Measurement of Iron Oxide Nanoparticulate Size and Clustering Behaviour in Iron Loaded Rodent Tissues using Novel Small Angle Synchrotron X-Ray Scattering Techniques

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Abstract

Iron overload diseases such as hereditary haemochromatosis and thalassaemia affect a large fraction of the world’s population. During iron overload, iron is deposited in tissues of the body in the form of ultrafine particles of hydrated iron(III) oxyhydroxide (5Fe$_2$O$_3$.9H$_2$O) associated with the iron storage compounds haemosiderin and ferritin. The physical form of the mineral cores of ferritin and haemosiderin is of particular importance since it is expected to reflect the biological and environmental conditions of deposition. For example, the size distribution determines the surface iron to core iron ratios, which in turn is expected to determine the molar toxicity of cellular iron deposits.

A methodology for quantifying the morphology and clustering behaviour of iron oxide nanoparticles in bulk samples of mammalian tissue has been developed and tested in a controlled biological system. The methodology has been applied to elucidate iron oxide particle size and clustering behaviour in the tissues of rats under a number of different iron loading conditions. Liver and spleen specimens from rats experiencing one of three iron loading regimes were available for the study. Control rats were fed a normal rodent diet, dietary iron loaded rats received supplementation with carbonyl iron and transfusional iron loaded rats received transfusions of packed red blood cells. Tissue iron concentrations ranged from 1 mg.g$^{-1}$ dry weight for control liver to 70 mg.g$^{-1}$ dry weight for the most highly loaded transfusional iron loaded spleen.

Iron oxide cores of the iron storage compounds ferritin and haemosiderin were imaged in unstained ultrathin sections of rat tissue by transmission electron microscopy (TEM). TEM studies showed both dispersed and clustered iron-containing nanoparticles. The clusters of nanoparticles were of the order of a micron in size and exhibited varying degrees of order and density. The dispersed particles were found to have a mean (± SD) particle diameter of 5.27 ± 0.83 nm. No global correlation was found between diameter and tissue iron concentration or organ type. The effective $d$-spacings of arrays of clustered particles were measured from the radially averaged fast Fourier transform (FFT) of digital images to be 9.0 ± 0.5 nm. No significant difference was found between the different iron loading states. The $d$-spacings and locations of spots in the
FFT were consistent with close packed 12 nm diameter ferritin protein shells with a face centred cubic (FCC) unit cell of dimension 17 nm.

Small angle x-ray scattering (SAXS) was employed to achieve an increase in sample volume of nine orders of magnitude over TEM. For analysis by small angle x-ray scattering the preferred tissue preparation was freeze-drying, followed by grinding to a fine powder and compacting to a pellet. The sample pellets were homogeneous and representative of the bulk. Scattering from iron oxide deposits was compounded with scattering from tissue structure. Anomalous SAXS (ASAXS) near the iron K-edge was employed to identify iron specific scattering features. Complex contrast variation could be observed in all tissues and iron specific scattering data displayed good signal to noise beyond tissue iron concentrations of 2 mg.g\(^{-1}\) dry weight.

Modelling of the iron specific scattering data facilitated quantification of particle diameter and clustering behaviour in the bulk and returned similar data to those found by TEM. ASAXS from the most highly loaded rat liver and spleen tissue indicate that the most likely particle diameter for spherical iron oxide deposits is 6.4 ± (SD) 0.3 nm. No appreciable difference in particle size with iron concentration, iron loading pathway or organ type could be identified. The scattering data indicated that the number rather than size of iron oxide nanoparticles increases with iron loading to accommodate the iron burden.

Spatial correlation between particles was required to achieve a satisfactory fit of theoretical models to the iron specific scattering data from iron loaded tissue. Spatial correlation was not required for fits to most data from control tissues. As such, clustering is likely to be extensive in the iron loaded but not control tissues. The most likely distance between planes of packed spheres from the iron specific scattering data was 8.6 ± 0.3 nm. Again, no significant change in packing distance could be observed with iron loading. The effective d-spacings observed by ASAXS are consistent with TEM data and a FCC unit cell of dimension 17 nm. The unit cell dimension and occupancy revealed no significant change with iron loading, therefore the number or size of micron scale clusters of iron laden cellular organelles must increase to accommodate the increasing storage requirements of heavily iron loaded tissue.

The iron oxide particle size and clustering behaviour measured by TEM and ASAXS remained remarkably similar under the wide variety of iron loading conditions in this study. As such, the primary determinant of the size and packing distance of iron oxide cores deposited in rat liver and spleen tissue is most likely the ferritin protein shell.
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Table 5.1: Sequence of steps involved in the data collection strategies A, B and C. $J_{TR}$ is the flux measured at the transmission detector and $J_{CCD}$ is the scattered flux measured at the CCD detector.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d\sigma$</td>
<td>differential scattering cross section</td>
</tr>
<tr>
<td>$d\Omega$</td>
<td>solid angle</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>solid angle</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
</tr>
<tr>
<td>$\eta$</td>
<td>detector efficiency</td>
</tr>
<tr>
<td>$\phi$</td>
<td>volume fraction</td>
</tr>
<tr>
<td>$f$</td>
<td>atomic scattering factor</td>
</tr>
<tr>
<td>$f'$</td>
<td>real anomalous dispersion correction factor</td>
</tr>
<tr>
<td>$f''$</td>
<td>complex anomalous dispersion correction factor</td>
</tr>
<tr>
<td>$f_0$</td>
<td>atomic scattering factor for normal dispersion</td>
</tr>
<tr>
<td>$\Delta \rho$</td>
<td>contrast / scattering length density difference</td>
</tr>
<tr>
<td>$A$</td>
<td>cross sectional area of incident beam</td>
</tr>
<tr>
<td>AgBeh</td>
<td>silver behenate</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>protein shell devoid of iron core</td>
</tr>
<tr>
<td>APS</td>
<td>Advanced Photon Source</td>
</tr>
<tr>
<td>ASAXS</td>
<td>anomalous small angle x-ray scattering</td>
</tr>
<tr>
<td>$B$</td>
<td>constant prefactor for power law term</td>
</tr>
<tr>
<td>$B$</td>
<td>scattering length</td>
</tr>
<tr>
<td>$b_e$</td>
<td>scattering length of a single electron</td>
</tr>
<tr>
<td>$C$</td>
<td>unit cell size</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CTF</td>
<td>contrast transfer function</td>
</tr>
<tr>
<td>$d$</td>
<td>real space dimension or distance between atomic planes</td>
</tr>
<tr>
<td>E</td>
<td>energy</td>
</tr>
<tr>
<td>EELS</td>
<td>electron energy loss spectroscopy</td>
</tr>
<tr>
<td>EFTEM</td>
<td>energy filtered TEM</td>
</tr>
<tr>
<td>$Erf$</td>
<td>error function</td>
</tr>
<tr>
<td>ETA</td>
<td>distance between layers of packed spheres</td>
</tr>
</tbody>
</table>
FCC
Fe
FEGTEM
Ferritin
FFT
G
$g(r)$
GIF
Haemosiderin
Hb
Hepatic
Hepatocyte
HRTEM
HSF
I($Q$)
$I(Q)_{arb}$
$I_s$
$I_{STD}$
$J$
$J_0$
$J_{0,S}$
$J_{0,STD}$
$J_{0,TR}$
$J_{0,TR,E}$
$J_B$
$J_{CCD}$
$J_{CCD,B}$
$J_{CCD,S}$
$J_{CCD,STD}$
$J_{TR}$
$J_{TR,B}$
$J_{TR,S}$
K-edge

face-centred cubic
Iron
field emission gun TEM
iron storage protein
fast Fourier transform
prefactor for Guinier term of Unified scattering function
scattering length density distribution
Gatan image filter
iron storage compound
haemoglobin
of the liver
Perform main functions of the liver
high resolution TEM
horse spleen ferritin
absolute scattering intensity
normalised intensity data in arbitrary units
scattering factor for a single electron
absolute scattering intensity of the standard at a particular $Q$
scattered flux (spherical wave)
Incident flux (plane wave)
incident beam flux measured by the incident beam monitor during scattering measurements on the sample
incident beam flux measured by the incident beam monitor during scattering measurements on the polyethylene standard
Incident flux measured by transmission detector
incident flux measured by the transmission detector with incident beam energy $E$
scattered flux from blank measured by CCD detector
scattered flux measured by CCD detector
scattered flux from the blank measured by the CCD detector
scattered flux from the sample measured by the CCD detector
scattered flux from the standard measured by the CCD detector
flux measured by transmission detector
flux through the blank measured by the transmission detector
flux through the sample measured by the transmission detector
inner electron shell (K) absorption edge

- xx -
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_f )</td>
<td>scattered wave vector</td>
</tr>
<tr>
<td>( k_i )</td>
<td>incident wave vector</td>
</tr>
<tr>
<td>Kupffer cells</td>
<td>non-parenchymal, highly mobile macrophages, 15% of liver cells</td>
</tr>
<tr>
<td>Lysosome</td>
<td>membrane bound organelle containing hydrolytic enzymes for intracellular digestion</td>
</tr>
<tr>
<td>Macrophage</td>
<td>cells of the reticuloendothelial system capable of engulfing and destroying cellular debris</td>
</tr>
<tr>
<td>MCM-41</td>
<td>mesoporous silicon dioxide</td>
</tr>
<tr>
<td>( n_e )</td>
<td>number of electrons</td>
</tr>
<tr>
<td>( N_p )</td>
<td>number of particles</td>
</tr>
<tr>
<td>( P )</td>
<td>exponent of power law</td>
</tr>
<tr>
<td>( P(Q) )</td>
<td>form factor</td>
</tr>
<tr>
<td>( PA )</td>
<td>net peak area</td>
</tr>
<tr>
<td>( Pack )</td>
<td>strength of spherical correlation</td>
</tr>
<tr>
<td>Parenchyma</td>
<td>main functional cells of organ eg: hepatocyte</td>
</tr>
<tr>
<td>Periportal</td>
<td>regions round the portal vein</td>
</tr>
<tr>
<td>( \mathbf{Q} )</td>
<td>scattering vector / momentum transfer</td>
</tr>
<tr>
<td>( Q )</td>
<td>magnitude of the scattering vector</td>
</tr>
<tr>
<td>( Q_{IN Variant} )</td>
<td>scattering invariant</td>
</tr>
<tr>
<td>( R )</td>
<td>distance between the electron and the point of observation</td>
</tr>
<tr>
<td>( R )</td>
<td>radial distance from the centre of any scattering body</td>
</tr>
<tr>
<td>( r_e )</td>
<td>classical radius of electron</td>
</tr>
<tr>
<td>reticuloendothelial cells</td>
<td>cells whose function is defence against infection and disposal of cellular debris</td>
</tr>
<tr>
<td>( R_g )</td>
<td>radius of gyration</td>
</tr>
<tr>
<td>( R_P )</td>
<td>radius of uniform sphere (particle)</td>
</tr>
<tr>
<td>( S(Q) )</td>
<td>structure factor</td>
</tr>
<tr>
<td>SAXS</td>
<td>small angle x-ray scattering</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error on mean</td>
</tr>
<tr>
<td>Siderosome</td>
<td>iron laden lysosome</td>
</tr>
<tr>
<td>( S_P )</td>
<td>surface area of particle</td>
</tr>
<tr>
<td>Splenic</td>
<td>of the spleen</td>
</tr>
<tr>
<td>( T )</td>
<td>thickness</td>
</tr>
<tr>
<td>( T_B )</td>
<td>transmission of blank</td>
</tr>
</tbody>
</table>
TEM: transmission electron microscopy

$t_s$: sample thickness

$T_s$: sample transmission

$t_{STD}$: thickness of the standard

$T_{STD}$: transmission of the standard

$V$: irradiated sample volume (scattering volume)

$V_P$: Volume of a particle

$Z$: atomic number

$A$: shape parameter for $P(Q)$

$\Theta$: half the scattering angle, $2\theta$

$P$: scattering length density

$\rho_M$: scattering length density of the matrix

$\rho_P$: scattering length density of the scatterer
Acknowledgments

My first vote of thanks is reserved for Associate Professor Tim St Pierre for his supervision of this body of work. This research would not have been possible without Tim’s creativity and enthusiasm. Thank you, Tim, for taking the risk (or having the faith) to support me in this opportunity of a lifetime. Your dedication and tact are to be admired.

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I am grateful to Dr Ralph James for being an Igor Pro and devising the computer model of particles for me. The x-radiography was made possible by Bruce Stock and Giselle
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There were times when I thought I might never achieve this goal and if it weren’t for my family, friends and faith I am quite sure it would have been unattainable. A very special thanks to the Solar Powered Superheroes Joany J-Co Connolly and Nicole N-Go Gorham and honorary member Holly H-Ro Rose. What can I say - you three are stuck with me (and unfortunately your yaws) for life. G’day from WA (and thanks) to Mikey Zee, the walking grammar book with a flip-flop soundtrack. To the Dodgy Parmenter Small Group, for your uplifting friendship. To my very dear friends Sanda, Ali and Belinda for keeping me afloat with your meals-on-wheels on the home stretch.

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My Peter... that’s one down and one to go. I look forward to it :) Thank you for everything.

And to Him who truly makes the impossible possible... thank you.

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1 Iron Loaded Mammalian Tissue: 
Iron Oxide Particle Size and Clustering Behaviour

1.1 Iron storage in mammals

Iron is an essential trace element for nearly all forms of life. In humans, iron is an important constituent of many biomolecules such as haemoglobin and myoglobin for oxygen transport and storage, and cytochrome c for cell respiration and ATP formation [1, 2]. Iron is lost by the body at a rate of about 1 mg/day through bleeding and epithelial cell desquamation [3]. Since no mechanism for excretion of excess iron exists, homeostasis is achieved by finely controlled dietary absorption, coupled with deposition of or mobilisation from iron stores [4].

There are two types of cellular iron storage compounds in mammals, the soluble form, ferritin and the insoluble type, haemosiderin. These compounds are normally found in man in several organs including liver, spleen, bone marrow, brain, heart, kidney, muscle and intestinal mucosa. Iron associated with these proteins is bioavailable and in the form of ultrafine hydrous ferric oxide or oxyhydroxide, herein referred to collectively as iron oxides (see reviews [3, 5]). Free iron is toxic and both compounds provide some protection by segregating iron from the cytosol [6]. Additionally, storing iron in ferric forms renders the deposits relatively inert, as it is ferrous iron that more significantly promotes formation of reactive oxygen species leading to oxidative damage at physiological pH [3].
1.1.1 Ferritin

Ferritin is the main form of iron storage in normal tissues. It consists of a hydrophilic protein shell of 24 subunits which renders it soluble. The roughly spherical protein has external and internal diameters of 12 nm and 8 nm (see Figure 1.1) [4, 7]. The chemical speciation of iron in ferritin is similar to the mineral ferrihydrite (5Fe₂O₃·9H₂O) [4]. The iron cores of ferritin are regularly sized (see Figure 1.2a) and can store up to 4500 Fe(III) atoms [8]. If the cavity is vacant, the protein shell is termed apoferritin.

![Figure 1.1: Schematic diagrams of ferritin a) 24 subunits comprise the protein cage b) cross section reveals iron oxide core attached to inner surface of protein shell (after [4, 9])](image)

1.1.2 Haemosiderin

The biochemical compound haemosiderin is also involved in iron storage and, unlike ferritin, it is poorly characterised. Haemosiderin is insoluble and the often incomplete protein shell gives rise to a lower protein to iron ratio than ferritin [3]. Iron cores show irregular morphology and a greater size distribution than ferritin ranging from 0.8 – 7 nm in diameter [10] (see Figure 1.2 and Table 1.1).

The wide size distribution that appears not to be limited by the ferritin cage suggests that haemosiderin deposition is not as controlled as that of ferritin [11]. Haemosiderin is normally found clumped in cells and though always iron(III), the chemical form varies [12]. Haemosiderin is thought to both arise from the breakdown products of ferritin [13] and also to be independently deposited iron [14]. As such, haemosiderin is considered to be both used for iron storage and cellular debris [5].
1.2 Iron overload diseases

Iron imbalance in man can lead to iron deficiency or iron overload. Iron overload diseases affect a large fraction of the world's population including 1 in 200 Australians [16-18]. During iron overload, iron is deposited in tissues of the body in the form of ultrafine particles of hydrated iron oxide associated with the iron storage compounds haemosiderin and ferritin. Accumulation of iron saturates ferritin synthesis. Ferritin deposits can increase ten-fold whereas haemosiderin deposits can increase 100-fold [19].

Organs particularly affected by iron overload are the liver, spleen, heart, pancreas, and adrenal gland. For example, normal hepatic iron concentrations are typically in the range 0.5 to 2 mg Fe.g\(^{-1}\) dry tissue [20, 21] whereas concentrations in iron loaded liver may exceed 40 mg Fe.g\(^{-1}\) dry tissue [22]. The excess iron is toxic, leading to cell death and organ dysfunction [23]. There is considerably risk of liver fibrosis for hepatic iron concentration in excess of 20 mg.g\(^{-1}\) dry weight [20]. Severe cases of iron overload can result in fatal liver and heart failure [24]. Knowledge of body iron loading is therefore of primary clinical importance in the management of haemochromatosis and thalassaemia.

Many different pathologies result in excess iron deposition and they may be classified by the route of iron acquisition; dietary or transfusional.
1.2.1 Dietary iron overload diseases

Dietary absorption of iron is normally finely regulated to control body iron [25, 26]. A genetic defect in hereditary haemochromatosis causes a loss of control over dietary absorption and results in iron loading [27]. With uncertain genetic element, Africans have presented with dietary iron overload owing to consumption of beer, traditionally home-brewed in steel drums [17, 28]. These conditions are examples of primary iron overload.

Dietary absorption and thus deposition of iron is increased in the transfusion independent anaemias in attempt to increase erythropoiesis and alleviate the anaemia [29]. These conditions, such as thalassaemia minor, thalassaemia intermedia and haemoglobin E disease are termed secondary iron overload since the iron loading is not the primary aetiology.

Regular phlebotomy is effective in reducing excess body iron stores and is the treatment for hereditary haemochromatosis [30, 31].

1.2.2 Transfusional iron overload diseases

In affluent countries, the treatment for several anaemic conditions involves regular blood transfusions to relieve the anaemia [32]. This treatment results in a large influx of iron into the patients’ bloodstream and thus iron overload. In these cases of secondary transfusional iron overload, chelation therapy is employed to reduce the tissue iron burden [33]. Thalassaemia major (β-thalassaemia), the non-thalassaemic hyperplastic refractory anaemias and the hypoplastic refractory anaemias are examples of secondary transfusional iron overload diseases [34-37].
1.3 Iron oxide particle size

The structure, function and composition of ferritin and haemosiderin have been the focus of much medical research. The research is important to improve our understanding of the mechanisms of iron processing and storage in the diseases described above, with a view to treatment and better clinical outcomes. In addition, ferritin with its self limiting nanoscale protein cage has attracted attention as a tracer, ideal model system and more recently with applications in nanotechnology [38-42].

In particular, there has been much interest in measuring the mineral core size of ferritin and haemosiderin from mammals as summarised in Table 1.1. A variety of measurement techniques have been employed and these are discussed in section 1.3.1. Studies of particle diameter have supported investigations of chemical form by Mössbauer spectroscopy, crystallinity by high resolution transmission electron microscopy, electron and x-ray diffraction, and structural and compositional studies by inductively coupled plasma spectrometry [43-48].

The studies summarised in Table 1.1 have established that particle size can vary according to biological mechanisms associated with its formation. Particle sizes studied to date have implicated relationships with several significant iron loading parameters. Particle sizes are affected by hydration and iron content. Differences in diameter are observed between the two iron storage compounds, ferritin and haemosiderin. The chemical form and crystallinity may have implications for the physical form. Mobilisation of iron and thus bioavailability and toxicity may be affected by the particle size. Particle size may be related to the tissue, cell or organelle in which it is deposited. Furthermore, the iron loading pathway or pathology may affect the physical size of deposits. These biological and environmental parameters are discussed in section 1.3.2, with particular reference to the iron oxide particle sizes.

1.3.1 Measurement techniques

Particle sizes have been measured primarily by transmission electron microscopy (TEM) but Mössbauer spectroscopy, small angle x-ray and neutron scattering (SAXS and SANS) and x-ray diffraction have been employed. Ferritin and haemosiderin core sizes have been studied in situ and, more commonly, following isolation from tissue.
Transmission electron microscopy exploits the electron density of the iron core to visualise ferritin and haemosiderin without the need for staining [49]. The technique has been useful in visualising the iron cores and facilitating measurement of sizes [45, 50, 51]. Between 50 – 100 iron cores are typically measured per sample, although some exceed 200 to improve sampling [52]. The estimation of particle size is affected by the focus of the microscope which is a somewhat subjective setting. The image of a sphere has no sharp change in contrast at its perimeter, thus definition of the particle edge is also subjective.

The precision of particle size measurement can be improved with high resolution TEM (HRTEM) [53, 54]. This technique measures individual cores rather than a population of particles to the detriment of sampling.

