensity that has been scattered away from the main beam. In most cases this is a very small effect. The usual corrections for SAXS data, described by Narayanan et al. (2001), were applied to the data including CCD flat field correction, dark current subtraction and geometrical corrections.

8.4 Acquisition of Diffraction Enhanced Images

The images shown in Article III were acquired using the diffraction enhanced imaging (DEI) set-up at ID17, the Medical beamline at ESRF. The SR source of this beamline is a wigglar, situated ~150 meters from the experimental set-up (Elleaume et al., 1999). A fan beam of 100 mm x 1 mm (Hor. x Vert.) was separated by slits, and a fixed-exit double-Laue monochromator was used for the selection of a broad band of radiation ($\Delta E/E > 10^{-4}$) (Suortti et al., 2000) centered at 51.5 keV. The band-width was reduced to less than $10^{-5}$ by the imaging monochromator. In order to obtain images, the samples were scanned through the flat beam. The whole set-up is very sensitive to vibrations and much care was devoted to damping of the vibrations.

The DEI is an analyzer-based imaging system (see Figure 13). It consists of a pair of perfect crystals arranged as a double-crystal diffractometer in a non-dispersive disposition (Chapman et al., 1997; Keyriläinen et al., 2002). The object to be imaged is placed between the crystals. The first crystal, situated before the sample is called the monochromator, and the crystal after the sample, the analyzer. The two crystals define a common reflectivity curve, or rocking curve (RC), which is the auto-convolution of the reflectivity curve of a single crystal. Therefore, the total width of the RC will be $w_{RC} \approx 1.2w_{D}$, where $w_{D}$ is the Darwin’s width (Zachariasen, 1945).

The DEI imaging system is in fact a simple version of the Bonse-Hart camera (Bonse and Hart, 1966). DEI can be used equally to image the sample, as to scan its scattering. Some examples of images can be seen in Article III. In DEI there is a single reflection per crystal whilst three to five reflections are possible in a Bonse-Hart set-up. DEI has limited sensitivity to weak scattering since the tails of the rocking curve are not as reduced as in the Bonse-Hart camera (Diat et al., 1997). Like in the Bonse-Hart camera, the DEI imaging is only sensitive to beam deviations in the plane of diffraction of the crystals.
Figure 13: Schematic representation of a DEI set-up. The analyzer is tuned to its working position with a piezoelectric actuator, installed at the extreme of a 0.5 m long arm. The resolution of the angle scale is 0.02 μrad. The sample is scanned in the z direction, and the images are acquired line-by-line with the FReLoN CCD.

The images were acquired using a 2048×2048 pixel FReLoN CCD camera coupled to a fluorescence screen by an optic fiber taper. The CCD was operated in the so called pipe-line mode, where a few lines of the detector are exposed and the accumulated charges are transferred to the adjacent lines for read-out. The read-out of the CCD is synchronized with the sample scan motor, so the images are built line-by-line. The acquisition time of a single image was a couple of seconds. Moreover, the delivered doses were very low (Bravin et al., 2002). The CCD’s pixel size is 47 μm. This, together with optimal point spread function of the fluorescence screen, allows a very good spatial resolution in the images.

Two symmetrical refraction images and a TOP images were acquired for every sample in the works reported in Article III. The refraction images were obtained tuning the analyzer to an angle off from the Bragg reflection by $\frac{1}{2}$ of the RC’s FWHM. The TOP image is acquired setting the analyzer to its nominal position, at the exact Bragg reflection angle. The images were then reconstructed with the algorithm introduced by Chapman et al. (1997).
9 Summary of the Results I-IV

In this Section, a summary of the results of this study is presented. The different aspects of the investigation will be briefly recalled and the findings from the publications exposed.

Scattering Studies

Fibrillar collagen, found in breast connective tissue, can be used as an indicator of the pathological state of the tissues. Breast cancer tissue presents an aberrant form of collagen, which has notable structural differences compared with collagen in healthy tissues. This phenomenon is understood as a possible break-up of the structure of the collagen fibers caused by collagenase, a metalloproteinase present in breast tumours that degrades collagen. The structure of the collagen can be studied with the aid of small-angle x-ray scattering, SAXS, which gives information about the size and the packing of the fibrils, as well as the axial periodicity of collagen, or the $d$-spacing. Moreover, SAXS patterns from fibrillar collagen can be fairly well modeled. Simulated models of structural degradation of the fibers are in good agreement with the observed scattering patterns.

