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Human breast cancer *in vitro*: matching histo-pathology with small-angle x-ray scattering and diffraction enhanced x-ray imaging

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Abstract

Twenty-eight human breast tumour specimens were studied with small-angle x-ray scattering (SAXS), and 10 of those were imaged by the diffraction enhanced x-ray imaging (DEI) technique. The sample diameter was 20 mm and the thickness 1 mm. Two examples of ductal carcinoma are illustrated by histology images, DEI, and maps of the collagen \(d\)-spacing and scattered intensity in the Porod regime, which characterize the SAXS patterns from collagen-rich regions of the samples. Histo-pathology reveals the cancer-invaded regions, and the maps of the SAXS parameters show that in these regions the scattering signal differs significantly from scattering by the surrounding tissue, indicating a degradation of the collagen structure in the invaded regions. The DEI images show the borders between collagen and adipose tissue and provide a co-ordinate system for tissue mapping by SAXS. In addition, degradation of the collagen structure in an invaded region is revealed by fading contrast of the DEI refraction image. The 28 samples include fresh, defrosted tissue and formalin-fixed tissue. The \(d\)-values with their standard deviations are given. In the fresh samples there is a systematic 0.76% increase of the \(d\)-value in the invaded regions, averaged over 11 samples. Only intra-sample comparisons are made for the formalin-fixed samples, and with a long fixation time, the difference in the \(d\)-value stabilizes at about 0.7%. The correspondence between the DEI images, the
SAXS maps and the histo-pathology suggests that definitive information on tumour growth and malignancy is obtained by combining these x-ray methods.

1. Introduction

Breast cancer is one of the principal causes of death among women in developed countries (Parkin et al 2001). Early detection of malignant tumours is closely related to a better survival rate (Michaelson et al 2002). Mammographic screening programmes are carried out in many countries as part of their public health care systems, and diagnostic mammography is usually the first step in clinical examinations of suspicious findings, such as an abnormal screening mammogram or a palpable lump. Diagnostic mammography has rather high sensitivity, order of 90% (Saarenmaa et al 2001), but the method has some inherent limitations. Mammography gives purely morphological information about the breast tissue, and the nature of tumours is determined by their shape, size, margins, density and location of the lesion and by the characterization of possible associated calcifications. Occasionally, tumours are seen as opaque masses, and these are especially difficult to discern in dense breasts.

While scattering is a nuisance and a limiting factor in clinical mammography, it carries much information about the composition and molecular structure of the breast tissue. In particular, the scattering amplitude of coherent scattering is the Fourier transform of the electron density of the object. The observable quantity is the intensity, but still the scattering pattern yields a detailed image of the structure of the object on the atomic and molecular level. Recently, there has been increasing interest in studying tumour-carrying breast tissue samples by scattering for imaging purposes (Evans et al 1991, Speller 1999, Leclair and Johns 2002, Cardoso et al 2003). It is known from earlier studies that collagen fibres in pathological breast tissues are different from those in healthy ones (Kauppila et al 1998, Pucci-Minafra et al 1998), and the corresponding signals in the x-ray scattering patterns have been searched for (Lewis et al 2000, Fernández et al 2002).

Another way to study the soft tissue, and in particular that from the breast, is by DEI, or diffraction enhanced imaging (Chapman et al 1997). This method is based on the use of a double crystal diffractometer in a non-dispersive arrangement. The sample is situated between the crystals, and very small refraction and scattering effects at the tissue interfaces can be measured. The angular range where these effects take place is as small as a few micro-radians, i.e. less than one arc-second, and the effects can be seen as intensity variations of the beam diffracted by the perfect crystal analyser. The analyser acts as a tuneable narrow angular slit, which is used to resolve scattering distributions and deviations of the transmitted beam due to refraction at tissue interfaces. Many morphological features in soft tissues that are difficult or impossible to see in clinical mammography can be detected with this method (Lewis et al 2003, Fiedler et al 2004, Keyriläinen et al 2005).