Estimates of particle size have been made indirectly with Mössbauer spectroscopy. The distribution of superparamagnetic blocking temperatures measured by Mössbauer spectroscopy provides an estimate of nanoparticle size [55, 56]. Mössbauer spectroscopy has been applied to study the diameter of iron oxide cores [47, 57] however variation in chemical form and crystallinity confound the measurement [58].

Electron and x-ray diffraction studies of iron storage compounds are chiefly concerned with the crystallinity of the core. The diffraction line width from single crystal cores has been used to provide indirect estimates of particle size [4, 44, 59] but these measurements are once again confounded by degree of crystallinity.

Small angle scattering techniques overcome the sampling limitations of microscopy and have been applied to study solutions of ferritin [7, 60-62]. Small angle x-ray and neutron scattering (SAXS and SANS) usually provide featureless monotonically decreasing intensity vs inverse space data. There are multiple solutions to modelling of the scattering data and prior knowledge of the sample is required. However, scattering studies have proved to be useful in confirming TEM observations in larger sample volumes.

1.3.2 Biological implications

Hydration

Ferritin cores were first observed in the TEM by Farrant in 1954 [49]. Early preparations returned core sizes of 5.5 nm and ferritin shell external diameters of 10 to
11 nm [49, 63, 64]. Harrison later showed with x-ray diffraction that the degree of hydration affected the measured size and reported a shell diameter of 11.2 nm which was swollen to 13.0 nm when wet [65]. Air drying of early preparations was thought to result in contraction of the protein shell. Scattering studies of ferritin in solution confirmed the larger shell sizes of 12 nm [7, 61] and the initial underestimates are attributable to differences in sample preparation.

Iron concentration

From early scattering data it was thought that the particle size remained unchanged with iron content [66]. More recent interpretations clarified that the core was always adhered to the inside of the ferritin shell independent of its iron content and size [62, 67].

The iron oxide particle size is determined by the number of iron atoms in the ferritin core, ranging from zero to a maximum of 4500 atoms. Horse spleen ferritin (HSF) reconstituted with approximately 1000, 2000 and 3000 atoms returned particle sizes of 5.22 ± 0.93 nm, 6.3 nm and 6.87 ± 0.33 nm respectively [47, 68]. As such, particle size has been used to estimate the number of iron atoms stored in HSF.

Chemical form

The chemical speciation of iron in ferritin is similar to the mineral ferrihydrite (5Fe₂O₃.9H₂O) [4]. Between two and six of the characteristic ferrihydrite diffraction peaks are observed for ferritin depending on the degree of crystallinity [4, 50].

Three forms of haemosiderin have been identified in mammalian tissues [58, 69]: 1) Ferrihydrite (5Fe₂O₃.9H₂O), as found in ferritin, is poorly crystalline and relatively soluble. 2) A second more crystalline, and consequently less soluble form has a structure similar to the mineral goethite (α-FeOOH). These two types of deposits are so named as their electron diffraction patterns show diffraction lines similar to those of the mineral phases ferrihydrite and goethite [70]. 3) Non-crystalline amorphous iron(III) oxyhydroxide deposits giving weak or no diffraction lines have also been identified.

It is generally agreed that haemosiderin particles are smaller than ferritin cores however recent studies suggest that the particle size is related to the chemical form [11]. In the case of the more uncommon goethite-like haemosiderin particle sizes may be larger than the other forms of haemosiderin and the ferrihydrite-like ferritin. Goethite-like deposits are typically found in the haemosiderin isolated from β-thalassaemic patients receiving
transfusions and chelation therapy and are as large as $6.76 \pm 3.98$ nm in diameter [11]. The larger size may indicate a mechanism of deposition independent of ferritin and as such the size is not limited by the protein cage. The relationship between particle size and chemical form has been recently identified as an area of iron overload research warranting further investigation [11].

**Crystallinity of core**

For a given number of Fe atoms, a non-crystalline core, with vacancies, would be larger than a crystalline one without vacancies. Various degrees of crystallinity have been observed for the mineral iron cores of ferritin and haemosiderin [58, 71]. Furthermore, iron(III) oxide systems can become more crystalline over time [72].

**Mobilisation**

The availability of stored iron is related to the chemical and physical form in which it is deposited. The iron release from the relatively large goethite-like haemosiderin deposits has been shown to be slower than from ferrihydrite-like haemosiderin cores [11]. Studies with iron chelators have revealed a preferential mobilisation from ferrihydrite-like haemosiderin cores smaller than ferritin cores [73-75].

With the exception of goethite-like deposits, haemosiderin releases iron more quickly than ferritin and the haemosiderin iron pool is generally considered more labile than ferritin [76]. The availability of iron appears inversely related to the crystallinity and the diameter is believed to have an additional effect. Smaller particles have a higher surface to volume ratio which is likely to increase their reactivity [11].

**Toxicity of deposits**

Ferritin and haemosiderin may exhibit cytotoxicity by a number of mechanisms. Iron oxide deposits appear to catalyse lipid peroxidation [77, 78]. Haemosiderin accumulation within lysosomes correlates with enhanced lysosomal fragility [19]. Release of hydrolytic enzymes from these membrane bound particles can be self-destructive to the cell. Iron stimulates the formation of hydroxyl radicals which can then mediate oxidative damage in the tissue [79, 80]. Protein modification [81] and oxidative DNA damage [82] are some other reported effects.
Variations in physical forms of deposited iron may be a key factor determining its relative toxicity. Catalytic reactions involve iron atoms at exposed particle surfaces, hence particle size distribution and resulting surface to volume ratios are likely to have implications for the toxicity of deposits.

**Sub-cellular distribution**

Cellular iron oxide deposits are commonly found in the cytosol and lysosomes with smaller amounts in the endoplasmic reticulum and Golgi apparatus [5, 83].

Lysosomes are digestive vesicles filled with hydrolytic enzymes [84]. Lysosomes sequester cytosolic iron and further isolate the iron core from the cytosol. Iron-laden lysosomes are termed siderosomes [85]. Iron deposits appear to exhibit some differences in physical form depending on the cellular organelle in which they are contained.

Ferritin can be found in the cytosol of normal and iron loaded tissue. With iron loading, the iron-rich ferritin molecules are sequestered by lysosomes in preference to less saturated protein shells [75, 86]. As such, the largest ferritin cores are commonly found in membrane-bound siderosomes. Smaller ferritin iron cores can also be found in siderosomes [73] but these organelles are more likely to have compromised membrane barriers and leak some iron cores back into the cytosol [87]. Haemosiderin particles, usually smaller than ferritin cores, are not likely to be found free in the cytosol with ultrastructural studies reporting haemosiderin aggregated in lysosomes [6].

The majority of quantitative studies have been performed on ferritin extracts so the cores are not identified by cellular origin. Some *in situ* studies indicate that lysosomal ferritin with diameters of 6.5 nm may be up to 1 nm larger in diameter than the cytosolic version at 5.5 – 6.0 nm [6, 75, 86]
Figure 1.3: Typical distribution of ferritin and haemosiderin in an iron loaded cell (after [5]).
Cell type

Ferritin is normally most prevalent in cells involved with iron metabolism such as normoblasts, hepatocytes, and splenic reticuloendothelial cells [5]. Particle size may vary according to the degree of degradation afforded by the cell type.

Several cell types comprise the liver, including parenchymal and Kupffer cells.

Parenchymal cells, or hepatocytes, perform the functions of the liver, including processing haemoglobin to recycle the iron and producing bile. Rat liver hepatocytes respond to iron loading with increased ferritin deposition. In the absence of many lysosomes, the cytosolic enzymes of the parenchyma cause little degradation of iron-rich ferritin in the hepatocytes [83].

Kupffer cells are part of the reticuloendothelial system and filter bacteria and foreign proteins from the blood. The numerous lysosomes of Kupffer cells sequester and degrade the iron cores. Hence, Kupffer cells predominantly show haemosiderin deposits and are relatively free from ferritin.

As a result, smaller cores are observed in Kupffer cells than parenchymal cells. These observations have been made for the liver of both dietary and transfusional iron loaded rats [88, 89].
**Tissue specificity**

Some tissue specificity has been observed in measurements of iron oxide particle size. Iron oxide cores isolated from a subject with β-thalassaemia/haemoglobin E disease showed larger sizes in the heart and pancreas over the liver and spleen [71]. Diameters (± SE) measured by TEM were 7.37 ± 0.09 nm for the heart, 7.18 ± 0.08 nm for the pancreas, 6.14 ± 0.10 nm for the spleen and 5.80 ± 0.13 nm for the liver. Mössbauer spectroscopic studies have also suggested the presence of relatively large (7.4 ± 1.2 nm) iron oxide particles in the heart [57] and supported the larger size found in the heart over the spleen [90].

It is thought these differences in size may reflect the roles the organs play in iron metabolism. The liver and spleen play active roles in the reprocessing of iron and rapid deposition forms smaller cores. Conversely, the heart and pancreas provide sites for more long term storage [91, 92] and the slow deposition results in the larger core size.

**Pathology**

Differences in core sizes have been observed between normal and disease states. Ferritin extracted from a normal human liver was significantly smaller than from a β-thalassaemia/haemoglobin E disease subject at (mean ± SE) 5.80 ± 0.13 nm and 7.01 ± 0.13 nm respectively [71].

Further, the particular iron overload pathology has been shown to influence the structure of iron oxide deposits. In particular the chemical speciation and crystallinity are variable depending on disease type [12, 69, 70, 93, 94] and presumably the physical form is affected. For example, transfused and chelated Australian β-thalassaemia patients have been shown by Mössbauer spectroscopy to exhibit more of the typically larger goethite-like deposits in the spleen than non-transfused non-chelated β-thalassaemia/Haemoglobin E patients in Thailand [95].

Differences in the genotype, iron loading pathway and nature of clinical treatment may affect the iron oxide particle sizes specific to the pathology. The relative contributions of these factors remain unclear and are difficult to distinguish in the complex human system.
Degree of iron loading

Differences in observed particle sizes may be a consequence of the degree of iron loading in the tissue.

Although some haemosiderin is likely, normal iron stores are primarily in the form of ferritin [5]. Individual ferritin cores appear to become larger with iron loading, seen as an increase in the population of iron-rich ferritin particles [1].

Observations indicate that with iron loading, cytosolic ferritin is formed first, then lysosomal ferritin followed by haemosiderin [96, 97]. The increase in haemosiderin deposition is more marked with the progression of iron loading than ferritin [19].

As discussed above, cytosolic ferritin, lysosomal ferritin and haemosiderin have different mean core sizes. As such, the distribution of particle sizes is likely to change with iron loading. With the deposition of haemosiderin, the particle size distribution is likely widened and with the exception of goethite-like haemosiderin, the mean particle size would likely reduce.
Table 1.1: Comparison of mammalian iron oxide particle diameter obtained by various methods for ferritin and haemosiderin. Most measurements are from isolated particles and in situ measurements are marked with an asterisk. Mean diameter ± SD reported, or range given in parentheses, unless otherwise stated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ref</th>
<th>Method</th>
<th>Particle diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haemosiderin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fe core</td>
</tr>
<tr>
<td>Human liver: GH</td>
<td>[94]</td>
<td>TEM</td>
<td>5.8</td>
</tr>
<tr>
<td>Human spleen: β-thal</td>
<td>[94]</td>
<td>TEM</td>
<td>5.6</td>
</tr>
<tr>
<td>Spitsbergen reindeer spleen</td>
<td>[50]</td>
<td>TEM</td>
<td>5.88 ± 0.81</td>
</tr>
<tr>
<td>Human spleen: normal</td>
<td>[50]</td>
<td>TEM</td>
<td>5.81 ± 0.59</td>
</tr>
<tr>
<td>Human spleen: β-thal (Aust)</td>
<td>[11]</td>
<td>TEM</td>
<td>1.79 ± 0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.58 ± 0.98</td>
</tr>
<tr>
<td>Rat liver: IP loaded cytosol</td>
<td>[73]</td>
<td>TEM</td>
<td>5.36 ± 1.31</td>
</tr>
<tr>
<td>Human spleen: β-thal</td>
<td>[98]</td>
<td>TEM</td>
<td>~ 5</td>
</tr>
<tr>
<td>Horse spleen</td>
<td>[50]</td>
<td>TEM</td>
<td>6.12 ± 0.51</td>
</tr>
<tr>
<td>Human liver: β-thal</td>
<td>[50]</td>
<td>TEM</td>
<td>5.49 ± 0.55</td>
</tr>
<tr>
<td>Human spleen: β-thal</td>
<td>[50]</td>
<td>TEM</td>
<td>5.57 ± 0.48</td>
</tr>
<tr>
<td>Human liver: GH</td>
<td>[50]</td>
<td>TEM</td>
<td>(5.36 – 6.31)</td>
</tr>
<tr>
<td>Human spleen: normal</td>
<td>[43]</td>
<td>TEM</td>
<td>(4.5 – 5.0)</td>
</tr>
<tr>
<td>HSF: native</td>
<td>[99]</td>
<td>TEM &amp;</td>
<td>(5 – 7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>x-diff</td>
<td>10-20% &lt; ferritin</td>
</tr>
<tr>
<td></td>
<td>[75]</td>
<td>TEM</td>
<td>(2.0 – 4.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5.5 – 6.0) cytosolic</td>
</tr>
<tr>
<td></td>
<td>[6]</td>
<td>TEM</td>
<td>1 – 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[10]</td>
<td>TEM</td>
<td>0.8 – 7</td>
</tr>
<tr>
<td>*Human heart: β –thal</td>
<td>[57]</td>
<td>Möss</td>
<td>7.4 ± 1.2</td>
</tr>
<tr>
<td>*Rat liver: dietary loaded</td>
<td>[86]</td>
<td>TEM</td>
<td>6.5 lysosomal</td>
</tr>
<tr>
<td>*Rat small intestine: dietary loaded</td>
<td>[86]</td>
<td>TEM</td>
<td>5.5 cytosolic</td>
</tr>
<tr>
<td>HSF: reconst 1000 Fe atoms</td>
<td>[51]</td>
<td>TEM</td>
<td>6.17 ± 0.94</td>
</tr>
<tr>
<td>HSF: reconst 3000 Fe atoms</td>
<td>[68]</td>
<td>TEM</td>
<td>5.22 ± 0.93</td>
</tr>
<tr>
<td>HSF: native</td>
<td>[54]</td>
<td>TEM</td>
<td>6.87 ± 0.33</td>
</tr>
<tr>
<td>HSF: reconst 3000 Fe atoms</td>
<td>[54]</td>
<td>TEM</td>
<td>(2.4 – 7.8) median 5 – 6</td>
</tr>
<tr>
<td>Human heart: β-thal/HbE</td>
<td>[71]</td>
<td>TEM</td>
<td>7.37 ± 0.09 (SE)</td>
</tr>
<tr>
<td>Human pancreas: β-thal/HbE</td>
<td>[71]</td>
<td>TEM</td>
<td>7.18 ± 0.08 (SE)</td>
</tr>
<tr>
<td>Human spleen: β-thal/HbE</td>
<td>[71]</td>
<td>TEM</td>
<td>6.19 ± 0.10 (SE)</td>
</tr>
<tr>
<td>Human liver: β-thal/HbE</td>
<td>[71]</td>
<td>TEM</td>
<td>5.80 ± 0.13 (SE)</td>
</tr>
<tr>
<td>Human liver: normal</td>
<td>[71]</td>
<td>TEM</td>
<td>7.01 ± 0.13 (SE)</td>
</tr>
<tr>
<td>Human spleen: normal</td>
<td>[71]</td>
<td>TEM</td>
<td>6.25 ± 0.11 (SE)</td>
</tr>
<tr>
<td>HSF: commercial solution</td>
<td>[53]</td>
<td>HRTEM</td>
<td>5</td>
</tr>
<tr>
<td>Human liver: commercial ferritin solution</td>
<td>[53]</td>
<td>HRTEM</td>
<td>6</td>
</tr>
<tr>
<td>Human brain: Alzheimer’s disease</td>
<td>[100]</td>
<td>TEM</td>
<td>5 – 7</td>
</tr>
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### Table 1.1: cont.

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Particle diameter (nm)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Haemosiderin</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fe core</td>
</tr>
<tr>
<td>Ferritin: 1730 - 2480 Fe atoms</td>
<td>[47]</td>
<td>Möss</td>
<td>6.3 (mode)</td>
</tr>
<tr>
<td>Ferritin</td>
<td>[49]</td>
<td>TEM</td>
<td>5.5</td>
</tr>
<tr>
<td>Ferritin</td>
<td>[59]</td>
<td>e-diff</td>
<td>4 - 5</td>
</tr>
<tr>
<td>HSF: denatured</td>
<td>[66]</td>
<td>SAXS</td>
<td>7.6 (± error 0.05)</td>
</tr>
<tr>
<td>HSF</td>
<td>[44]</td>
<td>x-diff</td>
<td>7.5 (± error 3)</td>
</tr>
<tr>
<td>Rat kidney: transfusional loaded</td>
<td>[85]</td>
<td>TEM</td>
<td>~ 6</td>
</tr>
<tr>
<td>Human liver:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse kidney glomerulus: IV</td>
<td>[38]</td>
<td>TEM negative stained</td>
<td>5.5 - 6.0</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>[101]</td>
<td>x-diff</td>
<td>7.4</td>
</tr>
<tr>
<td>HSF: fractionated</td>
<td>[7]</td>
<td>SAXS</td>
<td>7.4 internal</td>
</tr>
<tr>
<td>HSF: native</td>
<td>[60]</td>
<td>SAXS</td>
<td>7.6</td>
</tr>
<tr>
<td>Ferritin: dilute solutions</td>
<td>[102]</td>
<td>SAXS</td>
<td>6.8</td>
</tr>
<tr>
<td>HSF: solution</td>
<td>[62]</td>
<td>SANS</td>
<td>6</td>
</tr>
<tr>
<td>HSF: apoferritin &amp; ferritin</td>
<td>[67]</td>
<td>SANS</td>
<td>7.2 (± error 0.6)</td>
</tr>
<tr>
<td>Apoferritin crystals</td>
<td>[65]</td>
<td>x-diff</td>
<td>11.2 dry crystals</td>
</tr>
<tr>
<td>HSF: Ferritin</td>
<td>[103]</td>
<td>SANS</td>
<td>13.0 wet crystals</td>
</tr>
<tr>
<td>HSF: Apoferritin</td>
<td>[103]</td>
<td>SANS</td>
<td>7.64 internal</td>
</tr>
<tr>
<td>Apoferritin in solution</td>
<td>[61]</td>
<td>SAXS</td>
<td>12.34 external</td>
</tr>
<tr>
<td>HSF: solution</td>
<td>[104]</td>
<td>ASAXS</td>
<td>7.24 internal</td>
</tr>
<tr>
<td>Apoferritin solution</td>
<td>[105]</td>
<td>SAXS</td>
<td>12.86 external</td>
</tr>
</tbody>
</table>

**Abbreviations:** genetic haemochromatosis (GH), (β-thal) β-thalassaemia, (HbE) haemoglobin E disease, (HSF) horse spleen ferritin, intraperitoneal (IP), intravenous (IV), iron concentration [Fe], reconstituted (reconst), Mössbauer spectroscopy (Möss), x-ray diffraction (x-diff), electron diffraction (e-diff), Australian subject (Aust), Thai subject (Thai)
1.4 Iron oxide particle clustering behaviour

1.4.1 Measurement techniques

The clustering behaviour of iron oxide deposits has been studied both in situ and in extracts of ferritin and haemosiderin.

X-ray diffraction patterns have been used to quantify the unit cell of preparations of ferritin and apoferritin crystals [65, 101]. The unit cell has also been measured by TEM which is subject to the same limitations as measurement of particle size [106]. Fast Fourier transforms of TEM images have provided a more objective indication of the unit cell for a limited field of view [107]. Close packing in situ has been observed by TEM but no reference to quantification could be found.

Some TEM studies have been able to yield quantitative estimates for the centre-to-centre distance of neighbouring iron oxide particles [6, 75, 100]. As for particle size measurements, these estimates are subjective and limited to small sampling by their nature.

Observations of the degree of clustering in tissue have been qualitative with little ability to assess by TEM owing to the uneven distribution of iron on the micron length scale. Some systems have been devised to grade iron oxide deposition by optical microscopy [108-113]. Such histological scoring generally involves assigning a value to describe the extent of deposition in Kupffer cells. As shown in Figure 1.4, the accumulation of iron in Kupffer cells represents a change in clustering behaviour on the micron length scale. However, the histological techniques remain semi-qualitative at best and provide no information regarding the extent of clustering on the nanometre length scale.

1.4.2 Biological implications

Interesting relationships have been suggested between clustering behaviour and hydration, toxicity, cellular organelle and pathology and these relationships are discussed below.