![Graph showing scattering patterns](image)

Figure 14: Characterization of breast tissue by SAXS. Note the intensity difference between adipose tissue (healthy fat) and necrotic tissue. First and fifth orders of the collagen peaks are indicated. Adapted from Article II.
It is important to remind here that the formalin fixation of the samples did not alter the structural changes of the collagen fibrils produced by pathological degradation.

The first important finding of this study was the demonstration of the utility of the SAXS signal as a tissue-recognition tool. Article I and Article II compile the efforts that were done in this direction. The comparison of the patterns with the sample histology was very important during this phase of the study, as the scattering properties of the breast tissues were not known. The scattering from tissues (of inhomogeneous composition) is in general a mixture of different scattering components. Therefore, the histological comparison was also important for establishing scattering "standards" of the "pure" tissues.

SAXS patterns from healthy adipose tissue (fat) were found to be of relatively low intensity, particularly when compared with the patterns from necrotic tissue, which were several orders of magnitude more intense. The scattering patterns from collagen-rich tissues shown the characteristic collagen peaks.

In Section 8, two parameters taken from the SAXS patterns were introduced to determine the structural state of collagen fibers and therefore, obtain histo-pathological information about the tissues. One is the background intensity between the collagen peaks, which is related to the total surface area of the fibrils. The other is the position of the collagen peaks in the $Q$-space, which reflects the $d$-spacing along the axis of the fibrils. The parameters are complementary, so a structural break-up of the collagen fibrils will be seen both as an increase of the background intensity and as a shift of the $d$-spacing to larger values. A complete table displaying measured values of these parameters—which includes data from 28 samples—can be found in Article III. A couple of results can be pointed out:

- The $d$-spacing of invaded collagen was found to be about 0.7% larger than normal.
- The background scattering between the collagen peaks was from 1.7 to 2.5 times more intense in degraded collagen.

Surprisingly, some areas from samples labeled to be malignant revealed scattering characteristics similar to those from healthy samples. The consequent histological examination showed that these areas were free of cancer cells (i.e. healthy) despite the fact that the rest of the sample was malignant.

Further evidence of the degradation of the collagen fibrils is found in the equatorial direction of the scattering patterns. In this direction, information about the size and the packing of the fibrils is found. It can be seen in
Figure 14 that the position of the Bessel peaks \((s \sim 3 \cdot 10^{-2} \text{ nm}^{-1})\) and the interference peak \((s \sim 10^{-1} \text{ nm}^{-1})\) are shifted to larger values of \(s\), meaning that the fibrils become thinner and their distance smaller. The study of the behavior of the scattering patterns in the Porod’s regime gave also a hint about the structure of the tissues. For instance, SAXS from necrotic tissue showed a behavior close to \(Q^{-2}\), which is typical of coiled structures.

Maps

The SAXS patterns are shown to be particularly good as tissue characterization tool and they can be used to follow up pathological changes on the collagen-rich tissues. As it was described in Article II, the SAXS signal from a single measurement spot can be represented by a couple of parameters (see above), like the background intensity and the \(d\)-spacing length. These parameters can be converted into colour-coded maps and compared with the histo-pathology of the samples. As a result, a correlation between them can be established. Two of these examples are presented in Article III.

\[\text{Figure 15: Spott} \text{ing invasion of stroma by cancer cells with SAXS. a) Histology of an area of a carcinoma ductale. Stained pink, the collagen-rich stroma; pink-brownish, the areas that are invaded by cancer cells. b) the colour-coded map of the same region. Red: shift of the d-spacing to values larger than the mean. Green: mean value of all the measured points. The solid circle points a spot with a microcalcification. The dotted ellipse encircles an invaded region.}\]

The first colour map example of Article III shows that the background intensity is an excellent probe for tissue recognition. The colour map depicts the boundaries of the stroma (basically collagen) and the adipose tissue (fatty cells). Moreover, a region of invaded collagen is clearly revealed by the increase of the scattered intensity. It is an interesting fact that some collagen areas around \textit{in situ} islands of cancer cells appear to be healthy in terms
SAXS (i.e. they scatter as much intensity as the healthy). This was a corroboration of the previous observation, where healthy tissues were found close (or around) malignant tumours.

Another clear example of healthy collagen in the vicinity of invaded tissues is presented in Figure 15. The histology shows that only certain areas of the stroma were invaded by cancer cells (brownish pink in Figure 15a). In this case, the d-spacing signal is used to build a colour map of a region from a carcinoma ductale. Every colour dot represents a single SAXS measurement point. The dots were coded as follows: red, larger d-spacing; green, normal d-spacing. It was evident that the area presenting larger d-spacing in the collagen fibrils was coincident with the more invaded region of the stroma.