The main motivation of the present work is that of a better understanding of the changes in tissue structure taking place in breast cancer growth. The different methods probe the structures in length scales that cover many orders of magnitude. By DEI, details of the order of 0.1 nm can be visualized, while the structures reflected in the SAXS patterns have dimensions of a few tens of nanometres. Optical microscopy of histology sections differentiates tissues on the 1 µm level and reveals the presence and action of cancer cells, and this makes a link between the effects of cancer growth on the molecular and the macroscopic morphological levels. Quite extensive data have been cumulated in this study. Two cases, which exhibit typical pathological features, are illustrated by maps of histo-pathology, DEI and parameters...
extracted from the SAXS patterns. Relevant structural parameters of healthy and cancer-invaded collagen are given numerically for a large number of other samples. It will be seen that a one-to-one correlation can be established between the DEI images, the scattering maps and the histology of the samples, suggesting that x-ray imaging may be used as a non-invasive histo-pathological examination of breast tissue samples. It is clear that the method may have diagnostic applications, but at this stage we concentrate on elucidation of the signs and mechanisms of cancer growth.

2. Small-angle x-ray scattering (SAXS) and diffraction enhanced imaging (DEI)

Elastic photon scattering is one of the main scattering processes at the energies where this work has been carried out. The waves scattered from different parts of the object interfere and form an intensity pattern that is determined by the atomic and molecular structure of the object. The scattering amplitude is the Fourier transform of the electron density $\rho(r)$ of the object (Guinier 1955),

$$F(h) = \int \rho(r) \exp(ih \cdot r) \, d^3r.$$  \hspace{1cm} (1)

The scattering vector $h$ is defined by the wave vectors of the incident and scattered beams, $k_1$ and $k_2$.

$$h = (k_2 - k_1) = 2\pi s.$$  \hspace{1cm} (2)

It is evident that for two scatterers separated by vector $r$ constructive interference occurs when $h \cdot r = 2\pi m$ or $s \cdot r = m$, where $m$ is an integer, and a periodical variation $d$ in the direction of the scattering vector gives rise to interference maxima at $s = md^{-1}$. Inter-atomic distances (order of nanometres) cause interference at large values of the scattering vector, and this is called wide-angle x-ray scattering (WAXS), whilst supramolecular structures (tens to hundreds of nanometres) scatter in phase at small angles (SAXS). Finally, scattering from larger structures (from hundreds of nanometres to a few microns) is seen at even lower angles, in the regime of ultra small-angle x-ray scattering (USAXS). In the following, we concentrate on the SAXS regime, where the scattering pattern is determined by the supra-molecular structures of tissues.

2.1. Small-angle x-ray scattering

The principles and techniques of SAXS have been reviewed recently (Glatter 2002), and only a few results that are important in the present context are given here. First, the structure of the object, i.e., its electron density, cannot be derived from the measured scattering pattern by simple Fourier inversion; in fact, only the auto-correlation function is so obtained. When there is independent information about the structure, e.g., from electron microscopy, a structural model can be introduced and the calculated SAXS pattern is fitted to the experimental pattern. When there is sufficient ordering in the structure the parameters of the model can be resolved from different parts of the scattering pattern. It will be seen that this is the case of scattering from collagen present in breast tissue.

Second, the strongest interference effects are seen in first order, i.e., when $s \cdot r = 1$ ($h \cdot r = 2\pi$), when there is no long-range ordering in the sample. This means that the interference effects are washed out at sufficiently large values of the scattering vector. In this regime, Porod’s law applies, and for three-dimensional non-fractal objects the asymptotic behaviour of the scattered intensity is (Porod 1951)

$$I(h)_{as} = 2\pi(\Delta\rho)^2S \times h^{-4}.$$  \hspace{1cm} (3)
Here $\Delta \rho$ is the density difference between the object and its surroundings, and $S$ is the specific surface area, i.e. the surface area per unit volume. Porod’s law indicates that the intensity of scattering increases with non-uniformity of the sample, and this turns out to be an important parameter of the tissue structures.

The main components of breast tissue are the adipose tissue of large fatty cells, and connective tissue of fibrous collagen. In the case of in situ carcinoma there are also regions of necrotic tissue. Each one of these tissue components gives rise to a characteristic SAXS pattern. SAXS from healthy adipose tissue is of low intensity and has very little structure, while scattering from necrotic tissue is intense and falls off as $h^{-2}$, suggesting a structure of disordered molecular coils (Fernández et al. 2002). Between these two extremes, there is an intensity gap of two orders of magnitude, which makes it easy to identify adipose and necrotic tissues by their SAXS patterns. SAXS from collagen-rich tissue falls in this wide gap, and it is the only component of scattering that has much structural detail.