Centre-to-centre distance

As has been mentioned, ferritin and haemosiderin are often found packed into membrane limited lysosomes. The closest distance by which the ferritin iron core may be separated is limited by the 2 – 3 nm diameter subunits of the protein shell. Centre-
to-centre distances for iron oxide cores have been measured for human brain (12 to 14 nm) [100] and cultured rat heart cells (11.0 ± 0.5 nm) [75]. In the presence of a complete protein shell, spacing between ferritin particles has been observed as low as 3 to 4 nm where 5 nm is expected from the subunit size [6]. Molecular contraction on dehydration was the putative cause as had been established by Harrison. Harrison reported centre-to-centre distances of 10.5 nm, 11.2 nm and 13.0 nm for several sample preparations in order of increasing hydration [101]. The closest possible centre-to-centre distance for wet ferritin crystals is then given by the diameter of the molecule at 12 nm. Haemosiderin with incomplete protein shells may be packed at variable closer distances.

_Close packed unit cell_

Ordered arrays of iron-rich ferritin have been identified in the lysosomes of iron loaded cells [87]. The close packing occurs over a short range and most likely approximates a face-centred cubic (FCC) crystal structure [101]. The unit cell has been measured at 15.4 nm [106] and 15.8 nm for dry ferritin [65], 17 nm for apoferritin crystals in resin embedded cataracts [107] and 18.4 nm for wet isoferritin crystals [101].

_Haemosiderin and ferritin_

The clustering behaviour of ferritin differs from that of haemosiderin in tissue. Whilst ferritin is likely to be observed dispersed in the cytosol and clustered in lysosomes, haemosiderin is only found clustered in tissue [6]. Ferritin clusters can display a higher degree of order, attributable to the well defined protein shell. Haemosiderin, on the other hand, is more irregular and close packed arrays can not be found in their aggregates [99].

Extracts of ferritin and haemosiderin also display differing clustering behaviour. Haemosiderin tends to aggregate together in large clumps whereas ferritin preparations show discrete iron oxide cores [43].

_Sub-cellular distribution_

As has been discussed above, ferritin and haemosiderin clusters are usually membrane-bound within lysosomes whilst dispersed ferritin is found primarily in the cytosol. As such, the clustering behaviour is intimately controlled by the sub-cellular structure.
Cell type

Different clustering behaviour has been observed qualitatively in various cell types. As discussed above, some cell-types contain more lysosomes than others. In the liver, cells such as Kupffer cells are more likely to contain clustered arrangements of iron oxide particles, whereas the hepatocytes display dispersed deposits. The presence of siderosomes in the Kupffer cells reflects the cell's high capacity for storing iron [87]. In the spleen, cytosiderosis is observed in macrophages of the highly vascularised red pulp, whereas the parenchymal cells of the lymphatic white pulp remain relatively free from iron [86].

Toxicity of deposits

The manner in which iron oxide particles are clustered within a lysosome may have implications for the cellular toxicity. Hulcrantz et al. [114] have isolated two different populations of lysosomes, varying in iron content and proteolytic activity. They propose that the high iron content is the leading cause of membrane fragility. As such, it is likely that the most heavily clustered lysosomes would be more likely to leech proteolytic enzymes into the cytosol causing cell damage.

Tissue specificity

Some tissue specificity has been observed in the cellular distribution of iron oxide deposits. Iancu and Shiloh noted the striking ability of the spleen to transfer dispersed particles into lysosomes [87]. Almost all iron was found within siderosomal clusters in the sinusoidal epithelial cells of the spleen. In contrast, few clusters of haemosiderin were observed in the sinusoidal Kupffer cells of the liver.

Pathology

Structural differences between iron oxide cores isolated from different iron overload diseases have been well established [12, 69, 70, 93, 94]. It is interesting to note that on preparing extracts of haemosiderin, Mann et al. report that some deposits aggregated more than others [94]. The cores extracted from genetic haemochromatosis subjects clumped together whereas the material derived from β-thalassaemia subjects remained dispersed on the microscope grid.
Iron loading pathway

With dietary iron loading, such as genetic haemochromatosis, iron is first delivered to the liver via the portal vein. At the onset, iron is deposited in the periportal hepatocytes. Once parenchymal saturation is reached, the iron begins to accumulate in the Kupffer cells of the reticuloendothelial system [115]. In contrast, iron delivered via the transfusional route, is initially deposited in the reticuloendothelial system of the spleen. Parenchymal cells begin to scavenge iron as the loading progresses [115]. Since the different cell types exhibit different clustering behaviour, the iron loading pathway is likely to impact the distribution of iron within the cells.

Degree of iron loading

The presence of clusters is indicative of accumulation of iron in the cell and ordered arrays of ferritin become more prominent with iron loading [5]. As the iron loading progresses and different cell types become involved, there is likely to be a shift in the clustering behaviour of iron. A change in the distribution of iron has been observed on the micron length scale by optical microscopy [116]. At low levels of iron loading, iron is observed mainly in the periportal hepatocytes (Figure 1.4a). Following saturation of the parenchyma, iron accumulates in the Kupffer cells which can be seen forming clumps throughout the liver in Figure 1.4b. Kupffer cells contain many lysosomes, capable of sequestering the iron oxide particles into clusters. Thereby, the presence of micron-scale clusters of Kupffer cells may indicate changes in the clustering behaviour on the nanometre length scale. As such, there may be changes in the clustering behaviour of iron oxide nanoparticles with the progression of iron loading.
Figure 1.4: Optical micrographs of dietary iron loaded liver stained by Perls' reaction where iron is coloured blue a) rat sacrificed at 4 months shows iron laden hepatocytes in the periportal regions b) after 21 months of iron loading, clumps of iron laden Kupffer cells are distributed throughout the liver. Scale bars are 100 μm (from [116]).

1.5 Animal models

Animal models have made a significant contribution to the current state of understanding of erroneous iron metabolism [117]. Spontaneously iron loading mammalian systems have been studied including hypotransferrinaemic mice [118], Spitsbergen [50] and Svalbard reindeer [119] and the dugong [120]. Iron loading has been achieved in rodents with nutritional supplementation of carbonyl iron [121-123] and ferrocene [124]. Transfusional iron loading has included intramuscular injections to dogs [125] and baboons [126], parenteral administration to rats and rabbits [121, 122, 127] and subcutaneous iron dextran to gerbils [128].
1.6 Aims of the study

Evidence suggests that iron oxide particle sizes and their clustering behaviour are determined by their particular biological environment. Observed differences may reflect those environments, different mechanisms or rates of deposition and may have implications for the toxicity of deposits.

Most electron microscopy investigations to date have concentrated on preparations of ferritin and haemosiderin extracted from tissue. The process of isolation is discriminatory in its removal of iron oxide cores and is not likely to reflect the true mean size found in tissues. Furthermore, clustering behaviour in tissue can not be studied once the iron oxide cores are extracted.

To date, electron microscopic studies of iron loaded tissue have afforded only qualitative information regarding the degree of clustering in tissue. Quantitative information on particle diameter and interparticle spacing has been gleaned from electron microscopy. However, by nature, microscopy measurements are subjective, are limited to microscopic sample sizes and are not possible in cases of tight clustering of particles.

It is desirable that in situ studies of iron oxide particle sizes and clustering behaviour be conducted to enable assessment in their natural environment. A method for objectively quantifying iron oxide particle sizes and clustering behaviour in bulk tissue samples is lacking.

Small angle scattering has overcome some of these issues in that many ferritin cores are studied in solution thus improving the count statistics. However, determination of iron oxide particle size in situ is somewhat more difficult owing to the contribution of tissue structure to the scattering pattern which can be complicated and difficult to model.

The aims of this study were two-fold. Firstly, to develop and test a methodology to quantify iron oxide particle size and clustering behaviour in situ in bulk tissue samples. Second, to utilise the technique in a controlled biological system to elucidate iron oxide particle sizes and clustering behaviour in mammalian tissue under a number of different iron loading conditions.

The particular animal model chosen was the iron loaded rat, where dietary iron loading emulates the dietary iron loaded human conditions such as genetic haemochromatosis and transfusional iron loading emulates the iron loading observed from transfusion
dependent diseases such as β-thalassaemia. Both the liver and spleen from animals receiving iron loading for durations of up to 24 months were prepared in advance by Chua-anusorn [121] and available for the study. The iron loaded rat series is described in Chapter 2 with relevant principles of transmission electron microscopy (TEM) and small angle x-ray scattering (SAXS).

Chapter 3 describes quantitative assessment of iron oxide particle sizes and packing distances under a variety of iron loading conditions by TEM. In Chapter 4, laboratory benchtop SAXS was employed to trial methods of sample preparation and provide preliminary analysis of iron loaded tissue on the nanometre length scale.

The need to distinguish iron oxide particle scattering from complicated tissue scattering was established and experimental challenges faced in performing anomalous SAXS (ASAXS) are covered in Chapter 5. In Chapter 6, the ASAXS technique is demonstrated on dietary iron loaded rat liver. Quantitative information is extracted from the ASAXS data by modelling in Chapter 7. Chapter 8 covers the quantification of particle size and clustering behaviour in bulk tissue samples under a variety of iron loading conditions.
2 Materials and Techniques

2.1 Introduction

The size and clustering behaviour of iron oxide nanoparticles deposited in rat tissue were investigated in this study. The first section of this chapter discusses aspects of iron loading for the two rodent models used in this study; carbonyl-iron-fed rats and rats transfused with packed red blood cells. Next, the particular control and iron loaded rats from which the liver and spleen were removed for characterisation are described. The following sections describe relevant principles of the characterisation techniques; transmission electron microscopy (TEM) and small angle x-ray scattering (SAXS).

2.2 Rodent models

2.2.1 Dietary iron loading with carbonyl iron

Tissue iron concentrations

Elemental iron is bioavailable in the finely powdered form of carbonyl iron and significant tissue iron concentrations can be achieved in rats by dietary supplementation [121]. Iron deposition occurs primarily in the liver [129] where tissue iron concentrations increase steadily with duration of iron loading. Hepatic tissue iron concentrations in excess of 60 mg.g\(^{-1}\) dry weight have been observed for 24 months of iron loading. The liver was followed by the spleen where iron concentrations exceed 30 mg.g\(^{-1}\) dry weight after 24 months of iron loading [121]. Many other organs including intestines, pancreas and heart register increased tissue iron concentrations [36].

Cellular distribution

Within the liver, iron oxide deposition occurs predominantly in the periportal parenchymal cells [86]. As iron loading progresses, deposits can be found in all parenchymal cells. After several months, deposition begins in the Kupffer cells, continues to increase with iron loading and is marked after about 8 months [121, 129]. After 12 months of iron loading, aggregates of iron loaded Kupffer cells are observed.
On cessation of iron loading, parenchymal cells apparently mobilise iron more quickly than the Kupffer cells [121].

In the spleen, the major site of deposition is the macrophages of the red pulp. Some deposition is noted in the endothelial cells with no deposition in the lymphatic tissue of the white pulp [86].

*Subcellular distribution*

Hepatocytes and splenic macrophages demonstrate both lysosomal and cytosolic deposition. Iron is generally confined to the cytosol of hepatic Kupffer cells and found only in lysosomes of the splenic endothelial cells [86].

*Chemical form of deposits*

Mössbauer spectroscopy shows that the chemical form of iron oxide deposits in carbonyl iron loaded rat liver is predominantly poorly crystalline, either ferrihydrite-like or noncrystalline. Some proportion of the more crystalline goethite-like deposition can be seen to increase with iron loading [122].

A smaller quadrupole splitting observed for spleen tissue could indicate a more crystalline form of ferrihydrite, consistent with the slower rate of deposition in the spleen [122].

2.2.2 Transfusional iron loading with packed red blood cells

*Tissue iron concentrations*

Significant tissue iron concentrations can be achieved in rats by repeated parenteral transfusions of packed red blood cells [121, 130]. The principle site for iron deposition is the spleen where iron concentrations reach over 60 mg.g$^{-1}$ dry weight after only 12 months of loading. Hepatic iron loading is also significant at 20 mg.g$^{-1}$ dry weight after 12 months [121].

*Cellular distribution*

Hepatic cellular distribution of iron is similar to that observed for dietary iron loading although less dense. Periportal hepatocytes are first affected followed by the remaining parenchymal cells [121].
In the spleen, red pulp macrophages are the primary site of iron storage and tend to aggregate with high levels of iron loading. Macrophages increase in size and number with transfusional iron loading to accommodate the iron burden [121].

**Chemical form of deposits**

The liver from transfusional iron loaded rats shows similar Mössbauer spectral parameters to the dietary iron loaded spleen. These tissues may contain similar ferrihydrite-like deposition [122]. No significant amount of goethite-like iron oxide has been identified.

The rapidly deposited iron oxide of the transfusional loaded spleen shows no significant goethite-like form. As for the dietary iron loaded liver, Mössbauer spectroscopy indicates that deposition is likely to be predominantly ferrihydrite-like or non-crystalline [122].

### 2.3 Control and iron loaded rat tissue samples

Specimens were obtained from Female Porton rats, the offspring of iron loaded mothers which had been iron loaded during pregnancy and lactation. Rats were raised under one of the three regimes described below; control (6 rats), dietary iron loaded (18 rats) or transfusional iron loaded (8 rats). Rats were sacrificed at different ages, following which the liver and spleen were removed and rinsed with isotonic saline to remove excess blood. Full details of these procedures are available elsewhere [121].

#### 2.3.1 Control rats

Immediately after weaning (1 month after birth), six control rats were kept on a normal rodent diet and sacrificed at regular intervals from 2 months up to 12 months after birth.

#### 2.3.2 Dietary iron loaded rats

18 rats were loaded with iron by supplementation of the diet with carbonyl iron. 14 animals were sacrificed at regular intervals between 2 and 22 months after birth. At 22 months after birth, dietary iron supplementation to the four remaining rats ceased and they were returned to a regular diet for 2 to 8 weeks and then sacrificed.
2.3.3 Transfusional iron loaded rats

The 8 rats of the transfusional iron loaded series received weekly intraperitoneal injections of packed red blood cells. 6 rats were sacrificed at regular intervals between 2 and 12 months after birth. Injections to two rats were ceased at 10 months and then the rats were sacrificed after a further 1 or 2 months.

2.3.4 Tissue iron concentration

Tissue iron concentration for each rat liver and spleen sample was determined by atomic absorption spectroscopy (Figure 2.1). Iron concentration increased steadily with duration of iron loading and tissue iron concentrations comparable with human disease states were achieved.

![Figure 2.1: Tissue iron concentrations in the liver and spleen of the three experimental rat series sacrificed after months of iron loading a) control series (6 rats) b) dietary iron loaded series (18 rats) including four rats allowed to unload following cessation of iron supplementation at 22 months c) transfusional iron loaded series (8 rats) including 2 rats to which transfusions ceased at 10 months. (After Chua-anusorn et al [121])](image-url)
2.3.5 Purpose

This rodent model enabled the study of many different iron loaded states. Iron loading by dietary supplementation was designed to emulate the iron-loading pathway of human haemochromatosis. Iron loading by transfusion of packed red blood cells was designed to emulate secondary iron overload in transfusion dependent conditions such as β-thalassaemia. The three regimes facilitate investigation of the iron oxide deposits with degree of iron loading and iron loading pathway. Cessation of iron loading in some rats enables the study of the reversibility of iron loading in body tissues. Study of the liver and spleen enables the study of tissue specificity in iron deposition.
2.4 Transmission electron microscopy

A transmission electron microscope (TEM) provides images with subnanometre resolution and was used in this study to image iron oxide nanoparticles in sections of rat tissue. A description of relevant TEM operation follows.

2.4.1 Transmission electron microscope

A TEM consists of an electron beam, a variety of electromagnetic lenses, sample stage and detectors (Figure 2.2).

In a field emission gun TEM (FEGTEM), the electron source is a finely pointed crystal of zirconia coated tungsten. When subjected to an electric field in excess of $10^9 \, \text{V.m}^{-1}$, as can occur at a sharp point of metal, the work function barrier is reduced sufficiently such that an applied voltage can extract electrons from the tungsten by quantum tunnelling. The result is a bright, monochromatic beam of electrons [131]. A potential difference between the extraction anode and the acceleration anode of $10^2 \, \text{kV}$ accelerates the electrons down the microscope. To complete the illumination system, condenser lenses and an aperture control the size and thus intensity of the electron beam that interacts with the sample.

Further down the evacuated column of the TEM sits the sample stage where the beam interacts with the specimen just prior to focus (underfocussed conditions). The objective lens creates a real magnified image of the illuminated specimen. The objective aperture controls the angular range of scattered electrons that continue down the column. Since electrons interacting with electron-dense regions of the sample scatter at higher angles the objective aperture controls the contrast in the image.

In the imaging system, a series of intermediate and projector lenses further magnify the image. The image is projected onto a fluorescent screen which may be retracted to collect digital images on the charge coupled device (CCD) camera below.

For a TEM equipped with electron energy-loss spectroscopy (EELS) or energy filtered TEM (EFTEM) capabilities, a magnetic prism spectrometer is situated at the end of the microscope column. The spectrometer deflects the electrons according to their energy and the EELS spectrum is recorded. For EFTEM, the Gatan image filter (GIF) allows image formation at the CCD from a select range of energies.
2.4.2 Imaging with TEM

Mass-thickness contrast with brightfield imaging

In bright field imaging mode, electrons scattered through large angles are stopped from forming an image by the objective aperture. Electrons which pass through thin areas of the specimen or regions of low electron density will be undeflected and reach the detector. Electrons that pass close to heavy elements or through thicker regions of...
sample will scatter strongly, be removed by the objective aperture and will not reach the detector. Contrast owing to variation in sample thickness is indistinguishable from contrast owing to regions of increased electron density, leading to the term mass-thickness contrast [131]. In studies of biological material, features of interest are routinely stained with a heavy metal to provide contrast with the less dense tissue. Staining is not required for investigations of ferritin and haemosiderin in tissue since the iron oxide core itself provides good contrast with the surrounding tissue matrix.

**Contrast transfer function and Scherzer defocus conditions**

In an ideal microscope, the image is a 2-dimensional projection of all features of the sample differing only in magnification. Ideally all spatial frequencies in the sample will be recreated with equal amplitude and phase in the image. Owing to limitations of electromagnetic lenses, some spatial frequencies are not transferred equally through the optical system of the microscope (see Figure 2.3). The contrast transfer function (CTF) is independent of the specimen structure and defines how faithfully all spatial frequencies are transferred by the microscope. [132, 133]

In an electron microscope, the resolution is not limited by the wavelength of an electron (0.002 nm at 200 - 300 keV) but by the resolution limit defined by the CTF (approximately 2 nm\(^{-1}\)). Up to the resolution limit of the CTF, spatial frequencies in the image are representative of the sample. The microscope is a band-stop filter for frequencies near the resolution limit where the CTF crosses the abscissa.

It is desirable to maximise the resolution limit which can be visualised by inspection of the fast Fourier transform (FFT) of the image as a dark ring at high spatial frequencies. The focus and stigmation affect the CTF and can be manipulated to maximise the size of the dark ring in the FFT and thus the resolution limit. Optimising the spatial information transferred to the image in this way results in slightly underfocussed conditions known as Scherzer defocus. Furthermore, producing equal defocus conditions from image to image ensures equivalent magnification and facilitates quantitative comparison of morphological features in each case. [132, 133]
2.4.3 Fourier techniques

A biological specimen can be considered to be a combination of periodic and nonperiodic variations in density. In an ideal microscope the image is a 2-dimensional projection of the 3-dimensional sample density. A digital image of a sample can then be defined by a 2-dimensional Fourier transform:

\[ T(h, k) = \int_{x_1}^{x_2} \int_{y_1}^{y_2} D(x, y) e^{i(2\pi(hx + ky))} \, dx \, dy \]

Equation 2.1

or its summation

\[ T(h, k) = \sum_{x_1}^{x_2} \sum_{y_1}^{y_2} D(x, y) \{ \cos 2\pi(hk + ky) + i[\sin 2\pi(hx + ky)] \} \]

Equation 2.2

where values \( x_1, x_2, y_1 \) and \( y_2 \) define the limits of the real space positions on the sample, \( D(x, y) \) is the density at each sample position \((x, y)\) and \( h \) and \( k \) are integers defining the inverse space positions \((h, k)\) on the Fourier transform [133]. Typically, a fast Fourier transform (FFT) algorithm is employed to expedite the computation of the Fourier transform.

The Fourier transform represents the distribution of spatial frequencies that constitute the image. For an amorphous sample, the Fourier transform is symmetrical and radially averaging the Fourier transform results in the spectrum of spatial frequencies that comprise the sample density distribution. Any regular structural features in the
specimen increase the FFT signal at the spatial frequency corresponding to the real space dimension of the structure.

In practice, the FFT of a TEM image is a convolution of the CTF described above and the spatial information contained in the specimen. Features in the FFT attributable to the microscope can be clearly distinguished since they are altered significantly by slight modifications of focus or stigmation. Conversely, features attributable to the sample are somewhat more stable with minor manipulations of the instrument [133].

2.4.4 Analytical TEM

Two related analytical techniques, electron energy-loss spectroscopy (EELS) and energy filtered TEM (EFTEM), provide information on the elemental composition and distribution in a specimen. The basic principles governing these techniques are covered here and the reader is referred to [134] for a thorough introduction.

Electron energy loss spectroscopy

As the electron beam interacts with the specimen, elastic and inelastic scattering events occur. Inelastic interaction can occur with core, valence or conduction electrons in the sample. Inelastic scattering from core shell electrons results in energy loss signified by core shell electronic transitions. These transitions are characteristic of the elemental components of the specimen and allow for the chemical identity of the sample or its constituents to be determined.