**Imaging**

Furthermore, it has been demonstrated that the DEI images are an important aid for the interpretation of the SAXS data and the histology, because they clearly reveal the contours of the tissues. A good example of this is found in Article III and in Figure 16.

![Figure 16: Histology, a), and DEI slope image, b), of a sample from a carcinoma ductale of the breast. The morphological details shown in the histology are clearly visible in the DEI image. The border line between the collagen (pink in the histology) and the fat is clearly delimited. This is pointed out by two solid lines. As further reference, a microcalcification has been encircled. The roundish structures at the lower part are a mixture of microcalcifications and necrotic tissue located in a lobule.](image-url)
Simulations

The SAXS pattern from collagen was interpreted in Article I using a simple structure model. It was realized that the pattern includes much detailed information, which could be extracted from a more specific and realistic model. The model introduced in Article IV includes distributions of the fibril sizes and orientation, and it is demonstrated how these parameters can be determined from the experimental SAXS patterns. Figure 17 demonstrates that the average fibril diameter can be calculated from the minima of the Bessel function, which describes the equatorial pattern, even when the distribution is wide.

![Figure 17: Calculated equatorial scattering from perfectly oriented cylinders with three different radius distributions (a) and closeup on the first Bessel minimum (b). The radius distributions are represented in the inset of figure (a): the mean value is 30 nm and the standard deviations are 1, 3 and 5 nm respectively. Adapted from Article IV.](image)

The successful simulation of the small-angle x-ray scattering patterns from collagen-rich human breast tissue samples indicates that the simplified model for the electron density distribution is basically correct on the supramolecular level. When the model is applied to simulation of scattering from cancer-invaded tissue, it is observed that the parameters of the model change from those found when modeling healthy tissue. The differences in the model parameters correspond to structural changes of the fibrillar collagen, which may elucidate the mechanisms of cancer growth and invasion.
10 Conclusions

The results of the Articles I to IV fulfilled all the expectations of the research. It has been seen that the SAXS patterns have a clear utility in breast cancer diagnosis and the scattering signal from the tissues can be mapped throughout the samples. Moreover, it was seen in Article III that the fixation of the samples, despite changing the tissue structures, does not alter the results.

It is true that this work is an *in vitro* study with thin excised samples. Obviously, acquiring SAXS patterns from thicker samples, like complete breasts, is not practical. One direct application of SAXS for breast diagnosis was proposed by Lewis *et al.* (2000) and the technique is being developed. It consists of the acquisition of SAXS patterns from needle-core biopsies of breast tumours and this may be the application of SAXS closest to clinical practice.

The results of Article III suggest that DEI and imaging by scattering may be combined. It is important to realize that the DEI set-up is essentially a Bonse-Hart camera, so that the scattering signal may be analyzed at the same time. Preliminary results suggest that a quantitative separation of scattering is possible (Fernández *et al.*, 2006).

Is it important to note that the study of the SAXS signal allows revealing the structural changes that collagen suffers in the invaded stroma (i.e. in the presence of cancer cells). This is interesting in the case of certain tumours, which are difficult to spot mammographically in dense collagenous tissue (like carcinoma lobulare), especially in the absence of other tumour indicators like microcalcifications. For these particular cases, the SAXS imaging could be of great interest.

10.1 Future Prospects

The primary goal of this work has been to understand the changes of the supra-molecular structure of collagen and their possible correlation with tumour development. These changes have been evaluated from comparisons of the SAXS patterns with histo-pathology of the samples. These observations suggest that SAXS patterns may be used as diagnostic indicators. The morphology of the tissue is revealed in DEI and it may be possible to evaluate characteristic structural changes of the tissues from the scattering contribution in the DEI images. This would allow describing the effects of cancer growth in a range of many length scales.

Clearly this study needs to be continued including new tumour examples (especially those of difficult diagnosis) and other kinds of tissues also present
in the breast like skin, muscle or scar tissue. The skin is very rich in collagen and a clear source of scattering that must be taken into account in whole-breast scattering imaging. Scar tissue is another interesting example to be considered, since it is also collagen-rich and a potential source of specific scattering signal. Imaging thick samples with the DEI method is, on the other hand, something that can be easily achieved (Keyriläinen et al., 2005). Therefore, it opens the possibility to extend the studies from DEI to scattering imaging.

The experimental work is based on the use of synchrotron radiation (SR), although in principle, the methods can be used with conventional x-ray sources. New compact SR sources may become available in a few years and these would bring the new methods presented in this work to clinical environment.
References