Collagen is a fibrous protein, which is the main component of breast connective tissue. It is made of three $\alpha$-helixes, which are coiled around each other to form a long semi-rigid molecule. Due to intermolecular interactions there is, in the axial direction, a well-defined shift between neighbouring molecules, which results in a nearly parallel staggered array (Chapman 1989, Wess et al. 1998). There are of the order of 1000 molecules in the approximately circular cross section of the fibril. The fibrils have a periodical variation of the axial electron density variation, and the period is called the collagen $d$-spacing. It can vary (even strongly) depending on the type of collagen (i.e. the composition of the $\alpha$-helixes) and on the physical conditions of the fibrils, like strain or humidity (Hulmes 2002). In healthy human breast, collagen is composed mainly of type I and type III (Kauppila et al. 1998), and the $d$-spacing is around 65 nm (Fernández et al. 2002).

Scattering from collagen can be simulated using a model where the fibrils are described as aligned and closely packed cylinders, which form fibres (Fernández et al. 2002). The fibre defines two directions of scattering, the meridional, parallel to the fibre axis, and equatorial, perpendicular to the axis. There are rather sharp Bragg reflections in the meridional direction, and the intensity in the equatorial direction shows modulations due to the size and packing of the collagen fibrils. The structural parameters of the model can be derived from two-dimensional SAXS patterns acquired by an area detector. In general, the fibres have a distribution of orientations with respect to the incident x-ray beam, and this distribution can be included in the model for the collagen structure (Suhonen 2004). It turns out that with such a model the total scattering patterns from collagen-rich tissue samples can be simulated very accurately.

Several earlier studies have shown distinct differences between the SAXS patterns from healthy and cancer-invaded collagen (Lewis et al. 2000, Fernández et al. 2002, 2004). The changes are attributed to structural degradation of invaded collagen or to a low degree of ordering of the newly formed collagen among the adipocytes in the fatty tissue, where collagen is accompanied by invading cancer cells. The presence of cancer cells between the collagen fibres seems to be a necessary condition for modification of the collagen structure. Collagen that surrounds an in situ island of carcinoma, which has an intact epithelial wall, has the same structure as collagen in healthy tissue.

The main differences between the collagen SAXS patterns from healthy and invaded tissues are in the intensity level between the low-order collagen Bragg reflections, and in the $d$-spacing. Model calculations with realistic fibril sizes proved that the Porod regime extends to the $h$-range between the third and fifth order collagen peaks, where the differences in intensity can be attributed to differences in the specific surface area of the bundles and fibrils. Differences between scattering patterns of collagen in healthy and invaded areas of
the same breast tissue sample are illustrated in figure 1, and discussed in detail in the results section.

The above characteristic features of the SAXS patterns, which are related to the differences in the collagen structure, can be used for tissue mapping and comparison of the maps with the corresponding histo-pathology. It is expected that a one-to-one correlation can be established, so that the signs of malignancy can be read from the maps of scattering parameters (Fernández et al 2004).

2.2. Diffraction enhanced imaging

DEI is an analyser-based imaging system, which is x-ray absorption and phase-sensitive. The set-up consists of a double-crystal diffractometer in the non-dispersive arrangement (see figure 2). The sample is placed between the two identical crystals, called the monochromator (before the sample) and the analyser (after the sample). When the analyser is rotated about the axis that is perpendicular to the incident beam and the plane of diffraction the so-called rocking curve (or reflectivity curve) is recorded. The rocking curve is the auto-correlation function of the one-crystal rocking curve. In the case of weakly absorbing perfect crystals in symmetrical reflection geometry the rocking curve is approximately triangular. In the ‘nominal’ or ‘zero’ position, where the crystals are completely parallel, the reflectivity curve has its maximum. Under this condition, the analyser crystal acts as a narrow angular slit and rejects all scattering outside that range. The analyser can be rocked around the
Figure 2. Scheme of the diffraction enhanced imaging set-up at the ID17 beamline of the ESRF. The sample, placed between a double crystal diffractometer, is scanned through the beam (Z-arrow). Fine-tuning of the analyser is performed with a piezoelectric actuator, which pushes a long arm attached to the crystal.

zero position by a piezo-electrically driven lever arm system with better than 0.1 μrad resolution.