An EELS spectrometer uses a magnetic prism to separate the transmitted electrons by energy since the force on a charged particle in a magnetic field is proportional to its velocity. The resultant EELS spectrum contains a strong zero-loss peak from the elastically scattered and transmitted electrons. The low-loss region up to 50 eV is dominated by inelastic scattering events from valence or conduction electrons. Ionisation of core shell electrons by the direct beam causes characteristic energy losses that are observed as absorption edges in the core-loss region, beyond 50 eV. From the absorption edges in the core-loss region, elements in the sample can be identified by comparison with known electron ionisation energies.

Energy filtered TEM

For EFTEM, an energy selecting slit after the magnetic prism is used to select a narrow energy-loss band in the transmitted beam which is then used to form an image. Three images are acquired; 1) the post-edge image is collected with the slit set just after the
absorption edge of interest, 2) two pre-edge images acquired with the slit set at two adjacent energy ranges below the edge of interest. The post-edge image contains electrons scattered from core electrons of the element of interest and an unwanted background from other non-core inelastic scattering events. The pre-edge images are used to extrapolate the background signal to be subtracted from the post-edge image. Following subtraction, electrons scattered from the core shell of the element of interest will dominate the image. From this 2-D elemental map, the distribution of the chosen element in the image can be identified with nanometre resolution.

2.5 Small angle x-ray scattering

2.5.1 Introduction

SAXS is concerned with the elastic scattering of x-rays from inhomogeneities of electron density in matter. Although scattering at small angles was observed during the 1930’s, the SAXS technique emerged around the 1950’s as an extension of x-ray diffraction [135, 136]. For the elastic interaction of x-rays of wavelength $\lambda$ with crystalline material, Bragg’s law describes the inverse relationship between diffraction angle $\theta$ and the distance $d$ between atomic planes.

$$d = \frac{\lambda}{2\sin \theta}$$

Equation 2.3

Bragg’s law predicts a minimum $d$-spacing of $\lambda/2$ that can be studied by elastic scattering of x-rays but no maximum length scale. Like x-ray diffraction, SAXS is governed by Fourier transform theory and the reciprocity implies that x-rays scattered in the small angle region originate from relatively large scale structures. Typically, scattering angles of $2\theta < 4^\circ$ are studied in SAXS. Analysis of the scattering intensity in this angular range affords morphological and structural studies on the length scales 1 – 100 nm. Many applications have been found in investigations of colloids, polymers, self-assembled structures and biological macromolecules such as proteins and nucleic acids.

2.5.2 Instrumentation

A schematic diagram of the SAXS apparatus is shown in Figure 2.4. X-ray tubes and synchrotrons are both suitable x-ray sources for SAXS experiments. The small angles
(20) of interest are in close proximity to the transmitted direct beam presenting an experimental challenge in collecting intensity data. To achieve good signal to noise ratios, large sample-to-detector distances and quality collimating optics are required. Sample-to-detector distances of 0.5 – 10 m are common.

Laboratory benchtop x-ray source

An x-ray tube with a metal target is used in laboratory benchtop SAXS apparatus. Electrons are emitted from a tungsten filament and accelerated towards an anode target by a potential difference of approximately 30 – 50 kV. Upon striking the target, the electrons decelerate rapidly and x-rays, both Bremsstrahlung and characteristic, are emitted. The characteristic radiation is intense with a narrow energy distribution and is desirable for SAXS. The x-rays generated by a copper target have a wavelength 0.154 nm (Kα) and an energy of 8.05 keV [137]. Before impinging on the sample, optical components exclude the continuous spectrum of Bremsstrahlung radiation and focus the beam of Kα radiation. The collimated, monochromatic beam will possess sufficient intensity to generate a scattering pattern from a specimen in a few hours.

Synchrotron x-ray source

In a synchrotron, high energy electrons of the order GeV are made to orbit in a storage ring approximately 1 km in circumference. A series of bending magnets accelerate the electrons towards the centre of the ring and as their orbit changes direction they emit
intense x-radiation tangentially to the storage ring. The tangential x-radiation is synchrotron radiation and can be extracted from the storage ring through beamlines towards an end station which can house any number of experimental setups. Insertion devices are used to further intensify the synchrotron radiation emitted from the storage ring. An undulator consists of a series of permanent magnets with alternating polarity moving the electrons in a sinusoidal trajectory through the device. The x-rays emitted from electrons as they change direction most rapidly at the peak of their trajectory are all emitted along the axis of the undulator and constructively interfere to produce an even more brilliant beam. The strength of the undulator magnets can be altered to modify the curvature of the sinusoidal trajectory and the wavelength of radiation produced [137]. Synchrotron radiation is many orders of magnitude more brilliant than from x-ray tubes and as such a SAXS pattern can be collected in only a few seconds.

2.5.3 Interaction of x-rays with matter

X-rays proceed through a uniform medium with a certain probability of interaction. Several interactions are possible including elastic (coherent) scattering, photoelectric effect, Compton (incoherent) scattering, pair production and nuclear absorption [138].

The probability of interaction is an intrinsic property of the elemental composition of the medium and is called the cross section (units = cm$^2$). The total cross-section is the sum of the cross section for each interaction. The different scattering mechanisms cause scatter in different directions and the cross section for each interaction is dependent on the incident x-ray energy.

The photoelectric effect is only significant for x-rays with energy near an absorption edge in the medium. Pair production and nuclear absorption are insignificant for SAXS energies. Compton scattering can occur for x-ray energies used in SAXS but the scatter is non-directional so it contributes to the constant background signal only. Elastic Thomson scattering is the most significant for SAXS [139].

Elastic scattering

Elastic scattering as described by Thomson [140] occurs for x-rays with wavelengths comparable with the size of an atom and correspondingly x-ray energies of the order $10^3$ eV are used for SAXS. In the classical description of Thomson scattering, the electric component of the incident electromagnetic wave interacts with a free electron. The electron oscillates and reemits a secondary wave with the same frequency and $\pi$ phase
shift from the incident x-ray [141]. Secondary waves constitute the scattered x-ray beam which exhibits the highest flux in the forward and backward directions with the least flux in the transverse directions [137, 139].

2.5.4 SAXS theory

The scattering vector, $Q$

Scattering intensity is usually reported in terms of $Q$, the scattering vector, shown in Figure 2.4. $Q$ is the magnitude of the difference between the incident, $k_i$, and scattered, $k_f$, wave vectors:

$$|Q| = |k_f - k_i| = Q = \frac{4\pi \sin \theta}{\lambda}$$

Equation 2.4

For a periodic structure, substituting Bragg's law (Equation 2.3) yields the relationship between $Q$ and real-space dimension $d$

$$Q = \frac{2\pi}{d}$$

Equation 2.5

$Q$ has dimensions of (length)$^{-1}$ and is usually reported in units of Å$^{-1}$.

Scattering length

The scattering length, $b$, provides a measure of the strength of interaction between an incident wave and a scattering body. The scattering length is sometimes referred to as scattering amplitude and is the square root of scattering intensity.

For scattering from a single electron, the scattering flux, $J_e$, reaching a detector element of one unit area at some distance $R$ is proportional to the flux of the incident plane wave, $J_0$, and is given by the Thomson formula:

$$J_e = \frac{J_0 r_e^2}{R^2} \left(1 + \cos^2 2\theta \right)$$

Equation 2.6

where $R$ is the distance between the electron and the point of observation and $r_e$ is the classical electron radius (2.82 x 10$^{-13}$ cm) and $2\theta$ is the scattering angle.
For small angles, the second term of Equation 2.6 is near unity and can be ignored. Thus the scattering flux into a unit solid angle \( \Omega (= 1/R^2) \) in the forward direction by a single electron is

\[
J_e = J_0 r_e^2
\]

Equation 2.7

and then \( b_e \), the scattering length for an electron, is equivalent to its classical radius, \( r_e \).

For an atom, scattering length \( b \) is dependent on the number of electrons and is simply

\[
b = n_e r_e
\]

Equation 2.8

where \( n_e \) is the number of electrons in the atom. Scattering length is usually quoted with the units cm\(^{-1}\).

The scattering length is often reported in electron units and called the atomic scattering factor, \( f_0 \). The atomic scattering factor in the forward direction (i.e. \( Q = 0 \)) is simply given by

\[
f_0 = \frac{b}{r_e} = n_e
\]

Equation 2.9

and is equal to the atomic number, \( Z \), quoted in electron units.

**Scattering length density**

The scattering length density of a particle comprising \( n \) atoms is given by:

\[
\rho = \frac{1}{V_p} \sum_{i=1}^{n} b_i
\]

Equation 2.10

where \( V_p \) is the volume of the particle.
Contrast

Differences in electron density in a sample give rise to the scattering contrast that enables morphological and structural determination by SAXS. The contrast \((\Delta \rho)^2\) is given by:

\[
(\Delta \rho)^2 = (\rho_p - \rho_m)^2
\]

Equation 2.11

where \(\rho_p\) is the scattering length density of the scattering body of interest and \(\rho_m\) is the scattering length density of the surrounding matrix.

If there are multiple components in a sample with equivalent scattering length densities, the components are said to be contrast matched and inhomogeneities between them will not contribute to the SAXS pattern. The technique of contrast variation involves altering \(\rho_m\) to match that of one component of the sample, thereby simplifying the scattering that is due to the remaining components.

Differential scattering cross-section

The differential scattering cross section, \(\frac{d\sigma}{d\Omega}\), is the probability per unit sample volume of scattering into unit solid angle about a direction corresponding to the scattering vector, \(Q\). The differential scattering cross-section depends on the number, volume, contrast, shape and structure of the scattering bodies in a sample and is given by:

\[
\frac{d\sigma}{d\Omega} = N_p V_p^2 (\Delta \rho)^2 P(Q) S(Q)
\]

Equation 2.12

where \(N_p\) is the number of scattering particles per unit volume, \(V_p\) is the volume of the particle, \(\Delta \rho\) is the contrast between the particle and the matrix (scattering length density difference), \(P(Q)\) is the form factor and \(S(Q)\) is the structure factor.

Form factor

The form factor for a particle accounts for the interference between x-rays scattered from different parts of the same particle [142]. As a result, the form factor depends on the shape of the particle and is given by:
\[ P(Q) = \frac{1}{V_p} \left| \int_{V_p} \exp[i f(Q\alpha)] dV_p \right| \]

Equation 2.13

where \( \alpha \) is a shape parameter. The analytical solution for the form factor for a uniform sphere of radius \( R_p \) is

\[ P(Q) = 9 \left( \frac{\sin(QR_p) - QR_p \cos(QR_p)}{(QR_p)^3} \right)^2 \]

Equation 2.14

**Structure factor**

The structure factor describes how the interference between x-rays scattered from different particles affects the differential scattering cross-section [142]:

\[ S(Q) = 1 + \frac{4\pi N_p}{QV} \int[g(r) - 1]r \sin(Qr) dr \]

Equation 2.15

where \( r \) is a radial distance from the centre of any scattering body in the sample and \( g(r) \) is the scattering length density distribution function.

For a dilute system (\( N_p \to 0 \)) the structure factor is near unity and becomes more significant as the particle concentration increases. For interacting particles, the structure factor results in a series of maxima in the scattering pattern corresponding to the distance between successive neighbouring scattering bodies.

**Absolute intensity**

In a SAXS experiment, the flux measured at a detector is affected by the incident flux, the transmission of the sample, the volume of the sample irradiated and the detector efficiency. To facilitate quantitative analysis, it is desirable to correct for these effects by conversion of the measured scattered flux to absolute intensity, \( I(Q) \), which is equivalent to the differential scattering cross section:

\[ I(Q) = \frac{d\sigma}{d\Omega} = \frac{J_{CCD}}{J_o} \left( \frac{1}{T_s T_A A \eta} \right) \]

Equation 2.16
where $J_{\text{CCD}}$ is the scattered flux measured at the CCD detector, $J_0$ is the incident flux, $T_s$ is sample transmission, $t_s$ is the sample thickness, $A$ is the cross sectional area of the incident beam and $\eta$ the detector efficiency.

If the detector efficiency is unknown then $I(Q)$ can be determined by comparison with an absolute standard. An absolute standard has known scattering intensity $I_{\text{STD}}$ at a particular $Q$ and is given by Equation 2.17 which follows from Equation 2.16.

$$I_{\text{STD}} = \frac{J_{\text{CCD,STD}}}{J_{0,\text{STD}}} \left( \frac{1}{T_{\text{STD}}^\eta A \eta} \right)$$

Equation 2.17

where $J_{\text{CCD,STD}}$ is the scattered flux measured by the CCD at the particular $Q$ at which the absolute scattering intensity of the standard is known, $J_{0,\text{STD}}$ is the incident flux and $T_{\text{STD}}$ and $t_{\text{STD}}$ are the transmission and thickness of the standard. Solving Equation 2.17 for $A \eta$ and substituting into Equation 2.16 yields the absolute scattering intensity for the sample:

$$I(Q) = J_{\text{CCD,S}} \left( \frac{I_{\text{STD}} J_{0,\text{STD}}}{J_{0,S} J_{\text{CCD,STD}}} \right) \left( \frac{T_{\text{STD}}^\eta}{t_s} \right)$$

Equation 2.18

For the case of a source with constant incident flux, Equation 2.18 simplifies to:

$$I(Q) = J_{\text{CCD,S}} \left( \frac{I_{\text{STD}}}{J_{\text{CCD,STD}}} \right) \left( \frac{T_{\text{STD}}^\eta}{T_s t_s} \right)$$

Equation 2.19

The absolute scattering intensity, $I(Q)$, and differential cross section, $\frac{d\sigma}{d\Omega}$, are used interchangeably in SAXS analysis and are loosely termed “intensity” with units cm$^{-1}$.

2.5.5 Direct methods of SAXS data analysis

With information on the composition, size and shape of scattering bodies, it is possible to solve the structure and form factor from the differential scattering cross section. However, some direct methods of data analysis have been developed to enable interpretation with little a priori knowledge of the sample.
**Guinier analysis**

For a dilute system, the differential cross section is simplified in the low $Q$ region according to the Guinier approximation. The Guinier approximation of the scattering curve in the vicinity of $Q = 0$ is:

$$I(Q) = I(0) \exp \left( -\frac{Q^2 R_g^2}{3} \right)$$

Equation 2.20

where $R_g$ is the radius of gyration of the scattering bodies [143].

The radius of gyration is related to the distribution of scattering centres in a particle around its centre of mass, irrespective of its shape. To determine the shape of a scattering body from the scattering curve requires knowledge of the scattering length density distribution in the scattering body. However, the dimensions of well-defined geometric shapes can be determined from the $R_g$ and the radius $R_P$ of a uniform sphere is given by:

$$R_P = \frac{5}{3} R_g$$

Equation 2.21

Taking the natural log of Equation 2.20 yields:

$$\ln I(Q) = \ln I(0) - \frac{Q^2 R_g^2}{3}$$

Equation 2.22

and the radius of gyration, $R_g$, can be found from the linear portion of the Guinier plot, $\ln I(Q)$ against $Q^2$.

The Guinier method of determining $R_g$ is only valid for dilute, monodisperse particles in a region of the Guinier plot where $Q_{\text{max}} R_g \leq 1$

**Invariant**

The scattering invariant, $Q_{\text{INVARIANT}}$, provides a measure of the total scattering power of a sample, irrespective of the structure and morphology of the scattering bodies. The invariant is obtained by integrating the differential cross section over all $Q$:

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Equation 2.23

\[ Q_{\text{INVARIANT}} = \frac{1}{2\pi^2} \int_0^\infty Q^2 I(Q) dQ \]

For an ideal system with two phases of volume fractions \( \phi_1 \) and \( \phi_2 \) (= 1 - \( \phi_1 \)) the invariant is given by

\[ Q_{\text{INVARIANT}} = V(\Delta \rho)^2 \phi_1 \phi_2 \]

Equation 2.24

which increases approximately linearly with increasing small values of \( \phi_1 \) or \( \phi_2 \).

In the context of SAXS, the summation over the low \( Q \) range measured is used rather than the integral to infinity [137].

2.5.6 Data modelling methods of SAXS data analysis

Several programs are available which can generate theoretical scattering curves from any combination of structures and scattering contrasts. Given some prior knowledge of the sample, it is possible to generate theoretical scattering curves and model them with a good fit to the collected data. Ilavsky has developed the IRENA package of SAXS analysis macros for Igor Pro (Wavemetrics) available from UNICAT at the Advanced Photon Source [144]. This package enables modelling of Beaucage’s theoretical Unified scattering function to experimental data obtained from a population of scatterers [145].
Unified scattering function

The Unified scattering function describes theoretical scattering over several orders of magnitude in $Q$ [145]. The Unified function can combine Guinier “knees” and associated power law scattering from multiple structural features in a scattering curve. The Unified function is given in Equation 2.25 where the first term describes the Guinier contribution and the second term describes the power law contribution which is structurally limited by the error function ($erf$).

$$I(Q) = G \exp\left(-\frac{Q^2 R_g^2}{3}\right) + B \left[\frac{\text{erf}\left(\frac{QR_g}{\sqrt{6}}\right)}{Q}\right]^{\beta}$$

Equation 2.25

In Equation 2.25, the exponent of the power law $\beta$ is a constant, $G = n_e^2 N_p I_e$ and $B = 2\pi N_p \rho_e^2 S_p I_e$, where $n_e$ is the number of electrons in a particle, $N_p$ the number of particles, $I_e$ is the scattering factor for a single electron, $r_e$ is the scattering length for a single electron, $\rho_e$ is the electron density of a particle and $S_p$ the surface area of a particle.

Typically, local fits require confidence that a feature is the sole contributor to the scattering in that $Q$ range. This limitation can be overcome with the Unified scattering function which can be modified to incorporate scattering from multiple structural levels that overlap in $Q$ space.

2.6 Anomalous small angle x-ray scattering

Anomalous scattering

The Thomson scattering case is only valid when the x-ray energy is not near an x-ray absorption edge in the sample. SAXS typically uses x-rays with wavelength around 1 Å so for light elements the Thomson approximation is satisfactory. Near absorption edges, such as the K-edges of heavy metals, the atomic scattering factor deviates from $f_0$ owing to resonance phenomena. The deviation in the vicinity of an absorption edge is called anomalous scattering or anomalous dispersion [146].
Anomalous dispersion correction factors

Anomalous dispersion correction factors are applied to modify the atomic scattering factor to include its wavelength dependence near an absorption edge. The atomic scattering factor for a given element, \( f(\lambda) \) is complex and given by

\[
f(\lambda) = f_0 + f'(\lambda) + if''(\lambda)
\]

Equation 2.26

where \( f' \) is the real and \( f'' \) the imaginary correction factor [147]. The dispersion corrections decrease more quickly with scattering angle compared with \( f_0 \) [148]. However, similar to \( f_0 \), the angular dependence may be neglected at small scattering angles [146]. Figure 2.5 shows the theoretical variation of \( f' \) and \( f'' \) in the vicinity of the iron K-edge.

The complex scattering factor, \( f'' \), represents scattering with a \( \pi/2 \) phase shift. \( f'' \) is near zero below the energy edge and its effect is abrupt at the edge related to photoelectric absorption. \( f'' \) can be readily calculated from the sharp increase in the absorption spectrum. The real component, \( f' \), represents scattering in phase with normal dispersion and drops to a sharp minimum through the edge. \( f' \) is related to \( f'' \) as the sharp minimum of the real factor coincides with the greatest rate of change of absorption due to \( f'' \) [146].

![Figure 2.5: Anomalous scattering factors of iron at the K-edge (from [149] by way of [150])](image)

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Complex contrast variation

The net effect of the anomalous corrections is to decrease the scattering length of an atom as the energy approaches the edge and alter the scattering contrast of a sample, \( \Delta \rho \). The essence of anomalous SAXS lies in altering the incident x-ray energy near an absorption edge to produce wavelength dependence in the scattering curve. This alteration is called complex contrast variation [146].

Anomalous SAXS

If a heavy element exists in a sample, the change in contrast with anomalous scattering can be exploited. To perform an ASAXS experiment, the anomalous element should be present in either the scattering body or the matrix. SAXS patterns are collected at several energies just below the edge and well below the edge where dispersion is normal. Comparison of the curves identifies scattering features due to the element of interest, since their scattering power is altered with energy.

Sample fluorescence due to \( f'' \) precludes the use of energies above the edge. Synchrotron sources with good energy resolution are required to provide the variable energy x-rays. The energy dependence of the instrument needs to be well understood and change in sample transmission accounted for (see Chapter 5).

Differential SAXS

Subtraction of the SAXS curve collected near the edge from that collected well below the edge results in a differential SAXS curve. Any changes in the scattering pattern are revealed in the differential curve and element specific scattering remains. Thus the scattering curve is simplified and can be more readily analysed by standard techniques.
2.7 Concluding remarks

TEM and SAXS are complementary techniques in the characterisation of nanoscale structures. Some aspects are analogous such as the radially averaged FFT of TEM and $I(Q) \text{ vs } Q$ SAXS curves, and EFTEM realspace elemental maps and the inverse space differential SAXS. TEM and SAXS have both been employed in this study of iron oxide deposits in rat tissue having a number of different iron loading states.