For x-rays travelling through matter, the index of refraction can be written as

$$ n = 1 - \delta - i\beta, $$

where the real part $\delta$ corresponds to the phase shift due to scattering and the imaginary part $\beta$ to the absorption. In DEI the effects of the x-ray phase variation are converted to variation of intensity by the analyser crystal either by scatter rejection or by refraction. When the surface normal of the interface, where the density of the object changes from $\rho_1$ to $\rho_2$, makes an angle $\alpha$ with the incident beam the beam deviates by $\Delta\phi = (\delta_1 - \delta_2)\tan\alpha = \Delta\delta\tan\alpha$. The refraction effects are strongest when $\alpha$ is close to 90°, i.e. at the lateral interfaces of the object. It should be noted, however, that the analyser ‘sees’ only that component $\Delta\phi_n$ that is parallel to the plane of diffraction, which is vertical in the DEI images, so that the refraction contrast tends to zero in the horizontal direction. In terms of density variations, $\Delta\delta \approx 1.35 \times 10^{10} \Delta\rho$ [g cm$^{-3}$] $\lambda^2$[cm$^2$].

The DEI method is very versatile due to the simultaneous effects of refraction and scatter rejection, but there are several subtleties that must be understood for interpreting the images correctly (Chapman et al 1997, Hasnah et al 2002). Successful utilization of the DEI method requires an extreme stability of the system. This is achieved by vibration damping, separation of the scan stage from the monochromator-analyser mount, and by including a feed-back loop in the fine-tuning of the analyser rocking angle (Fiedler et al 2004).

3. Experiment

The experiments reported in this paper consist of two different and consecutive experiments: imaging and small-angle scattering. The imaging part of the experiment was limited to acquiring DEI images of the samples at three positions of the rocking curve, and retrieving the contributions of apparent absorption and the pure refraction. Once the samples were imaged, they were brought to the small-angle scattering beamline, and maps of SAXS patterns were acquired over the sample area.
3.1. Samples

The samples were obtained from excised breast specimens. These specimens were first deep frozen in LN₂ and stored at −80 °C and their size was of several cubic cm. Altogether 28 samples were used in the SAXS experiments, and DEI images were acquired from 10 samples. While frozen, a few slices that were 1 mm thick and 20 mm in diameter, were cut from them and with the help of a hypodermic needle, dye colours were introduced into the samples. These colours were used as spatial reference markings. Surgical dyeing colours appear to have no significant effect on the scattering signal in the regions studied, but nevertheless, dyed spots were avoided during the experiment.

Two adjacent slices were cut from each specimen. They were let thaw and submerged in formalin⁷ for fixation. The first slice was prepared for histological examination, and it was used as a guide for selection of the interesting regions of the sample for the SAXS experiments. The second slice was placed between two thin Kapton⁸ foils, and mounted leak-tight into an aluminium frame.

After the experiment, the samples were processed for histo-pathological examination by embedding them in paraffin blocks for cutting about 5 µm thick slices, which were stained (Herovici 1963) and placed between microscope glasses. Special care was exercised to preserve the sample orientation.

Formalin fixation reduces the water content of the tissues. Collagen fibrils suffer structural changes as a consequence of this dehydration: the basic structure remains intact, but the d-spacing becomes shorter. This effect is discussed in section 4.3, where numerical results are given in table 1.

3.2. Imaging

The samples were imaged using a DEI set-up installed in the experimental hutch at ESRF’s ID17 Medical Beamline (see figure 2). First, a double crystal Laue monochromator at 140 m from a wiggler source gives a broad-band monochromatic flat beam (33 keV), which is limited by slits to be approximately 1 mm high and 100 mm wide. Two samples next to each other were placed between the narrow-band Si(333) monochromator and the identical analyser. The samples were scanned vertically through the beam, and a high-resolution area detector was used to record the beam reflected by the analyser. The detector (FreLoN camera) has a fluorescent screen for conversion of x-rays to visible light, which is guided by tapered fibre optics to a CCD (Bravin et al 2003). The image is recorded line-by-line, and the pixel size is 47 µm. All the samples were imaged at three angular positions of the analyser: at the middle of the slope of the rocking curve on the low-angle side, at the corresponding position on the high-angle side and at the top of the rocking curve. The rocking angle from the top position to the mid-slope position was 1.1 µrad. Radiation doses were of the order of 1 mGy.