Both TEM and SAXS have their benefits and disadvantages. By TEM, the size and shape of nanoparticles are made visible. Only isolated particles are easy to measure. The eye is drawn to the larger of these particles, but with careful measurement, representative particle size distributions can be obtained.

Studies have reported good agreement between parameters measured with TEM and SAXS [151]. Others have exploited the limited sample preparation required for SAXS to confirm that observations by TEM were artefacts [152]. In the case of porous particles, Guinier predicts that SAXS may yield smaller diameters than TEM since inhomogeneities within particles can be seen more readily by scattering [135]. The SAXS technique is volume weighted so if polydispersity exists in the sample the measured diameter will be biased towards the larger particles. As such, SAXS provides most reliable results when a monodisperse dilute solution is present. Corrections can be made to incorporate polydispersity and increased concentrations with some degree of accuracy. A particularly valuable benefit of SAXS is the increase in volume of many orders of magnitude that can be sampled hence reducing sampling errors.
3 Transmission Electron Microscopy of Iron Oxide Deposits in Iron Loaded Rat Tissue

3.1 Introduction

Farrant’s pioneering work in 1954 was the first to exploit the electron density of iron oxide cores to image ferritin in the electron microscope [49]. Since then, transmission electron microscopy (TEM) has been used extensively to study unstained preparations of ferritin and haemosiderin both following isolation from tissue and in situ. Imaging and microanalysis by TEM has played an important role in determining the structure and composition of ferritin and haemosiderin and their distribution in tissue [49, 115, 153]. A summary of particle sizes reported in the literature measured by TEM was presented in Table 1.1.

In the current study, TEM was used to elucidate iron oxide particle sizes in a series of rats under various iron loading conditions. Fast Fourier transform techniques were employed to provide objective measurements of interparticle distances in the same series of rats.

3.2 Materials and methods

3.2.1 Sample preparation

Liver and spleen samples from each series of rat described in Chapter 2 were removed for TEM. Two control rats (6 and 12 months), four dietary iron loaded (2, 8, 21.5 and 23 months) and four transfusional iron loaded animals (4, 8, 12 and 12 months) were selected. The eldest of each of the iron loaded series had been allowed to unload following cessation of iron loading (refer to Chapter 2).

Small samples of the 20 tissues were prepared in epoxy resin prior to ultrathin (100 nm) sectioning using a Reichart ultramicrotome for TEM. Unstained sections of rat tissue were supported on a formvar coated titanium grid.
3.2.2 Instrument specifications

TEM images were acquired on a Jeol 3000 TEM, equipped with a Gatan image filter (GIF) and digital imaging system and operating at 200 kV acceleration voltage. Scherzer focus conditions were used to ensure consistent magnification. Magnification was chosen to maximise both contrast and resolution. The magnification chosen ensured measurements of particle size would comprise approximately 10 pixels to limit uncertainty on a measurement to within 10 %.

3.2.3 Analytical microscopy

Electron energy loss spectroscopy and energy filtered TEM were performed at 300 kV. To obtain an oxygen elemental map, EFTEM was conducted using the GIF with the spectrometer slit width of 40 eV at the oxygen K-edge. Pre-edge images were collected with the slit centred at 484 eV and 514 eV and the post-edge image was collected at 567 eV. To obtain an iron elemental map, EFTEM was conducted at the iron L2,3-edge. The pre-edge images were collected at 643 eV and 683 eV and the post-edge image was collected at 728 eV.

3.2.4 Quantitative measurements

Particle size measurement

For both the liver and spleen from two controls, four dietary iron loaded and four transfusional iron loaded animals, an area of the ultrathin tissue section 600 nm by 600 nm was imaged. The size of every discrete particle within this area was measured. Particle size was measured in two orthogonal dimensions using a scale tool on the digital image of the particles. Any obvious long axis and its perpendicular axis were measured to determine average particle size and aspect ratio. Approximately 150 to 200 particles were measured in two dimensions for each of the 20 samples. Cores were difficult to locate in the spleen sample from the control animal sacrificed at 12 months. As such there is a considerably smaller count of 14 particles for this sample.

The relationship between particle size and tissue iron concentration was assessed with a Spearman rank order correlation. Differences in particle size between the liver and spleen samples from each rat were assessed by a Paired Two Sample t-Test.
Fourier analysis

Images of dispersed and clustered iron oxide nanoparticles were fast Fourier transformed (FFT) to assess repeat distances in the images. FFT were radially averaged and converted to scattering vector, $Q$, where $Q = 2\pi/d$ ($\text{Å}^{-1}$) to facilitate comparison with small angle scattering data in subsequent chapters.

To assess the most common repeat distance in images obtained from each series of rat samples, radially averaged FFT for all images collected for each series were averaged. Linear background fits were performed to the radially averaged data. Following background subtraction, Gaussian peaks were fitted to the data to identify position and width of features in the radially averaged FFT data.

3.3 Results

3.3.1 Qualitative observations

Transmission electron microscopy of unstained sections of rat tissue revealed the presence of micron-scale clusters of electron dense nanoparticles. The clusters of nanoparticles exhibited various sizes, degrees of order and density. The particles within disordered clusters appeared generally smaller than those found in ordered clusters. Similarly sized particles were found dispersed in the regions between the clusters (see Figure 3.1).
Iron oxide nanoparticles form micron scale clusters of various degrees of order and density. Similarly sized nanoparticles are dispersed between clusters.

3.3.2 Analytical microscopy

The EELS spectrum showed features at the oxygen K-edge and the iron L-edge confirming the presence of oxygen and iron in the sample (Figure 3.2a). EFTEM elemental maps showed that the location of both oxygen and iron in the sample coincided with the location of the electron dense nanoparticles (Figure 3.2).
3.3.3 Particle size measurement

Owing to their dispersed nature, the discrete particles scattered between the micron-sized clusters were chosen for further particle size definition. Particles were considered to be spherical with an average measured aspect ratio $1.17 \pm 0.13$. Size distributions of the measured dispersed particles are shown in Figure 3.3.
Figure 3.3: Dispersed iron oxide particle size distributions measured by TEM in rat samples with tissue iron concentration in mg.g\(^{-1}\) dry weight given in the key;
a) & b) Control liver;
c) & d) Control spleen;
e), f) & g) Dietary iron loaded liver; h) iron loaded liver following cessation of supplementation;
i), j) & k) Dietary iron loaded spleen; l) iron loaded spleen following cessation of supplementation;
m), n) & o) Transfusional iron loaded liver; p) iron loaded liver following cessation of transfusion;
q), r) & s) Transfusional iron loaded spleen; t) iron loaded spleen following cessation of transfusion.
Mean diameters for each of the 20 samples studied are summarised in Table 3.1. The overall mean (± SE) dispersed iron oxide particle diameter found for rat tissue was 5.27 ± 0.08 nm.

Table 3.1: Dispersed iron oxide particle diameters in rat tissue measured by transmission electron microscopy. The number of particles counted for each sample, the average diameter and standard error (SE) on the mean and the standard deviation (SD) are given for samples from each rat series. Samples experiencing unloading following cessation of iron loading by dietary supplementation or transfusion are italicised.

<table>
<thead>
<tr>
<th>Sample series</th>
<th>Age (months)</th>
<th>Tissue iron concentration (mg.g⁻¹ dry weight)</th>
<th>Number of cores measured</th>
<th>Mean core diameter ± SE (nm)</th>
<th>SD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td>4</td>
<td>1.39</td>
<td>168</td>
<td>5.54 ± 0.06</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.96</td>
<td>154</td>
<td>5.43 ± 0.06</td>
<td>0.74</td>
</tr>
<tr>
<td>Control spleen</td>
<td>4</td>
<td>6.0</td>
<td>160</td>
<td>5.18 ± 0.07</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>11.4</td>
<td>14</td>
<td>5.62 ± 0.38</td>
<td>1.41</td>
</tr>
<tr>
<td>Dietary liver</td>
<td>2</td>
<td>14.0</td>
<td>158</td>
<td>5.59 ± 0.07</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>40.6</td>
<td>214</td>
<td>5.30 ± 0.06</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>21.5</td>
<td>64.9</td>
<td>158</td>
<td>5.14 ± 0.06</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>49.6</td>
<td>212</td>
<td>5.64 ± 0.05</td>
<td>0.69</td>
</tr>
<tr>
<td>Dietary spleen</td>
<td>2</td>
<td>3.0</td>
<td>151</td>
<td>5.24 ± 0.08</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>13.6</td>
<td>195</td>
<td>5.59 ± 0.05</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>21.5</td>
<td>33.7</td>
<td>163</td>
<td>5.35 ± 0.05</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>30.5</td>
<td>160</td>
<td>5.87 ± 0.05</td>
<td>0.66</td>
</tr>
<tr>
<td>Transfusional liver</td>
<td>4</td>
<td>4.5</td>
<td>150</td>
<td>5.27 ± 0.07</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>11.6</td>
<td>151</td>
<td>4.70 ± 0.06</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20.6</td>
<td>215</td>
<td>5.00 ± 0.05</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>19.4</td>
<td>176</td>
<td>5.18 ± 0.07</td>
<td>0.96</td>
</tr>
<tr>
<td>Transfusional spleen</td>
<td>4</td>
<td>18.1</td>
<td>215</td>
<td>4.65 ± 0.06</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>48.9</td>
<td>201</td>
<td>5.05 ± 0.06</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>66.4</td>
<td>215</td>
<td>5.11 ± 0.06</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>73.4</td>
<td>176</td>
<td>5.01 ± 0.06</td>
<td>0.79</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>5.27 ± 0.08</td>
<td>0.83</td>
</tr>
</tbody>
</table>

*Spearman rank-order correlation test*

Spearman rank-order correlation was performed for the mean diameter for dispersed particles from each of the 20 samples in Table 3.1 and found no correlation with tissue iron concentration at the 0.05 level of significance (p-value 0.16).
**Paired two sample t-test**

A paired two sample t-test between the mean particle diameter for the liver and spleen of each animal returned parameters reported in Table 3.2. The test returned P-values that did not exceed the critical t values for the 95% confidence interval. Thus, the mean particle size for the liver was not found to be systematically different from the mean particle size for the spleen of the same animal.

Table 3.2: Paired two sample t-test between the mean iron oxide particle diameters measured for the liver and spleen from each animal.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (nm)</td>
<td>5.28</td>
<td>5.27</td>
</tr>
<tr>
<td>Variance (nm)</td>
<td>0.08</td>
<td>0.13</td>
</tr>
<tr>
<td>Observations</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Standard Error on Mean (nm)</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Hypothesized Mean Difference (nm)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Degrees of Freedom</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Test Statistic</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>P(T&lt; t) one-tail</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td>P(T&lt; t) two-tail</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.26</td>
<td></td>
</tr>
</tbody>
</table>

**3.3.4 Fourier analysis**

Inspection of the radially averaged FFT from TEM images identified three types of clustering behaviour. 1) Dispersed particles (Figure 3.4 a & d) yielded radially averaged FFT with a monotonic decrease in intensity with increasing “Q”. 2) Images of disordered clusters (Figure 3.4 b & d) produced a broad increase in intensity in the radially averaged FFT around 0.04 – 0.10 Å⁻¹. 3) A peak at approximately 0.07 Å⁻¹ could be resolved in images of clusters with regions of regular ordering of particles (Figure 3.4 c & d).
In some cases a particularly high degree of order could be observed and rows of close packed particles could be identified within a cluster. Clusters with ordered regions were most easily located in the transfusional iron loaded spleen series and an example of such ordering is shown in Figure 3.5. Rows of particles are clearly observed in Figure 3.5 a. Three pairs of spots in the first ring of the corresponding FFT (Figure 3.5 b) indicate periodicity in at least three directions. The brightest pair of spots in the FFT occurs at 0.067 Å⁻¹ corresponding to a real space distance of 9.3 nm. The other two pairs of spots can be observed at similar distance from the centre of the FFT at 0.073 Å⁻¹ and 0.075 Å⁻¹ corresponding to real space dimensions of 8.6 nm and 8.4 nm respectively. Second and third order spots are also visible at “Q” values of 0.113 Å⁻¹ and 0.173 Å⁻¹.
arrangement of particles is consistent with viewing a face-centred cubic (FCC) crystal system along a direction close to the (110) axis. A line projection taken across the solid white line of Figure 3.5a resulted in the image intensity profile in Figure 3.5c. The distance between these four rows of particles was approximately 35 nm so the distance between each row was approximately 9 nm. These data taken together suggest an FCC unit cell size of approximately 17 nm.

Figure 3.5: Ordered rows of iron oxide particles; a) example TEM image taken from transfusional iron loaded spleen ([Fe] = 72.4 mg.g⁻¹ dry weight), b) FFT of image a), c) line projection (solid white line in a) of image intensity across four rows of particles in image, d) guide to locations of spots in FFT from b.

Background subtracted radially averaged FFT transform data from all images from each series of rat samples are shown in Figure 3.6. Gaussian functions were fitted to the radially averaged FFTs for each series of rats except for the control spleen for which no images of clusters were available.
Position and width of Gaussian peaks fitted to the radially averaged FFT data for each series are summarised in Table 3.3. No significant differences were observed between the peak positions. The average peak position was 0.070 Å⁻¹ with a width of 0.020 Å⁻¹, corresponding to real space position of 9.0 nm.

Figure 3.6: Radially averaged FFT averaged for all images taken from each series following linear background subtraction; a) Control liver, 10 images; b) Dietary iron loaded liver, 41 images; c) Dietary iron loaded spleen, 37 images; d) Transfusional iron loaded liver, 37 images; e) Transfusional iron loaded spleen, 57 images. Note: control spleen is absent since the 7 images available for this series contained no clusters and did not produce a peak in the radially averaged FFT.
Table 3.3: Gaussian peak fitted to background subtracted radially averaged FFT summed for all images taken from each series (curves shown in Figure 3.6). The effective d-spacings from peak position in real space (nm) and the peak position in "Q" space are given to facilitate comparison with SAXS data. Mean measured core diameter for each series shown in italics for comparison.

<table>
<thead>
<tr>
<th>Sample series</th>
<th>Number of images used</th>
<th>Mean core diameter ± SE (nm)</th>
<th>Peak &quot;Q&quot; position (Å⁻¹)</th>
<th>Peak width (Å⁻¹)</th>
<th>Effective d-spacings (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td>10</td>
<td>5.48 ± 0.06</td>
<td>0.065</td>
<td>0.012</td>
<td>9.7</td>
</tr>
<tr>
<td>Control spleen</td>
<td>7</td>
<td>5.40 ± 0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dietary liver</td>
<td>41</td>
<td>5.42 ± 0.06</td>
<td>0.069</td>
<td>0.018</td>
<td>9.1</td>
</tr>
<tr>
<td>Dietary spleen</td>
<td>37</td>
<td>5.51 ± 0.06</td>
<td>0.071</td>
<td>0.023</td>
<td>8.8</td>
</tr>
<tr>
<td>Transfusional liver</td>
<td>37</td>
<td>5.04 ± 0.06</td>
<td>0.071</td>
<td>0.023</td>
<td>8.9</td>
</tr>
<tr>
<td>Transfusional spleen</td>
<td>57</td>
<td>4.95 ± 0.06</td>
<td>0.075</td>
<td>0.025</td>
<td>8.4</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>5.27 ± 0.08</td>
<td>0.070</td>
<td>0.020</td>
<td>9.0 ± 0.5 (SD)</td>
</tr>
</tbody>
</table>

3.4 Discussion

Transmission electron microscopy of unstained sections of rat tissue revealed the presence of electron dense nanoparticles. The EELS spectrum and elemental maps confirmed that the contrast was due to iron oxide nanoparticles.

Hepatic and splenic deposition of ferritin and haemosiderin was observed. Dispersed particles of ferritin were distributed in the cytosol of liver and spleen cells. Micron-scale clusters of particles are likely to be membrane bound ferritin and haemosiderin sequestered within lysosomes. The clusters of ferritin and haemosiderin exhibited varying degrees of density and order. These observations are typical of normal and iron loaded rat tissue [1, 121].

3.4.1 Particle size measurement

The histograms in Figure 3.3 show a similar distribution of dispersed particle diameters in each rat tissue sample analysed and no clear difference in mean or distribution can be observed. A Spearman rank order correlation test failed to identify correlation between the mean diameter and tissue iron concentration. Although there were variables other than iron concentration in the sample series, there appears no global change in dispersed particle size with the amount of stored iron. The amount of iron stored in each ferritin molecule has been said to increase with iron overload [1]. In particular, Iancu’s study identified that individual ferritin particles in clusters appeared more iron-rich in iron
loaded tissue. The present study was unable to quantify the particle size within clusters since the clustering obscured particle edges. For the dispersed particles, no systematic increase in particle size with iron loading could be observed.

Previous studies have reported tissue specificity in the size of iron oxide deposits[71]. Ferritin particles isolated from the organs of a β-thalassaemia/Haemoglobin E (β-thal/Hb-E) disease subject showed that the heart and pancreas cores were larger than those removed from the liver and spleen. The liver and spleen ferritins from β-thal/Hb E were comparable at (mean ± SE) 5.80 ± 0.13 nm and 6.14 ± 0.10 nm respectively. In the current study, the Paired Two Sample t-Test showed that the diameter of dispersed iron oxide particles in the liver tissues samples were not systematically different from those found in the corresponding spleen tissue removed from the same animal. As such, similarity between particle sizes in the liver and spleen was confirmed in situ in the current study with (mean ± SE) particle sizes of 5.28 ± 0.09 nm and 5.27 ± 0.11 nm respectively.

There is no evidence in this study to indicate that iron oxide particles dispersed in the cytosol differ under a variety of iron loading conditions. We report an iron oxide core size of (mean ± SD) 5.27 ± 0.83 nm for cytosolic ferritin in iron loaded rat liver and spleen. This diameter is similar to other reported ferritin cores from iron loaded tissue measured by TEM as summarised in Table 1.1. For example, cytosolic ferritin cores have been estimated at 5.5 nm [6, 86]. Slightly larger particle sizes of 5.5 nm to 7.5 nm are generally reported for ferritin cores extracted from iron loaded liver and spleen [11, 50]. Ferritin within lysosomes is putatively larger than cytosolic ferritin [75, 86]. The inclusion of iron-rich lysosomal ferritin in extracts is likely to account for the larger size measured for isolates compared with the smaller cytosolic ferritin in this study. Under quite different environmental conditions in each sample studied here, the iron oxide cores remain remarkably similar. The primary determinant of particle size is therefore likely to be the ferritin protein shell.

3.4.2 Fourier analysis

Three types of clustering behaviour were observed by TEM and could be separated on the basis of the radially averaged FFT their images produced. Dispersed particles are likely to be cytosolic ferritin which has been observed for normal and iron loaded liver and spleen [5, 115]. The disordered clusters of slightly smaller cores are likely to be ferritin or haemosiderin filled siderosomes [87]. The clusters with ordered regions in
the rat tissue consist of slightly larger cores and are likely to be iron-rich ferritin cores bound within siderosomes. The most densely packed clusters would be the most heavily laden lysosomes.

Figure 3.4 demonstrates that whilst particles in all clustering configurations contribute to the radially averaged FFT signal, it is dominated by regular arrays of particles in the regions of order in some clusters. The most prominent feature in the radially averaged FFT is a peak at approximately 0.07 Å⁻¹, which is attributable only to ordering of particles in clusters in the TEM images.

Closer inspection of some images (Figure 3.5) reveals a close packed arrangement of particles within the siderosomes. The spot pattern in the FFT in Figure 3.5b further indicates periodicity in at least three directions. The two brightest spots occur at 0.067 Å⁻¹, giving an effective d-spacing of 9.3 nm in the image Figure 3.5a. The distances between rows of packed particles in the two other directions were 8.6 nm and 8.4 nm. Similar row spacing was crudely obtained with a line projection which measured the distance between rows of particles to be approximately 9 nm.

Peaks could be found in the radially averaged FFT from all series of rat tissues bar the control spleen for which no clustered particles could be found. Gaussian peak fits showed no significant difference in row spacings between the series. The mean peak position, dominated by interparticle spacings of particles in packed clusters, was 0.070 Å⁻¹ corresponding to a most common repeat distance of 9.0 nm in the TEM images. As such, we report average effective d-spacings of 9.0 ± 0.5 (SD) nm between rows of iron-rich ferritin particles packed in the siderosomes of rat tissue. Such row spacings would be observed for a FCC arrangement of particles in the (110) direction with a unit cell of dimension of 18.0 nm.

Face-centred cubic systems have been observed for preparations of wet apoferritin crystals [101]. The study reported a unit cell of 18.4 nm from 13.0 nm apoferritin spheres. Similarly, a unit cell of size 18.5 nm has been observed for ferritin removed from the liver and spleen of a variety of mammals including rat, mouse and human [4]. These data compare favourably with the current in situ study where the unit cell of approximately 17 nm implies close packed particles of diameter 12 nm. An external diameter of 12 nm for the ferritin protein shell is consistent with that observed previously by TEM, x-ray diffraction and small angle scattering (see Table 1.1). The centre-to-centre distance for close-packed ferritin in iron loaded rat tissue remains remarkably similar under a variety of iron loading conditions. For clusters displaying
regions of order, the packing distance is likely to be determined first and foremost by the size of the intact ferritin protein shell.

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### 3.5 Conclusions

Transmission electron microscopy of unstained sections of rat tissue revealed the presence of electron dense nanoparticles displaying three types of clustering behaviour; 1) dispersed particles, 2) disordered clusters of particles 3) clusters of particles with regions of order.

The micron-scale clusters of iron oxide nanoparticles exhibited varying degrees of density as well as order. Disordered clusters appeared to be comprised of generally smaller cores than the clusters with regions of order. Similarly sized particles were found dispersed in the regions between the clusters.

Dispersed particle sizes were similar for liver and spleen samples from all series of rats and no global correlation was found with tissue iron concentration or between the organ types. The mean (± SD) iron oxide core diameter for cytosolic rat liver and spleen ferritin was measured to be 5.27 ± 0.83 nm. Particles were considered to be spherical with an average measured aspect ratio of 1.17 ± 0.13.

Fourier transforms of images of clusters of iron oxide deposits suggested a close packed arrangement of particles. Radially averaged FFTs showed no significant difference in effective $d$-spacings (9.0 nm) between the rat series. Such $d$-spacings would be expected for 12 nm ferritin spheres comprising a FCC unit cell of dimension 17 nm.
4 Laboratory Benchtop Source
Small Angle X-ray Scattering
from Iron Loaded Mammalian Tissue

4.1 Introduction

As discussed in Chapter 3, iron oxide nanoparticles can be visualised without staining by transmission electron microscopy (TEM) and ferritin and haemosiderin have been extensively studied by this technique (see summary in Table 1.1). Owing to the uneven distribution of iron on the micron length scale, it is not feasible to quantitatively characterise iron oxide deposits in a complete specimen by electron microscopy. With TEM, a very small region of tissue is studied and interpretation of particle size is somewhat subjective. Some of these difficulties may be overcome with small angle scattering techniques.

The benefits of small angle x-ray scattering (SAXS) have been recognised with wide application in the biological and medical sciences. Soon after diffraction principles were expanded to study nanoscale structure in the 1950’s, researchers began investigating biological macromolecules in solution by SAXS. Kratky attributes development of some aspects of the SAXS technique to early studies of proteins [136]. For a review of early studies of various proteins, virus molecules and nucleic acids see [136].

More recently the application of small angle X-ray scattering has been extended to include the study of intact biological tissue. Structural properties of bone [154-160], human tooth dentin [161] magnetosomes of magnetotactic bacteria [162] and wood [154] have been revealed. Coherent scattering profiles of blood, muscle, fat, liver, kidney, breast, blood, tendon, bone and white and grey matter of the brain have been reported [163, 164]. With the exception of fat and bone, intensity was largely dominated by scattering from water in the hydrated tissues.

SAXS presents an opportunity to quantitatively measure structural change in molecules and tissue accompanying various pathological conditions. Studies of breast tissue have demonstrated structural differences between adipose, glandular, malignant and benign
tumour tissue and support the use of SAXS in breast cancer research and diagnosis [165-168]. Somewhat more controversial is the suggestion that the structure of hair may indicate breast cancer [169]. X-ray scattering at small angles has also shown promise in the study of urinary calculi [170], osteoporosis [158], cirrhosis and hepatocellular carcinoma [171].

Preservation of tissue in formalin for SAXS has previously been used as a sample preparation technique [164]. Formalin can leach iron from tissue and therefore may not be suitable for studying iron deposits [172]. Bielig et al. [61] warn against lyophilising apoferritin solutions but freeze drying whole tissue provides the benefit of eliminating water scattering [173, 174].

The structures of ferritin and apoferritin in solution have previously been studied by small angle x-ray scattering, the results of which were presented with TEM data in Table 1.1. Kleinwächter reports an average diameter of 7.6 nm for the core of denatured ferritin [66]. Apoferritin was given an external spherical diameter of 12.2 nm and internal diameter 7.4 nm [7]. In the same study, Fischbach and Anderegg dissolve ferritin in a contrast matched sucrose solution and attribute the radius of gyration of 28.3 Å resulting from ferritin cores of sphere diameter 7.3 nm. These results are in good agreement with other studies of ferritin in sucrose solution to eliminate protein scatter to yield a core diameter of 7.6 nm [60, 102]. Bielig et al. [61] determined the radius of gyration of apoferritin by two methods of SANS analysis, Guinier method and from the electron density distribution function obtained by Fourier transformation. The two methods were in good agreement yielding radii of gyration of 56 Å and 52 Å, or diameters of 14 nm and 12.5 nm respectively. The second method yielded an internal diameter of 7.1 nm which is comparable with ferritin core diameters described above.

Furthermore, the well defined structure of apoferritin lends itself well as an ideal sample and has found use in demonstrating SANS instrument improvements [39, 41].

The current study extends the application of SANS to characterise ferritin and haemosiderin in situ in control and iron loaded tissues. For electron microscopy (Chapter 3) the electron density of iron affords good contrast with the lighter elements that comprise biological tissue, carbon, hydrogen, oxygen and nitrogen. Similarly, the differing electron density of a scattering body and its surrounding matrix provides the contrast for SANS. As introduced in Chapter 2, the scattering length density, \( \rho \), of a particle of \( n \) atoms is given by
\[ \rho = \frac{\sum_{i=1}^{n} Z r_e}{V_p} \]

Equation 4.1

where \( r_e = 2.81 \times 10^{-13} \) cm is the classical radius of the electron and \( Z \) is the atomic number of the \( i^{th} \) atom in the particle of volume \( V_p \).

The scattered intensity from iron oxide particles in a tissue matrix is proportional to \((\rho_{\text{particle}} - \rho_{\text{tissue}})^2\) and hence dependent on the electronic contrast of iron oxide with the less electron dense tissue.

The scattering technique is employed to increase the sample size from that of TEM to quantify particle sizes. Studying ferritin and haemosiderin \textit{in situ} eliminates the possibility of particle bias or modification that may occur from an extraction process. Furthermore, by applying scattering techniques \textit{in situ}, ordering such as observed in clusters of iron oxide deposits by TEM can be quantified.

### 4.2 Materials and methods

#### 4.2.1 Sample preparation

Two methods of sample preparation were explored for suitability for SAXS analysis. Both methods involved dehydrating and preserving samples of rat liver from the dietary iron loaded and control series described in Chapter 2.

\textit{Wax embedded}

A sample of each rat tissue was fixed, dehydrated and embedded in a paraffin wax block. A custom made "millitome" device was constructed in order to section the wax blocks containing the tissue samples. A photograph of the device is given in Figure 4.1. The paraffin wax blocks were mounted onto the motor driven clamp. The cutting blade did not rotate but could be advanced manually onto the rotating sample for sectioning. The section thickness was controlled by a micrometer attached to the blade.

1.0 mm thick sections of each rat tissue embedded in wax were prepared. The thickness of each section was measured at a minimum of six sample positions to account for variation in thickness which occurred due to the varying hardness of samples compared with wax.
Figure 4.1: a) Photograph of the millitome device, custom built to section blocks of wax embedded tissue samples to a thickness of 1 mm b) the motor driven sample block rotates (arrow) as the stationary blade is advanced by hand. The sectioned sample lies on the foil catchment (circled).

Pellets

Approximately half of each organ remained following preparation for TEM and wax embedding. This remainder of each sample was freeze-dried and ground to a fine powder with a mortar and pestle. In order to compact samples of finely ground lyophilised tissue, a tablet maker was modified to produce cylindrical pellets with parallel faces and a diameter of 3 mm. Between 8 – 10 mg of powder was used to prepare pellets of thickness 0.8 – 1 mm. The thickness of the pellets was measured with a micrometer and pellets weighed with an electronic balance to assess pellet density. Multiple pellets were made for some samples to test uniformity and homogeneity of sample preparation and to determine if one pellet was representative of the bulk tissue. The pellets were held between two layers of adhesive tape when mounted in the sample chamber.

Pig fat

In addition to the rat liver, a sample of fresh pig fat was cut from the rind of sliced bacon and mounted between two layers of adhesive tape for immediate SAXS analysis to investigate the scattering from adipose tissue.
4.2.2 Small angle x-ray scattering

*Instrument*

SAXS measurements on the rat liver were obtained using a Bruker NanoSTAR SAXS instrument with a copper source. The Cu Kα line provides x-rays of wavelength $\lambda = 0.1542$ nm and energy $E = 8046$ eV [141]. The SAXS pattern was recorded on a position-sensitive 2-D detector at 65 cm distance from the sample. The $Q$ range covered was $0.01 \: \text{Å}^{-1} < Q < 0.34 \: \text{Å}^{-1}$, where $Q = (4\pi/\lambda)\sin \theta$ is the magnitude of the scattering vector. The beam diameter was 0.13 mm resulting in scattering from tissue samples of volume $1.6 \times 10^{-3}$ mm$^3$, nine orders of magnitude greater than the volume of tissue examined by TEM as described in Chapter 3.

*Data collection*

Sample transmission measurements were made over 100 seconds, and scattering data collected for 3 hours for each of the wax embedded and pelletised tissue samples. Transmission and scattering measurements were also made on a plain wax section. Background transmission and scattering measurements were made, in the evacuated chamber for the wax sections, and for a double thickness of adhesive tape for the pellets. To evaluate the pellet sample preparation method, multiple transmission and scattering measurements were made on pellets prepared from the same sample.

*Data reduction*

2-D scattering data showed no evidence of asymmetry, so patterns were radially averaged and background subtracted. Data were corrected for transmission and normalised to absolute scattering cross sections using a highly cross-linked polyethylene S-2907 standard from Oak Ridge National Laboratories via Equation 2.19.

*Data analysis*

Bragg peaks in the radially averaged data were analysed for peak position, height, full width half maximum, and peak area. A power law fit was made to the data points surrounding the peak to approximate the scattering background on which the peak was superimposed. The fitted background intensity was subtracted from the measured intensity at each $Q$ to estimate the intensity attributable to the Bragg peak.
4.3 Results

4.3.1 Scattering features from wax embedded rat liver tissue

Figure 4.2a shows the radially averaged SAXS intensity data for the wax and wax embedded rat liver tissue.

There is a large Bragg peak present for the wax sample in the range $0.113 \, \text{Å}^{-1} \leq Q \leq 0.202 \, \text{Å}^{-1}$ and since the rat tissue samples are embedded in wax the peak is present in their data as well.

A distinct change in slope occurs in the scattering data from the iron loaded liver in the $Q$ range $0.05 \, \text{Å}^{-1} \leq Q \leq 0.08 \, \text{Å}^{-1}$. The change in slope forms a "shoulder" feature that is not present in the control liver or wax scattering data.

Figure 4.2b shows the wax embedded rat liver tissue scattering following subtraction of the wax scattering intensity. The remaining intensity data show an incomplete subtraction of the Bragg peak.
Figure 4.2 a) Example SAXS data from wax embedded dietary iron loaded rat liver sample with tissue iron concentration 40.6 mg.g⁻¹ dry weight (×), wax embedded control rat liver sample with tissue iron concentration 1.4 mg.g⁻¹ dry weight (+) and the wax sample (○). b) SAXS from the same wax embedded dietary iron loaded rat liver sample and wax embedded control rat liver sample following subtraction of wax scattering data.

4.3.2 Assessment of pellet sample preparation

Density

Measurement of the thickness and mass of the pellets (see Figure 4.3) confirmed that each of the rat tissue pellets for bulk analysis by SAXS were prepared with consistent density. Constant density ensures that following thickness correction, comparison on an absolute scale between samples is possible.
Figure 4.3: Thickness and mass of pellets prepared from control and iron loaded rat liver tissue samples. All pellets have a diameter of 3 mm.

**Pellet lateral uniformity**

Multiple x-ray transmission measurements made at 0.2 mm lateral increments on a pellet of control rat liver tissue and pellet of dietary iron loaded liver tissue are shown in Figure 4.4. Within the reproducibility of the measurement, the transmission is unvaried across the sample, confirming sample uniformity by the pellet method of sample preparation.

Figure 4.4: X-ray transmission measurements at 0.2 mm increments across a control rat liver pellet (+) with \([\text{Fe}] = 1.4 \text{ mg.g}^{-1}\) dry weight and dietary iron loaded liver pellet (\(\times\)) with \([\text{Fe}] = 40.6 \text{ mg.g}^{-1}\) dry weight.
Sample transmission

Multiple transmission measurements for pellets of each control and dietary iron loaded liver samples are given in Figure 4.5. The thickness corrected x-ray transmission of each sample decreased with increased tissue iron concentration, the electron dense scattering material in the tissue.

![Graph](image)

Figure 4.5: Multiple thickness-corrected sample transmission measurements for control liver tissue pellets (+) and dietary iron loaded liver tissue pellets (×).
Sample homogeneity

Multiple scattering measurements made from multiple positions on up to three pellets of the same rat liver tissue were in excellent agreement. An example of the reproducible nature of the scattering data is shown in Figure 4.6.

Figure 4.6: Radially averaged SAXS data from a control liver tissue sample with tissue iron concentration 1.4 mg.g⁻¹ dry weight a) five scattering curves were obtained from three different pellets (one pellet was studied at three different positions) and are in good agreement b) enlargement of Bragg peaks

4.3.3 Scattering features from pellets of rat liver tissue

Typical SAXS data obtained from control and dietary iron loaded liver tissue are shown in Figure 4.7. A number of features were observed in the scattering data from the pelletised liver tissue and are summarised in Table 4.1. With reference to Figure 4.7, the dashed arrow marks the position of the moderately intense Bragg peak observed in control tissues. The solid arrows mark the two possible Q-positions of the more intense Bragg peak, observed in both control and iron loaded liver tissue.
Figure 4.7: Small angle X-ray scattering data from liver tissue of three dietary iron loaded rats (red, yellow, green) and two control rats (blue, purple). Tissue iron concentrations are given in the key in mg.g\(^{-1}\) dry weight. The dashed arrow marks the position of the moderately intense Bragg peak observed in control tissues. The solid arrows mark the two possible \(Q\)-positions for the more intense Bragg peak, observed in both control and iron loaded liver tissue.

Table 4.1: Features observed in the SAXS data from six control and 18 dietary iron loaded pelletised rat liver samples identified by \(Q\)-position. Real space dimensions are given from the relationship \(d = 2\pi/Q\). From Chapter 3, the peak from the FFT of TEM images of clustered iron oxide deposits is included for comparison.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Strength</th>
<th>(Q)-position (Å(^{-1}))</th>
<th>(d)-spacing (nm)</th>
<th>Control liver (n=6)</th>
<th>Iron loaded liver (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoulder</td>
<td>prominent</td>
<td>0.05 - 0.08</td>
<td></td>
<td>Not prominent</td>
<td>Prominent (n=18)</td>
</tr>
<tr>
<td>Bragg peak</td>
<td>moderate</td>
<td>0.103</td>
<td>6.10</td>
<td>Present (n=6)</td>
<td>Not present</td>
</tr>
<tr>
<td>Bragg peak</td>
<td>strong</td>
<td>0.128 or 0.136</td>
<td>4.91 or 4.62</td>
<td>Prominent (n=6)</td>
<td>Present (n=10)</td>
</tr>
<tr>
<td>Bragg peak</td>
<td>weak</td>
<td>0.235</td>
<td>2.67</td>
<td>Present (n=3)</td>
<td>Present (n=5)</td>
</tr>
<tr>
<td>Bragg peak</td>
<td>weak</td>
<td>0.260</td>
<td>2.42</td>
<td>Present (n=2)</td>
<td>Not present</td>
</tr>
<tr>
<td>FFT of TEM</td>
<td></td>
<td>0.07</td>
<td>9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD core size from TEM</td>
<td></td>
<td></td>
<td>5.27 ± 0.83</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Shoulder**

The shoulder feature in the \(Q\) range 0.05 Å\(^{-1}\) - 0.08 Å\(^{-1}\) observed in the SAXS from wax embedded dietary iron loaded liver samples was present in the data from the pellets of the same sample series. Again, this shoulder was not prominent in the data from control liver tissue pellets. As shown in Figure 4.7, the shoulder intensity tended to increased with iron concentration.
Bragg peaks

A number of Bragg peaks could be resolved at higher $Q$ which were not visible by the previous sample preparation technique owing to the wax structure.

Peaks are seen in the SAXS data from many of the rat tissue samples at up to five positions in the $Q$-range 0.10 Å$^{-1}$ - 0.26 Å$^{-1}$. As summarised in Table 4.1, the peaks were more prominent in the control than the iron loaded liver tissue. The d-spacing of iron oxide deposits in clusters from the fast Fourier transform (FFT) of TEM data discussed in Chapter 3 is included for comparison.

The strongest intensity Bragg peak occurs at approximately 0.13 Å$^{-1}$ corresponding to a real space dimension of approximately 4.8 nm. The peak varies in position and intensity from sample to sample. These Bragg peaks are present in the SAXS data from all of the liver samples removed from control rats but can only be resolved in 10 of the 16 iron loaded samples studied. Furthermore, as shown in Figure 4.8, it appears that the peaks can occur at two possible $Q$-positions; 0.1286 ± (SD) 0.0009 Å$^{-1}$ or 0.1379 ± (SD) 0.0013 Å$^{-1}$.

Figure 4.7: The peaks were similar in shape and there was no clear correlation between peak position, height, width or area and iron loading.

The strongest intensity Bragg peak occurs at approximately 0.13 Å$^{-1}$ corresponding to a real space dimension of approximately 4.8 nm. The peak varies in position and intensity from sample to sample. These Bragg peaks are present in the SAXS data from all of the liver samples removed from control rats but can only be resolved in 10 of the 16 iron loaded samples studied. Furthermore, as shown in Figure 4.8, it appears that the peaks can occur at two possible $Q$-positions; 0.1286 ± (SD) 0.0009 Å$^{-1}$ or 0.1379 ± (SD) 0.0013 Å$^{-1}$. These two peak positions are also indicated with solid arrows in Figure 4.7.

The peaks were similar in shape and there was no clear correlation between peak position, height, width or area and iron loading.

A Bragg peak of moderate intensity could be observed at a $Q$ position of 0.103 Å$^{-1}$ from all of the control but none of the iron loaded liver samples. A very weak peak feature could be resolved in a small minority of control and iron loaded liver samples at $Q$ positions of 0.235 Å$^{-1}$ or 0.260 Å$^{-1}$. Again, there appeared no clear correlation between peak position or intensity and iron loading.
4.3.4 Scattering features from pig fat

Scattering data collected from the fresh bacon fat tissue were dominated by two intense Bragg peaks at 0.145 Å⁻¹ and 0.185 Å⁻¹ and are shown in Figure 4.9.

![Figure 4.9: SAXS from fresh bacon fat is dominated by two Bragg peaks.](image)

4.4 Discussion

4.4.1 Scattering from wax embedded rat liver tissue

Paraffin wax is comprised of elements of low atomic number. However the differential scattering cross section (see Equation 2.12) is dependent on the number and volume of scatterers as well as electron density. This dependence is exemplified by the presence of intense Bragg peaks in the SAXS from wax and wax embedded tissue (see Figure 4.2a). In principle, the presence of wax peaks in the Q-range of interest does not preclude the method of sample preparation since the wax scattering intensity can be subtracted from the data. However, in this instance, incomplete subtraction was observed resulting in ill defined intensity for the control and iron loaded liver tissue at the Q values around the wax peaks, 0.15 Å⁻¹ ≤ Q ≤ 0.18 Å⁻¹ (see Figure 4.2b).

From these data, it is difficult to determine if the incomplete subtraction is due to some order occurring over a similar length scale in the liver samples as that which occurs in the wax.

The presence of a shoulder feature at 0.05 Å⁻¹ – 0.08 Å⁻¹ in the dietary iron loaded but not control liver tissue suggests that the shoulder could be due to the increased iron concentration in the iron loaded tissue.
4.4.2 Scattering from pellets of rat liver tissue

Thickness and mass measurements confirmed that the modified tablet maker was capable of compacting each freeze-dried sample to an equivalent density (Figure 4.3). Transmission and scattering measurements across a pellet demonstrated the uniformity and homogeneity of the sample prepared by the pellet method (Figure 4.4 & Figure 4.6). Since scattering from multiple positions on one pellet and from different pellets of the same sample was in good agreement, the pellet method created samples representative of the bulk tissue. The sample volume by the wax method was limited by the beam size, however conducting SAXS from freeze-dried homogenised pelletised tissue effectively samples the whole organ volume. Without the interference of wax peaks, Bragg peaks in the scattering from pelletised tissue can be resolved over the \( Q \) range 0.10 Å\(^{-1}\) - 0.26 Å\(^{-1}\) (Figure 4.7). The consistency, homogeneity, larger effective sample volume and absence of background structure made the pellets of freeze-dried ground tissue the preferred method of sample preparation for SAXS. Further discussion of scattering features pertains to data collected by this preferred method.