3.3. Small-angle scattering

In the second phase of the experiment, small-angle scattering patterns of the samples were recorded at the High Brilliance Beamline ID02 of the ESRF (see figure 3). The x-ray source was an undulator, and the beam was monochromatic (12 keV) and focused on the sample as a pencil beam (200 × 200 µm²). The photon flux at the sample position was approximately

⁷ Formalin is a 10% formaldehyde solution.
⁸ Polyamide polymer film (Kapton, DuPont de Nemours, France).
Table 1. Summary of collagen $d$-spacing and intensity of SAXS. The samples are grouped according to the treatment, and the character of the sample is indicated. The letter in the code indicates the patient, and there may be several samples from one patient. The $d$-spacing is given with the standard deviation of the values taken from a well-defined benign or malignant region, where the number of points, $N$, varies from 3 to 190 (global average 75 points). Systematic analysis of intensity was carried out for four samples only, and given by $I_m/I_b$ (malignant/benign). The samples discussed in sections 4.1. and 4.2. are indicated by bold print.

<table>
<thead>
<tr>
<th>Diagnostics</th>
<th>$d$ (nm)</th>
<th>$N$</th>
<th>$d$ (nm)</th>
<th>Malign. $N$</th>
<th>$I_m/I_b$</th>
<th>Notes</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1 Fibroadenoma</td>
<td>64.89 ± 0.09</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>Benign lesion</td>
</tr>
<tr>
<td>A2 Fibroadenoma</td>
<td>64.88 ± 0.07</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td>Benign lesion</td>
</tr>
<tr>
<td>A3 Mastopathic</td>
<td>65.02 ± 0.04</td>
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<td>Benign lesion</td>
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<tr>
<td>B1 Ca ductale GIII</td>
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<td>81</td>
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<td></td>
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</tr>
<tr>
<td>B2 Ca ductale GIII</td>
<td>65.29 ± 0.21</td>
<td>28</td>
<td></td>
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<tr>
<td>B3 Ca ductale GIII</td>
<td>65.19 ± 0.06</td>
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<td>65.27 ± 0.23</td>
<td>73</td>
<td>2</td>
<td>Benign and malignant</td>
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<td>B4 Ca ductale GIII</td>
<td>65.24 ± 0.11</td>
<td>27</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B5 Ca ductale GIII</td>
<td>65.10 ± 0.06</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C Ca ductale GIII</td>
<td>64.87 ± 0.11</td>
<td>96</td>
<td></td>
<td></td>
<td></td>
<td>Benign region</td>
</tr>
<tr>
<td>D1 Ca lobulare</td>
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<td>160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2 Ca lobulare</td>
<td>65.39 ± 0.07</td>
<td>3</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B6 Ca ductale GIII</td>
<td>65.25 ± 0.25</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td>1–2 days fixation</td>
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<td>Short time</td>
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<tr>
<td>E1 Ca ductale GI</td>
<td>64.83 ± 0.30</td>
<td>121</td>
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<td></td>
<td>Histology benign</td>
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<tr>
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<td>64.84 ± 0.19</td>
<td>59</td>
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<tr>
<td>F2 Ca lobulare GIII</td>
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<td>106</td>
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<td>106</td>
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<td>Measurement repeated</td>
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<td></td>
<td></td>
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<td>Mainly healthy</td>
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<tr>
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<td>64.78 ± 0.06</td>
<td>16</td>
<td>64.81 ± 0.10</td>
<td>23</td>
<td>2.5</td>
<td>Benign and malignant</td>
</tr>
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<td>64.82 ± 0.04</td>
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<td></td>
<td></td>
<td></td>
<td>Near in situ (invasive?)</td>
</tr>
<tr>
<td>G4 Ca ductale GIII</td>
<td>64.79 ± 0.05</td>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td>Benign region</td>
</tr>
<tr>
<td>G5 Ca ductale GIII</td>
<td>64.75 ± 0.07</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>Collagen in invaded fat</td>
</tr>
<tr>
<td>Long time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H Ca ductale GIII</td>
<td>59.69 ± 0.12</td>
<td>75</td>
<td>60.06 ± 0.19</td>
<td>19</td>
<td>2.4</td>
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<tr>
<td>J Ca lobulare GIII</td>
<td>59.95 ± 0.23</td>
<td>90</td>
<td></td>
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<td></td>
</tr>
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<td></td>
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</tr>
<tr>
<td>K Ca ductale GIII</td>
<td>59.97 ± 0.10</td>
<td>11</td>
<td>60.47 ± 0.18</td>
<td>11</td>
<td>1.7</td>
<td>Near DCIS/invasive</td>
</tr>
<tr>
<td>L Ca ductale GIII</td>
<td>59.67 ± 0.64</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td>Calcifications present</td>
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<tr>
<td>M1 Ca ductale GIII</td>
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<td></td>
<td></td>
<td>DCIS</td>
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<tr>
<td>M2 Ca ductale GIII</td>
<td>59.89 ± 0.10</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
<td>Invasive</td>
</tr>
</tbody>
</table>