4.4.3 Shoulder scattering feature

The prominent shoulder at 0.05 - 0.08 Å\(^{-1}\) in the iron loaded liver but not in the control tissues and the increased intensity of the shoulder with increased iron concentration indicates that this scattering feature is due to iron in the tissue (Figure 4.7). The shoulder intensity increases with no significant change in position. This indicates that an increase in the number, rather than size, of nanoparticles accompanies the increase in iron concentration. To confirm this observation, quantitative analysis of the scattering data is required. Attempts to model polydisperse non-interacting spheres to the data were unsuccessful. Whilst Guinier regions where the slope of the Guinier plot yielded radii of gyration satisfying the Guinier criteria \( Q_{\text{max}} R_g \leq 1.2 \), the lack of ability to fit a non-interacting model suggested the iron oxide particles were too concentrated in the tissue to validate Guinier analysis.

4.4.4 Bragg peaks

The discovery of ordered structures in the rat liver tissues raises questions about their origin. Peaks are observed in iron-loaded tissues, yet more prominent in controls. Since there was no other clear correlation between scattering features and iron loading, the identity of the ordered scatterer is not clear.
**Iron as the origin**

Ordered clusters of iron oxyhydroxide particles in iron loaded liver have been observed previously by microscopy (Chapter 3). We know from FFTs of TEM images that in some clusters of iron oxide deposits, ordering is significant enough to cause a peak in the frequency spectrum. The strongest Bragg peak of the SAXS data \( (Q = 0.13 \text{ Å}^{-1}, d = 4.8 \text{ nm}) \) occurs on a length scale comparable to that of the iron oxide cores measured by TEM, \( 5.27 \pm 0.83 \text{ nm} \) (see Chapter 3). Close packed spheres of diameter \( 5.27 \text{ nm} \) could produce structure peaks at a distance comparable with the strongest observed Bragg peak. In the case of iron oxide cores of ferritin and haemosiderin, this would require considerable degradation of the \( 12 \text{ nm} \) protein shell.

Inspection and FFT of our microscopy images suggests two types of particle clusters, ordered and disordered. Were the peaks due to ordering of iron in the tissue this would suggest two types of clustering behaviour in iron loaded tissue i) those which produce Bragg scattering, ii) those clusters which do not produce Bragg scattering.

Two types of clustering behaviour would be physiologically meaningful. Bragg scattering may indicate ordered arrays of iron oxide particles, identifying organised packing of particles associated with iron storage. Disordered clusters, which would not contribute to the peak, may be related to the less organised sequestering of iron oxide particles by lysosomes in order to isolate toxic iron from the cytosol. The lack of order in these clusters may be due to the partial degradation of the protein shell or iron oxide particles by the low pH environment of the lysosomes.

The prominence of peaks in the control scattering data indicates that the ordered structures are more common in the control rats rather than the iron loaded animals. It should be noted that in this study the control rats were weaned from iron loaded mothers and contain higher than normal tissue iron concentrations. Should the Bragg peaks in the scattering data be due to ordering of iron oxide nanoparticles, the data would indicate that ordered clusters are present in control tissue and that as the tissue progressively loads, most clusters are disordered in nature. An increase in disorder with iron loading is consistent with the lysosomal degradation of iron that is a feature of iron overload. However, features present in the scattering data from some samples at other \( Q \)-values \( (0.103 \text{ Å}^{-1}, 0.235 \text{ Å}^{-1}, 0.260 \text{ Å}^{-1}) \) showed no correspondence with features in FFT of TEM images.
**Tissue as the origin**

We have seen Bragg peaks arise from scattering from large amounts of low electron density material in the case of the wax sections (Figure 4.2). In addition, biological tissue is known to exhibit sufficient order to produce scattering peaks in the low angle region. Desouky et al. [174] attribute 4.65° (0.66 Å⁻¹) peaks in their scattering data to proteins in protein-rich freeze-dried tissue such as muscle and bovine albumin. In the same study freeze-dried fat showed a sharp peak at 10° (1.46 Å⁻¹). Breast adipose tissue produces a sharp intense peak at 0.12 Å⁻¹ [167] and scattering peaks have been observed for pork adipose [164]. Kosanetzky et al. [163] attribute scattering peaks from adipose tissue to the alignment of fat cells over long range in the tissue. Several other studies have demonstrated that biomolecules or biological tissues possess sufficient periodic structure to produce scattering peaks [171, 173]. Fat is known to form crystals [175] and the scattering profile from bacon fat (Figure 4.9) with peaks at 0.145 Å⁻¹ and 0.185 Å⁻¹ clearly demonstrates a high degree of order in fatty tissue on the 1 - 10 nm length scale. Since the fat content of rat liver is of the order of 10% [176, 177] it is possible that tissue structure such as that of fat is the origin of Bragg peaks in the tissue.

**The requirement for complex contrast variation**

The increase in intensity of the shoulder feature with iron concentration provides evidence that this feature is due to scattering from iron. The nature of the peaks is less well understood and more information is required to identify structure of iron or tissue matrix as the origin of the SAXS peaks.

Contrast variation techniques allow modification of the scattering length density of the scattering bodies of interest or their matrix in order to vary the scattering contrast, (Δρ)², and the scattered intensity. By varying the scattering length of iron, changes in scattered intensity can be attributed to iron and thus iron dependent features resolved. In the following four ASAXS chapters, complex contrast variation is employed to separate iron scattering from tissue scattering and afford identification of the origin of the shoulder and peaks revealed by single energy SAXS in this chapter.
4.5 Conclusions

Pelletising freeze-dried ground tissue provides consistently dense, homogenous samples representative of the bulk and is the preferred method of sample preparation for SAXS analysis. Radially averaged scattering data from liver tissue pellets revealed a number of scattering features, namely a shoulder at 0.05 Å⁻¹ - 0.08 Å⁻¹ and up to five peaks in the Q-range 0.10 Å⁻¹ - 0.26 Å⁻¹.

The increased intensity of the shoulder with tissue iron concentration suggests that this feature is due to an increase in the number of iron oxide nanoparticles with iron loading. Bragg peaks are present in the scattering data from control and iron loaded liver tissue samples and show no clear correlation with iron loading. Further information is required to unambiguously determine the origin of scattering features.
5 Anomalous Small Angle X-Ray Scattering
Near the Iron K-Edge on
Beamline 15-ID-D at the Advanced Photon Source

5.1 Introduction

The limitations of microscopy and the benefits of scattering techniques for the analysis of iron oxide nanoparticles in the bulk were considered in Chapter 4. As discussed, small angle x-ray scattering (SAXS) has been successfully employed to study ferritin and apoferritin in solution. Determination of iron oxide particle size in situ is somewhat more difficult owing to the contribution of tissue structure to the scattering pattern which can be complicated and difficult to model [178]. Varying the energy of the incident beam in anomalous SAXS (ASAXS) allows us to vary the scattering length for iron to distinguish iron oxide particle scattering from complicated tissue scattering.

ASAXS was initially demonstrated from solutions of biological macromolecules in the 1980’s [104, 146]. Since then, the emerging technique has mainly found application in the materials sciences, such as catalysis, metallurgy and glass science [179-184].

ASAXS experiments require data collection at a number of different incident beam X-ray energies. As the incident beam energy approaches the Fe-K edge, the scattering length of iron is reduced. Changing the energy also introduces multiple undesirable effects on the instrument performance and calibration. Detector responses, beam position, beam flux and sample transmission will typically vary with energy. A thorough understanding of the instrument’s performance and limitations at all energies is required in order to accurately detect the changes in scattering cross section with energy. Experimental requirements for ASAXS include:

1. Variable energy x-ray source with high energy resolution, high angular collimation and relatively constant high flux as a function of energy
2. Reproducible selection of x-ray energy
3. Accurate measurement of incident flux, sample transmission and scattered flux at each energy
4. Determination of beam centre energy dependence
Prior to this study, iron K-edge ASAXS had not been performed on beamline 15-ID-D at the Advanced Photon Source (APS), Argonne National Laboratory. This chapter reports on the analysis of instrument performance, development of optimised data collection and processing strategies to facilitate ASAXS on the beamline.

### 5.2 Instrumentation

The SAXS instrument

ASAXS data were obtained in two sessions on beamline 15-ID-D at the Advanced Photon Source, Argonne National Laboratory. The 7 GeV synchrotron storage ring energy was operating in top-up mode on a 90 second cycle which produced beam currents of 100 ± 1 mA.

Beamline optics and detectors

A schematic diagram of the 15-ID-D beamline is shown in Figure 5.1. The insertion device which delivers the beamline from the storage ring is an APS Type A undulator. A cooled double-bounce diamond (111) crystal constitutes the high heat load monochromator. A series of mirrors and slits collimate the beam. The incident beam monitor used for routine analysis consists of a thin mica foil in the beam, whose fore-scattering is detected by an array of four photodiodes arranged orthogonally about the direct beam. The majority of the beam passes through the incident beam monitor and crosses the beam exit window to be incident on the sample which is maintained in ambient conditions. The beam size on the sample was 0.25 mm by 0.3 mm or 0.5 mm by 0.5 mm resulting in scattering from tissue sample volumes nine orders of magnitude greater than the volume of tissue examined by TEM. The standard mica exit and entrance windows were replaced with Kapton polymer since mica contained a small but troublesome amount of iron. The transmission detector is mounted on a retractable arm and is withdrawn during scattering measurements. The beamline is also equipped with a beam stop detector for instrument alignment and provides an indication of transmission during scattering measurements. 2D scattering patterns were collected on a Bruker 6000 CCD detector at the end of the beamline, approximately 10 cm behind the beam-stop. The CCD detector consisted of 1024 x 1024 pixels of dimension 92 x 92 μm. Two sample to detector distances were used; 1) short camera length with nominal
sample-to-detector distance 0.6 m delivering a $Q$-range of 0.02 - 0.61 Å$^{-1}$ with the beam centre offset 2) medium camera length, nominally 1.9 m and $Q$-range 0.004 - 0.019 Å$^{-1}$.

Further details of the beamline are available elsewhere (Cookson et al 2006 submitted).

**Software**

The beamline is equipped with saxs15id, a purpose-built software package for data processing and analysis, which runs under IDL Virtual Machine 6.0.

![](image)

**Figure 5.1**: Beamline optics and detectors for 15-ID-D at the Advanced Photon Source. The CCD detector behind the beam stop is not shown (after [185]).

### 5.3 Methods: Instrument performance and limitations

To prepare for the ASAXS investigation of iron oxide particles in iron loaded tissue, aspects of instrument performance were assessed at x-ray energies from 6.900 keV through the iron K-edge to 7.130 keV.

#### 5.3.1 Incident beam energy selection

The undulator and monochromator can be used to select the wavelength of synchrotron radiation delivered from storage ring to the sample. The undulator provides a narrow spectrum of x-ray wavelengths which peaks at a wavelength inversely related to the transverse distance between its alternating permanent magnets. After modifying the wavelength of the undulator, the spectrum is further sharpened by the diamond crystal monochromators with an energy resolution of approximately 0.7 eV.
Two methods of using the undulator and monochromator to select the x-ray energy were tested; 1) peak the undulator close to the iron K-edge and use the monochromator to select the energy from the undulator spectrum and 2) scan both the undulator peak and monochromator to select the energy. The incident flux and beam centre positions were measured for each method to compare their suitability.

5.3.2 Incident beam monitor

The photodiodes of the incident beam detector provide a relative measure of incident flux and are routinely used for data normalisation. For fixed wavelength operation, the incident beam monitor performs well and produces very little parasitic scattering. The performance of the incident beam monitor with varying energy was assessed by comparison with the response of the transmission detector to the direct beam.

5.3.3 Sample stage

In Chapter 4, the freeze-dried ground pellets of rat tissue were shown to be homogeneous. Nevertheless, for scattering data at each energy to be compared it is desirable that the data be collected at one same sample position. Fiducial positions were marked on the same stage to test the ability of the rotation and vertical translation drive motors to reproduce sample position.

5.3.4 Transmission detector

To estimate inherent uncertainty in the transmission detector, its response at one energy was compared with the simultaneous response of the incident beam monitor. Since the detectors were observing the same incident flux, the precision in the monitors could be estimated from the correlation between the two responses assuming equal variance.

As shown in Figure 5.1, the transmission detector sits on a retractable arm and is removed from the beam path when collecting scattering data. The sensitivity of the detector to repositioning errors of the retractable arm was assessed.

Transmission detector response was corrected for dark-current measured using its average response when in the retracted position.

5.3.5 Beam centre

A mesoporous silicon dioxide powder with highly ordered pores of narrow size distribution (MCM-41) was employed to determine beam centre. The sample of MCM-41 was mounted inside the camera chamber approximately 15 cm in front of the CCD.
detector. The beam centre was determined in the horizontal and vertical directions from
the average of six arc fits to the MCM-41 diffraction rings using saxs15id.

To determine the energy dependence of the beam centre position, diffraction from the
MCM-41 calibrant was collected with each of the incident beam energies used in the
ASAXS experiment on the medium camera length. To determine the temporal beam
centre stability, multiple beam centre measurements made at 6.900 keV over an 18
minute period were compared.

5.3.6 Camera length and Q-calibration

For each of the camera lengths, the sample-detector distance was calibrated using silver
behenate (AgBeh) with known d001-spacing of 58.380 Å [186], assuming accurate
energy scale calibration of the monochromator. As mentioned in section 5.3.1, the
photon energy is known to 0.7 eV which gives a precision of 0.01 % for energies
around 7 keV and a similar precision in camera length.

The pixels of the CCD detector represent a different set of Q-values for each x-ray
energy and AgBeh was used to calibrate Q each time the energy was changed.

Q interpolation

In order to generate differential SAXS data, scattering intensity values were required at
the same points in Q-space. Following radial averaging of the scattering pattern,
intensities at fixed Q-values were determined using a cubic spline interpolation plug-in
to Microsoft Excel.

5.4 Methods: Data collection strategies

A number of data collection strategies were trialled and refined as the instrument
performance and limitations became clear over the course of experiment. The three
strategies described herein represent increasing improvements and are relevant to the
data presented in subsequent chapters. Measurement times for scattering and
transmission were chosen according to the dynamic range of the detectors to ensure
sufficient count above the detector floor without saturating the detector.

5.4.1 Strategy A

This strategy was devised during the first allotment of beamtime and was used on the
short camera length. This was an extension of the Q range studied by Cu Kα source
SAXS described in Chapter 4. The strategy was devised to eliminate use of the incident beam monitor following analysis of its energy dependence (see section 5.6.2). The transmission detector was used to measure: 1) the flux of the transmitted beam with the sample in place and 2) the flux of the incident beam without the sample present. There is a short time delay between the two measurements and minor temporal variations in the incident flux were ignored in this method. In order to make the two adjacent measurements, the transmission detector is retracted and repositioned between measurements of transmitted flux for different samples. As such, the response of the transmission detector is assumed not to be sensitive to repositioning. An initial set of six samples were studied, one overlaid with MCM-41 at the sample position to act as a semi-internal standard. A highly cross-linked polyethylene S-2907 standard from Oak Ridge National Laboratories was also studied. The undulator was fixed to peak at 7.070 keV and the energy tuned by scanning the monochromator only. Scattering flux was acquired over 5 seconds and transmission measurements made for 10 seconds. The strategy involves the steps outlined in Table 5.1.

5.4.2 Strategy B

Strategy B was used during the second allotment of beamtime and was a refinement of Strategy A to account for fluctuations in incident beam flux (see section 5.6.4). The data were acquired on the medium camera length which truncated the \( Q \)-range from that in Chapter 4. Unlike the incident beam monitor, the transmission detector cannot measure incident flux whilst the CCD is measuring scattered flux from the sample. Although the response of the incident beam monitor was strongly energy dependent, it was used in this strategy to measure relative changes in incident flux since its sensitivity was constant at a fixed energy. Despite its energy dependence (see Figure 5.2), relative flux measurements from the incident beam monitor could still be used to account for temporal variation in incident flux. To remove all systematic errors from energy repeatability limits of the monochromator, the absolute scattering standard (to which all scattering measurements were compared) was remeasured each time the energy was varied. This was of particular importance very close to the iron K-edge because the sensitivity of the incident beam monitor changed very rapidly with energy (see Figure 5.2).

This strategy assumes that the sensitivity of the incident beam monitor is constant at a fixed energy and relies on good repeatability in transmission detector repositioning. Highly accurate sample stage repositioning is not required since the transmission and
scattering data for a sample are collected without it being moved. Data acquisition was limited to approximately 10 samples to minimise potential temporal variation in incident beam properties and instrument response. Scattering and transmission data were collected for 2 and 10 seconds respectively. The steps involved in Strategy B are shown in Table 5.1.

5.4.3 Strategy C

Strategy C was a refinement of Strategy B following recognition of the sensitivity of the transmission detector to repositioning (see section 5.3.4) and the accuracy of the sample stage (see section 5.3.3). To avoid repositioning the transmission detector between transmission measurements for different samples, twice the sample stage movements were performed. Accurate sample stage repositioning was required to ensure that transmission and scattering data are collected at the same sample position. The method did not require constant incident flux between measurements at each energy. The strategy was devised near the end of the beamtime and owing to time constraints was only performed for one sample, a dietary iron loaded liver. As for Strategy B, scattering and transmission data were collected for 2 and 10 seconds respectively. The strategy involves the steps outlined in Table 5.1.
Table 5.1: Sequence of steps involved in the data collection strategies A, B and C. \( J_{TR} \) is the flux measured at the transmission detector and \( J_{CCD} \) is the scattered flux measured at the CCD detector.

<table>
<thead>
<tr>
<th>Sample position</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STEP ONE:</strong> (short camera)</td>
<td>Select energy (fixed undulator)</td>
</tr>
<tr>
<td><strong>STEP TWO:</strong></td>
<td>Direct beam, Blank (adhesive tape), Polymer standard</td>
</tr>
<tr>
<td><strong>STEP THREE:</strong></td>
<td>Sample 1, Sample 6 with MCM-41</td>
</tr>
<tr>
<td><strong>STEP FOUR:</strong></td>
<td>Change energy and repeat from step two</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample position</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STEP ONE:</strong> (medium camera)</td>
<td>Select energy (scanned undulator)</td>
</tr>
<tr>
<td><strong>STEP TWO:</strong></td>
<td>Direct beam, Blank (adhesive tape), Polymer standard, AgBeh, MCM-41</td>
</tr>
<tr>
<td><strong>STEP THREE:</strong></td>
<td>Sample 1, Sample n-1, Sample n (n≤10)</td>
</tr>
<tr>
<td><strong>STEP FOUR:</strong></td>
<td>Change energy and repeat from step two</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample position</th>
<th>Actions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STEP ONE:</strong> (medium camera)</td>
<td>Select energy (scanned undulator)</td>
</tr>
<tr>
<td><strong>STEP TWO:</strong></td>
<td>Direct beam, Blank (adhesive tape), Polymer standard, AgBeh</td>
</tr>
<tr>
<td><strong>STEP THREE:</strong></td>
<td>Direct beam, Blank (adhesive tape), Polymer standard, AgBeh</td>
</tr>
<tr>
<td><strong>STEP FOUR:</strong></td>
<td>Change energy and repeat from step two</td>
</tr>
</tbody>
</table>

5.5 Methods: Data processing

Normalisation procedures to account for differences in sample transmission, thickness and incident flux were introduced in Chapter 2. As shown in Equation 2.18, a scattering standard is required in order to display scattering intensity on an absolute scale. The polyethylene standard utilised in the current study consists of low atomic number elements. As such, the scattering length is energy independent over the relatively narrow energy range of interest. A distinct scattering peak at \( Q = 0.0227 \) Å\(^{-1}\) is accessible at both 15-ID-D camera lengths for energies near 7 keV and the peak
intensity is known at 41.2 cm\(^{-1}\). Details of normalisation procedures differed for the
data collection strategies and are described for radially averaged data below.

### 5.5.1 Strategy A

This strategy aimed to eliminate use of the incident beam monitor which is normally
used to account for variation in incident beam flux. To avoid reliance on the incident
beam monitor, two alternative methods of normalisation were developed. The first
method employed a semi-internal standard and the second was a detector based method.

**Semi-internal standard**

The semi-internal standard method was used to normalise only one sample which had a
specimen of MCM-41 powder overlaid at the sample position during data collection.
Since scattering from the silicate is independent of energy changes near the iron K-edge,
the net area of silicate peaks could be used to normalise scattering intensity. A
Gaussian peak fitting routine in saxs15id was used to determine the net peak area above
a quadratic background for the sample at each energy. The scattering data at each
energy were normalised to equate silicate peak area to that measured at 7.07 keV in
each scattering pattern. In doing so, the scattering intensity was normalised for energy
dependence of incident flux, sample transmission and CCD detector response and given
by:

\[
I(Q)_{arb} = J_{CCD} \left( \frac{PA_{MCM,7.07}}{PA_{MCM,E}} \right)
\]

**Equation 5.1**

Where \(I(Q)_{arb}\) is the normalised scattering intensity in arbitrary units, \(J_{CCD}\) is the
scattered flux measured at the CCD detector, \(PA_{MCM,7.07}\) is the net silicate peak area
measured at 7.07 keV and \(PA_{MCM,E}\) is the peak area measured with incident beam
energy, \(E\).