$10^{12}$ photons s$^{-1}$. The sample was in air, close to the thin entrance window of the SAXS camera. Pinhole geometry was used, and the scattering patterns were recorded with a FReLoN detector where a fluorescence screen is coupled to a CCD camera with lens optics. The detector is placed in a big vacuum tank, where the sample-to-detector distance was varied from 3 to 10 m. The integration times for a single scattering pattern were from 20 to 50 ms, depending on the type of the tissue. The radiation dose was 200–500 Gy per image, which is about 1% of the dose needed for clear radiation damage to the collagen structure (Fernández et al 2002). The samples were moved by stepper motors along a co-ordinate system referred to the frame of the sample holder. A mesh scan was performed (steps varied from 0.25 to 1 mm), recording a complete scattering pattern for each position. The DEI images, together with the previous
3.4. Mapping by SAXS

The parameters of the collagen structure, which are obtained from the SAXS patterns, can be used to make quantitative maps over the sample area. As discussed earlier and seen in figure 1, the most obvious markers of the scattering patterns are the positions of the Bragg reflections and the intensity of scattering in the Porod regime. These correspond to the axial period and the specific surface area of the fibrils and fibres. Other characteristics of the patterns are the period of intensity fluctuations, which are determined by the fibril diameter and packing distance, and the slope of the intensity curve in the Porod regime, which indicates the dimensionality of the object. For mapping, the intensity between the third and fifth orders of the Bragg reflection, and the $d$-spacing are used.

For the analysis and data reduction, the SAXS patterns were azimuthally integrated, plotted as functions of $h$ or $s$, and normalized by the absorption factor. Well-established routines of the dedicated SAXS beamline were used for determination of the centre of the scattering pattern (Hammersley et al 1996). The effects of a possible residual error are eliminated in comparisons, because the same centre is used in all azimuthal integrations.

The attenuation in the sample was measured by two PIN diodes, which were located upstream of the sample and at the beamstop (see figure 3). The samples were about 1 mm thick, and the attenuation was approximately proportional to the sample thickness (Fernández et al 2002). With 12 keV incident radiation and 10 m sample-to-detector distance the $s$-values
Figure 4. First example, ductal carcinoma in situ with invasion (sample G): (a) histological slice of the sample. The dark pink areas are collagen-rich and the light pink areas contain adipose tissue (fat). A solid line marks the border of the invaded tissue. Note two islands of carcinoma in situ surrounded by healthy tissue. The arrows mark features visible in the refraction image and are used for orienting the sample; (b) refraction image of the sample. The arrows point the features visible in the histology. Note the perfect match despite the fact that histology is a small fraction of the sample thickness; (c) map of the background intensity, overlaid by the histology. Dots: blue = low intensity (fat in the histology), red = high intensity (collagen in the histology). The higher intensity is found from the invaded collagen. Note that the collagen surrounding the in situ islands shows healthy characteristics; (d) map of the measured axial period. The longer periods are found mainly in the invaded collagen; (e) typical SAXS patterns from this sample. The collagen peaks became fainter upon cancer invasion. Inset shows detail around the fifth collagen peak (marked with arrows). Vertical scale is compressed for a better comparison.

are covered from 0.004 nm$^{-1}$ to 0.114 nm$^{-1}$, corresponding to object sizes between 250 nm and 8.8 nm. The intensity fluctuations due to fibril size and packing, the first, third and fifth Bragg peaks, and much of the Porod regime are seen in this range.

Automatic routines were developed for calculation of the average intensity between $s = 0.052$ nm$^{-1}$ and 0.065 nm$^{-1}$, i.e. between the third and fourth (which is usually invisible) Bragg peaks. The position of the third Bragg reflection, the peak value and the integrated intensity were determined by fitting a Gaussian and a straight line to the sloping background and the reflection profile. The fit gives the centre of the reflection, not the apparent peak position, which is affected by the slope of the background. The numerical values of the
4. Results and discussion

Breast tissue samples carrying tumours were imaged in three ways, by optical microscopy of stained histology sections, by x-ray projection imaging with the DEI method and by the Fourier images of the SAXS patterns. The principal aim of this work is to overlay the histology images and the associated pathology analysis with the parameter maps of the SAXS patterns in order to see correlation between cancer growth and changes in the supra-molecular structure of collagen. The SAXS maps and histology images are also compared with the DEI images to look for morphological signs of breast cancer growth. The examples include two common forms of breast cancer: ductal carcinoma with in situ and invading regions, and ductal carcinoma (grade II) with a clear front towards healthy tissue. The SAXS patterns from 28 samples are described by the distributions of the d-values and intensities that correspond to scattering from healthy and cancer-invaded collagen-rich tissues.

4.1. Ductal carcinoma in situ with invasion, sample G

The sample was defrosted and fixed in formalin for two weeks. The histo-pathology of the sample showed a tumour with mainly healthy collagen and some regions of intensely invaded
collagen (figure 4(a)). The borders between collagen and adipose tissue show up very clearly in the DEI image that is the difference between the images taken at the opposite slope positions of the analyser (figure 4(b)). For instance, a thin collagen strand is seen in the upper part of the image with a perfect match with histology, and this helped in overlaying the images. There seems to be more structure in the regions of healthy collagen than in the invaded region. The spatial correlation of the SAXS intensity map with histology is good: low intensity in the regions of healthy adipose tissue, medium intensity in the regions of healthy collagen, and highest intensity in the invaded region (figure 4(c)). The comparison between histopathology and the d-spacing map is far less conclusive for this sample; although the d-spacing is generally larger in the invaded region, there are rather large variations (figure 4(d)). The Bragg peaks are small in the invaded regions, which hampers fitting of the Gaussian curve to the experimental reflection profile. A rather striking example of collagen degradation and fading of the Bragg reflections is shown in figure 4(e). In this angular range, the well-ordered, healthy collagen exhibits sharp reflections from the 5th order to the 12th order (note that the even order reflections are seen, too), but in the invaded regions the axial ordering gradually disappears, and the general intensity increases indicating breaking up of structures. The
Figure 5. (Continued.)
change of intensity becomes easier to see, but the Bragg reflections become more and more
difficult to separate from the background.

4.2. Ductal carcinoma (grade II), sample H

This sample was defrosted and fixed in formalin for 10 weeks. In this case, the pathology
examination revealed two well-defined areas: one with healthy collagen and the other with
invaded collagen. The areas were well defined and separated by the so-called ‘pushing-front’
(figure 5(a)). The DEI image (figure 5(b)) was used for establishing the co-ordinate system in
the SAXS measurement. Some other features of the DEI image are commented on later. The
maps of the SAXS intensity and the \(d\)-spacing have an excellent mutual correlation and also
with the histo-pathology. The scattered intensity of the Porod regime is distinctly higher in
the areas where pathology examination shows invaded collagen (figure 5(c)). In this case the
collagen peaks were easier to fit than in the previous example, since the tissue is not as heavily
invaded as in the previous example. The peak-fitting algorithm works well in most cases, and
the \(d\)-spacing map shows a clear result: the \(d\)-spacing is systematically longer in the area of
invaded collagen (figure 5(d)).

Figure 5(e) shows a detailed comparison of images along a line that crosses the ‘pushing-
front’. First, the jumps both in the intensity and \(d\)-spacing are seen, when the front is crossed
from the healthy region to the invaded one. At the same time the DEI slope image shows
much more structure in the healthy region than on the other side of the front. It appears
that ordered collagen has stronger internal boundaries than the invaded collagen in the length
scale that is visible in DEI. In the present case the smallest discernible details are a few tens
of micrometres. It looks that above that limit the structures in the invaded region are quite
homogeneous, i.e. ordering at the level of collagen bundles and fibres has disappeared.

4.3. Summary of structural parameters of collagen

Many samples have been studied by SAXS in the course of this work. Some of the results
have been reported earlier (Fernández et al 2002, 2004), and in the following we summarize
findings on 28 samples. In some cases the data are incomplete, but intra- and inter-sample
comparisons between healthy and cancer-invaded tissues can be made in terms of the collagen
\(d\)-spacing and the scattered intensity in the Porod regime. The results are collected in table 1.

Great care was exercised to ensure that the differences in the \(d\)-spacing were genuine, not
artefacts of the fitting procedure. Background subtraction and profile fitting are critical issues,
which have been studied extensively (Honkimäki and Suortti 1999). Comparisons between
benign and malignant regions are straightforward for a given sample or patient, but there are
also clear overall differences between similarly treated samples. In the case of fresh, defrosted
samples the \(d\)-values are pair-wise similar for all samples, and that there is a systematic
increase of 0.76% due to invasion of cancer. Formalin fixation reduces the \(d\)-value, and there
seems to be a threshold period, because up to 2 weeks the changes are small, but drastic at
10 weeks. Although only intra-sample comparisons can be done reliably for the samples of
short fixation time, there is a small overall difference of 0.2% in the \(d\)-values of benign and
malignant regions. On the other hand, the difference stabilizes again at 0.7% for the samples
that have been fixed for the longest time (10 weeks). The standard deviation of the \(d\)-values
is typically larger in the cases of invaded regions, because the Bragg reflections become weak
and the background level is higher. The intensity ratio \(I_m/I_b\) (malignant/benign) is given in
four cases only, but values between 1.5 and 2.5 were observed in all samples.
5. Conclusions

The results of this work show that the SAXS patterns reveal changes in the tissue structure that are caused by invasion of breast cancer. The collagen degradation is the key to the scattering-based mapping, which matches one-to-one with the histo-pathology of the samples. The degradation manifests itself in the breaking of the collagen fibrils, which results in an increase of the specific surface area of the fibrils and intensity of scattering. It is probable that the long-range intermolecular bonding is relaxed in shorter fibrils, which leads to an increase in the axial period of the fibrils. This is probably the most important observation of the present study, because it links the changes in the fibril structure to changes at a deeper level, and this observation will be followed up in future studies. The decrease of ordering is also seen in the reduction of the effects of refraction in the images obtained by the DEI method. The aim of this work was elucidation of the effects of cancer growth in different length scales by combining information from the Fourier images of molecular structures, from histo-pathology at the cell level, and from images of tissue morphology. The results suggest that x-ray methods might be used as a diagnostic tool, which provides the essential information now obtained by histo-pathology.

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References

Chapman J A 1989 The regulation of size and form in the assembly of collagen fibrils in vivo Biopolymers 28 1367–82
Hasnah M O et al 2002 Diffraction enhanced imaging contrast mechanisms in breast cancer specimens Med. Phys. 29 2216–21
Herovici C 1963 A polychrome stain for differentiating pre-collagen from collagen Stain Technol. 38 204–05
Honkininki V and Suortti P 1999 Effects of instrument function, crystallite size and strain in reflection profiles Defect and Microstructure Analysis by Diffraction ed R L Snyder, J Fiala and H J Bunge (New York: Oxford University Press) chapter 4
Keyriläinen I et al 2005 Visualization of calcifications and thin collagen strands in human breast tumour specimens by the diffraction enhanced imaging technique: a comparison with conventional mammography and histology Eur J. Radiol. 53 226–37
Lewis R A et al 2003 X-ray refraction effects: application to the imaging of biological tissues Br. J. Radiol. 76 301–8
Michaelson J S et al 2002 Predicting the survival of patients with breast carcinoma using tumor size Cancer 95 713–23
Pucci-Minafra I et al 1998 Absence of regular α2(I) collagen chains in colon carcinoma biopsy fragments Carcinogenesis 19 575–84
Saarenmaa I et al 2001 Validity of radiological examinations of patients with breast cancer in different age groups in a population based study Breast 10 78–81
Speller R 1999 Tissue analysis using x-ray scattering X-Ray Spectrom. 28 244–50
Suhonen H 2004 private communication