**Detector-based corrections**

This method was used to normalise scattering data from all six samples acquired at the
short camera length. The transmission detector was used to measure incident flux and
sample transmission. The data were corrected for sample transmission, background
scatter, energy dependence of the incident flux from the fixed undulator and energy
dependence of detectors by:
where \( J_{0,TR,7.07} \) was the incident flux measured by the transmission detector with incident beam energy 7.070 keV and \( J_{0,TR,E} \) as the incident flux measured by the transmission detector with incident beam energy \( E \). \( PA_{STD,7.07} \) was the net area (above background) of the 0.0227 \( \text{Å}^{-1} \) peak of the polyethylene standard at 7.070 keV and \( PA_{STD,E} \) was the net peak area at energy, \( E \).

The transmission of the sample was determined using the transmission detector to measure the flux through the sample \( J_{TR,S} \) and through the blank (adhesive tape) \( J_{TR,B} \):

\[
T_S = \frac{J_{TR,S}}{J_{TR,B}}
\]

Equation 5.3

Similarly, the transmission of the blank was determined using the transmission detector to measure the flux through the blank (adhesive tape) \( J_{TR,B} \) and the incident beam flux \( J_{0,TR} \):

\[
T_B = \frac{J_{TR,B}}{J_{0,TR}}
\]

Equation 5.4

The data were not normalised to an absolute scale.

5.5.2 Strategies B and C

Data collected by Strategies B and C were scaled according to changes in incident flux using the relative changes in the incident beam monitor. The data were converted to an absolute scale by comparison with the polyethylene standard collected alongside each group of samples at each energy. The data were scaled according to the method introduced in Equation 2.18 and incorporating background correction:

\[
I(Q) = \frac{J_{CCD,S} - J_{CCD,B}T_S}{T_B} \left( \frac{I_{STD}}{J_{0,ST,STD}} \right) \left( \frac{T_{STD}}{T_S} \right)
\]

Equation 5.5
Where $I(Q)$ is the absolute scattering intensity of the sample, $J_{CCD,S}$ is the scattering flux of the sample measured by the CCD detector and $J_{CCD,B}$ the scattering flux of the blank. $J_{0,STD}$ was the incident beam flux measured by the incident beam monitor during scattering measurements on the polyethylene standard and $J_{0,S}$ was the incident beam flux during scattering measurements on the sample. $I_{STD}$ is the known scattering intensity of the standard (42.1 cm$^{-1}$ at 0.0227 Å$^{-1}$) and $J_{CCD,STD}$ was the scattering flux measured at the CCD from the standard at 0.0227 Å$^{-1}$. $t_{STD}$ and $t_s$ are the thicknesses of the standard and sample. The transmissions of the sample, blank and standard are denoted by $T_s$, $T_B$ and $T_{STD}$ and were determined by the following:

$$T_s = \frac{J_{TR,S} J_{0,B}}{J_{TR,B} J_{0,S}} \quad T_B = \frac{J_{TR,B} J_0}{J_{0,TR} J_{0,B}} \quad \text{and similarly} \quad T_{STD} = \frac{J_{TR,STD} J_0}{J_{0,TR} J_{0,STD}}$$

Equations 5.6, 5.7 & 5.8

For data acquired at or below 7.100 keV, changes in sample transmission with energy were linear fitted to reduce random errors due to transmission detector repositioning. Above 7.100 keV, raw transmissions were used since these deviated from the linear fit.

**Uncertainty estimation**

The sasx15id software provides an estimate of two standard error uncertainty on the measured flux at the CCD detector. Uncertainty is obtained from the variation in the observed flux for individual pixels at each radial distance used for radial averaging. Uncertainties for the other variables of Equation 5.5 were estimated to one standard deviation. The uncertainties were combined and propagated through the normalisation procedure.

### 5.6 Results: Instrument performance and limitations

#### 5.6.1 Incident beam energy selection

*Incident beam flux*

The incident beam flux for two methods of x-ray energy selection is shown in Figure 5.2. For the first method with the fixed undulator wavelength, the transmission detector measured the rise and fall in flux of the direct beam across the undulator energy peak at approximately 7.09 keV. The right-skewed energy spectrum of the undulator had a full width a half maximum of 0.195 keV. The transmission detector shows a direct beam
with near constant high flux for the second method where the undulator and monochromator were both scanned to select the x-ray energy. The near constant flux of the second method provides improved count statistics at lower energies and a more consistent heat load on the beamline optics.

Figure 5.2: Incident flux of the direct beam near the iron K-edge measured with the transmission detector \( J_{0,TR} \) for the two methods of x-ray energy selection. For the first method, the undulator was fixed and the monochromators scanned to select the wavelength. For method two, the undulator and monochromators were both scanned to select the energy. The incident beam flux as measured by the incident beam monitor \( J_0 \) is also shown for method two.

**Beam centre**

The beam centre positions in the horizontal and vertical plane for the two methods of energy selection are shown in Figure 5.3. The fixed undulator method resulted in substantial deflection of the beam centre in the vertical plane, despite the operation of the beam position feedback system (Figure 5.3a). Moving the undulator and scanning the monochromator in the second method was found to provide a more stable beam centre.
Figure 5.3: Beam centre positions in the horizontal (●) and vertical directions (○) for each method of energy selection a) the undulator was fixed and the monochromators scanned to select the wavelength b) the undulator and monochromators were both scanned to select the energy. Measurements taken at a camera length of 1876 mm on the CCD detector where 1 pixel = 92 μm with beam position feedback control in operation.

**Preferred method of energy selection**

On the basis of near constant high flux and minimised beam centre variation, the second method of energy selection was chosen as the preferred method. As such, the undulator and monochromator were scanned to select the incident beam x-ray energy for the bulk of ASAXS experiments.

5.6.2 Incident beam monitor

The response of the incident beam monitor with photon energy is shown in Figure 5.2. There is a sudden change in the sensitivity of the detector at the iron K-edge owing to a small but troublesome level of iron in its mica foil. The magnitude of this problem rendered the detector difficult to use for measurement of incident flux across the range of energies required. Alternative methods of normalisation were devised for each data collection strategy (see section 5.5).

5.6.3 Sample stage

Regular inspection of fiducial markers in the sample stage confirmed accurate reproduction of rotational and y-translational positions over multiple stage movements. Given the uniformity of sample preparation demonstrated in Chapter 4 and the accuracy
of the stage drivers, repositioning between measurements on a sample would produce negligible uncertainty.

5.6.4 Transmission detector

Energy dependence

As shown in Figure 5.2, the transmission detector shows a more accurate response with changes in incident beam energy than the incident beam monitor. Using the transmission detector to measure the direct beam flux and the sample transmission at each energy removes any small energy dependence of the transmission detector response from the sample transmission measurement.

There is a small variation in beam flux of about ±0.5% over a 90 second time frame due to injections to boost the decaying storage ring current. The response of the transmission and incident beam detectors to these small changes in flux without changing x-ray energy are compared in Figure 5.4. When the transmission detector is held in position (Figure 5.4a & b), the responses of the two detectors compare favourably. Assuming equal inherent uncertainty, the standard deviation for both detectors was determined from the correlation in Figure 5.4b to be 0.04%.

Repositioning error

The correlation between the response of the incident and transmission detectors is reduced when the transmission detector is retracted and repositioned between measurements (Figure 5.4 c & d). Note that since repositioning the detector arm takes time, the 90 second period between injections can not be resolved in Figure 5.4c. The increased variation between the response of the two detectors in Figure 5.4c & d is attributable to sensitivity of the transmission detector to repositioning errors. From correlation analysis, the repositioning was estimated to contribute 0.9% error (1 SD) and was the most significant source of uncertainty for transmission measurements and intensity scaling of CCD images on this instrument. As such, it is advantageous not to move the transmission detector between measurement of the incident flux of the direct beam and measurement of sample transmission (as was incorporated into data collection Strategy C).
5.6.5 Beam centre

*Beam centre energy dependence*

As shown in Figure 5.3b, the horizontal beam position moved systematically from 0.3 pixels to 0.7 pixels with energy increasing from 6.900 keV to the Fe K-edge. For CCD pixels of 92 μm, this deflection corresponded to a deflection of less than 40 μm over a distance of nearly 2 m. Nevertheless, the horizontal deflection warranted correction and the raw beam centre positions were used for each energy. In the vertical direction, fluctuations in the beam centre position were random and within 0.3 of a pixel so the average obtained from six energies below the Fe K-edge was used.

*Beam centre stability over time*

Beam centre measurements for a sequence of MCM-41 scattering data acquired at one energy over 18 minutes were compared. The beam centre was found to be stable over
the time of collection (18 minutes) within arc fitting errors from saxs15id so temporal correction was not required.

5.6.6 Camera length and $Q$-range

The camera lengths determined from the AgBeh standard are shown with their corresponding $Q$-range in Table 5.2. The usable $Q$-range was slightly truncated at low $Q$ owing to poor count statistics and high $Q$ owing to low signal to noise ratio.

Table 5.2: Measured sample-detector distances and $Q$-range for two camera lengths on beamline 15-ID-D at APS.

<table>
<thead>
<tr>
<th>Camera</th>
<th>Sample-detector distance (mm)</th>
<th>$Q$-range ($\text{Å}^{-1}$)</th>
<th>Usable $Q$-range ($\text{Å}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short</td>
<td>547.2 ± 0.1</td>
<td>0.0118 – 0.643</td>
<td>0.02 – 0.45</td>
</tr>
<tr>
<td>Medium</td>
<td>1875.7 ± 0.2</td>
<td>0.00362 – 0.190</td>
<td>0.008 – 0.12</td>
</tr>
</tbody>
</table>

5.7 Results: Data collection and normalisation strategies

5.7.1 Strategy A

Strategy A yielded useful scattering data for a number of energies over the short camera length. The internal standard and detector-based methods of normalisation were in excellent agreement as shown for the sample with MCM-41 overlay in Figure 5.5.

![Figure 5.5: Scaling factors to normalise scattering from a highly iron loaded tissue sample as a function of beam energy. Scaling factors for both the detector-based and semi-internal standard normalisation methods are shown normalised to unity at 7.07 keV for comparison.](image)

The agreement in Figure 5.5 verified the accuracy of the detector based method which was used to normalise the remaining samples without semi-internal standard. As such,
the data were normalised for energy dependence of the incident beam flux and energy dependence of sample transmission. Data were not converted to absolute scale but the normalisation ensured that data obtained from individual samples could be compared with data obtained from the same sample at different energies. The data were not corrected for small fluctuations in incident beam flux brought about by changes in the storage ring current (see Figure 5.4). This contributed to ± 0.5 % uncertainty on the scattered intensity. Uncertainty (± 0.9 %) was also brought about by repositioning errors on the transmission detector (see Figure 5.4 c & d) which was used to measure both incident flux and sample transmission. The total uncertainty on scattering intensity was typically ~2 %. Although data collection by Strategy A was superseded, this short camera length data set was sufficient to be useful for initial ASAXS observations (see Chapter 6).

5.7.2 Strategy B

Strategy B yielded scattering data for a number of energies over the medium camera length and was superior to Strategy A. Measures of incident flux by the incident beam monitor were included and whilst they did not provide an accurate representation of the actual incident flux (see Figure 5.2), relative changes were able to account for small changes in incident beam flux with time (see Figure 5.4). Accounting for these changes in the incident flux reduced the uncertainty on the scattered intensity. Despite the strong spectral sensitivity of the incident beam monitor, high precision at each energy permitted normalisation of temporal variations in incident flux by way of Equation 5.5 over the entire energy range, even through the iron K-edge.

Furthermore, the data were corrected for sample thickness and differences in sample transmission due to both differing sample composition and energy of measurement. This was a significant improvement from Strategy A where individual samples were corrected only for their energy dependence and could not be compared with one another.

By this strategy, the major contributor to uncertainty on the absolute scattering intensity was the repositioning error (± 0.9 %) of the transmission detector on which transmission measurements were based. The total uncertainty on the absolute scattering intensity was typically less than 1 %. Inspection of ASAXS data from iron loaded mammalian tissue (see Chapter 6) and a typical uncertainty of ~1 % suggested a limit of detection of 2 – 5 mg Fe.g⁻¹ dry weight.
Unfortunately, the limitations of the transmission detector were not established until towards the end of the beamtime. Subsequent to these results, the transmission detector arm has been improved, from which future experiments at 15-ID-D will benefit.

5.7.3 Strategy C

Strategy C was only performed on one sample owing to time constraints. Collecting data by this method was deemed most reliable since the sample stage driver motors proved more consistent than the transmission detector arm. Thus, the data carry all the benefits of strategy B with a reduced uncertainty on the scattering intensity since random errors in the sample transmission were reduced. The total uncertainty on the absolute scattering intensity was typically ~0.5 %. The limit of detection by Strategy C may be as low as 1 -2 mg Fe.g⁻¹ dry weight, comparable with normal iron loadings.

For SAXS instruments with a transmission detector sensitive to repositioning errors and reliable sample stage, Strategy C is recommended.
5.8 Conclusions

Instrument performance and limitations

The performance of beamline 15-ID-D with varying wavelength near the iron K-edge has been investigated for the first time. The following conditions were observed to optimise ASAXS analysis near the iron K-edge:

1. The preferred method to select the incident beam energy is to scan the undulator and the monochromator. The photon energy is precise to 0.7 eV and can be accurately reproduced to within a few eV. Beam centre variation at the CCD detector is minimal (± 0.2 pixels) and the constant high flux places constant heat load on the monochromator.

2. The incident beam monitor displayed strong energy dependence near the iron K-edge owing to its mica foil. The response is constant at any one energy right through the edge to within 0.04 % (1 SD) and relative measurements are of high quality.

3. Fluctuations in the storage ring current cause incident beam flux variation of ± 0.5 % over 90 s intervals which should be accounted for by use of the incident beam monitor.

4. The driver motors of the sample stage are very precise and contribute negligible error.

5. The transmission detector in a fixed position displays some inherent uncertainty, estimated at 0.04 % (1 SD). Dark current correction was necessary and was measured with the detector in the retracted position.

6. The retractable transmission detector is sensitive to repositioning and movement of the detector contributes approximately 0.9 % random error to the flux. This was the greatest uncertainty on the scattering intensity.

7. Transmission data for one sample was acquired without moving the detector with subsequent reduction in uncertainty. In hindsight, acquisition of all transmission data without movement of the detector would have been an improvement. The detector arm has since been replaced which will benefit future experiments.

8. Beam centre was determined with mesoporous silicate MCM-41 and showed variation of approximately ± 0.2 pixels with energy. Following correction at
each energy, the beam centre was accurate to within the uncertainty on the fitting procedure so did not contribute significant uncertainty.

9. Silver behenate provided camera length and $Q$ calibration at each energy to within 0.02%.

Data collection and normalisation strategies

Strategies were developed to collect and normalise the following data from iron loaded mammalian tissue with tissue iron concentrations in excess of 2 mg.g$^{-1}$ dry weight:

1. Short camera length data for six rat tissue samples. Data were not on an absolute scale but corrected for energy dependence such that data collected for an individual sample at different energies can be compared.

2. Medium camera length data for all rat tissue samples. Data were normalised to absolute scattering cross sections using a highly cross-linked polyethylene S-2907 standard from Oak Ridge National Laboratories
6 Iron Loaded Mammalian Tissue: Anomalous Dispersion Resolves Iron Dependent Small Angle X-Ray Scattering

6.1 Introduction

Synchrotron sources are capable of producing radiation covering a broad range of the electromagnetic spectrum. The flux of radiation is many orders of magnitude greater than achieved from conventional x-ray tubes. The high flux of radiation greatly reduces experimental time and makes small angle x-ray scattering (SAXS) from large sample sets feasible on a synchrotron source. The ability to tune the x-ray energy from a synchrotron facilitates anomalous SAXS (ASAXS) experiments. Anomalous dispersion of x-rays near the iron K-edge has been demonstrated by Stuhrmann [104] for ferritin molecules in solution. However, in iron loaded tissue, the ferritin and haemosiderin particles are often aggregated in clusters further complicating the analysis of data.

The study reported here exploits the speed of data collection available on the synchrotron source to expand the SAXS study reported in Chapter 4 to include all control and iron loaded sample sets described in Chapter 2.

The study also aims to utilise ASAXS to elucidate particle size and clustering effects of iron oxide particles in mammalian liver tissue. The data collected and presented in this Chapter constitute the first ASAXS experiment performed on beamline 15-ID-D at the Advanced Photon Source (APS). The instrumentation, data acquisition and normalisation were described in Chapter 5. The results obtained demonstrate the ability to perform iron K-edge ASAXS from tissue samples and, with the use of complex contrast variation, resolve the origin of features in the Cu Kα SAXS data described in Chapter 4.
6.2 Materials and methods

6.2.1 Sample preparation

Rat tissue samples were studied in the form of pellets of freeze-dried ground tissue. The sample preparation was as described in Chapter 4. A total of 62 pellets from the control, dietary and transfusional iron loaded samples were studied. The tissue samples were mounted onto the sample holder between two layers of adhesive tape.

6.2.2 Anomalous small angle x-ray scattering

Instrument

ASAXS measurements were made on beamline 15-ID-D at APS as described in Chapter 5 using 16 incident photon energies between 6.900 and 7.140 keV. Data were acquired on a 2-D CCD detector at two different camera lengths. The short length at 0.55 m resulted in a \( Q \)-range of 0.02 to 0.45 Å\(^{-1} \) and the medium length at 1.9 m resulted in a \( Q \)-range of 0.008 to 0.12 Å\(^{-1} \). The beam size on the sample was 0.25 mm by 0.30 mm resulting in scattering from tissue sample volumes nine orders of magnitude greater than the volume of tissue examined by transmission electron microscopy (Chapter 3). MCM-41, a mesoporous silicon dioxide powder was studied for beam centring. Silver behenate (AgBeh) was used to calibrate sample-to-detector distance and detector \( Q \) values. A highly cross-linked polyethylene S-2907 standard from Oak Ridge National Laboratories was studied as the absolute standard.

Data collection

Transmission measurements were made with an exposure time 10 seconds and scattering data were acquired for 5 seconds for the short camera length and 10 seconds for the medium length. The 1000-fold increase in data collection speed with the synchrotron source compared to the Cu \( K_\alpha \) source (Chapter 4) afforded data collection from all 62 rat tissue sample pellets.

2-D scattering data showed no evidence of asymmetry, so patterns were radially averaged and background subtracted. Short camera length data were normalised for energy dependence of the sample transmission and instrument but were not scaled to absolute units. As part of the normalisation procedure (see Chapter 5) one dietary iron loaded liver sample was overlaid with MCM-41. Medium camera length data were
corrected for sample transmission and normalised to absolute scattering cross sections using the techniques described in Chapter 5.

Gaussian peaks on a power law background were fitted to scattering features to estimate peak position, full width half maximum and net peak area.

6.3 Results

6.3.1 Anomalous dispersion

The energy dependence of iron loaded tissue sample transmission is shown in Figure 6.1 with the real and complex anomalous dispersion correction factors, $f'$ and $f''$. The sample transmission increased smoothly and linearly with incident x-ray energy, up to 7.100 keV where pre-edge or edge structure was observed. A pre-edge drop at 7.114 keV was consistently observed in numerous iron-loaded samples. As the x-ray energy increased further, there was a sharp drop in transmission.
6.3.2 Synchrotron small angle x-ray scattering

Radially averaged synchrotron SAXS data collected at the short and medium camera lengths are shown in Figure 6.2 and Figure 6.3. The data were acquired with incident beam energy of 6.900 keV which provided the maximum scattering contrast between iron-rich and organic structures in the samples. The short camera length revealed scattering from the shoulder and Bragg peaks shown in Chapter 4. In addition, the extended $Q$-range facilitated identification of further structure in the scattering from rat tissue samples. The medium camera length $Q$-range is centred on the shoulder feature, increasing the $Q$ resolution and count statistics for this feature.

Figure 6.1: a) Anomalous dispersion correction factors near the iron K-edge (from [149] by way of [150]) b) Sample transmission for a control spleen (○) and dietary iron loaded spleen (●) with tissue iron concentrations as given in the key.
Figure 6.2: Typical SAXS data acquired on the short camera at 6.900 keV from control and iron loaded tissue samples. Solid arrows mark the $Q$-position of Bragg peak scattering features and dashed arrows identify broad shoulder features in the sample scattering. Tissue iron concentrations are given in the key in mg.g$^{-1}$ dry weight a) control liver b) dietary iron loaded liver with unmarked MCM-41 peaks c) transfusional iron loaded liver d) control spleen e) dietary iron loaded spleen f) transfusional iron loaded spleen.
Figure 6.3: Typical SAXS data acquired on the medium camera at 6.900 keV from control and iron loaded tissue samples. Tissue iron concentrations are given in the key in mg g\(^{-1}\) dry weight a) control liver b) dietary iron loaded liver c) transfusional iron loaded liver d) control spleen e) dietary iron loaded spleen f) transfusional iron loaded spleen.

**Control liver**

As shown in Figure 6.2a, the synchrotron SAXS from control liver displayed the same scattering features as identified by Cu-K\(_\alpha\) source SAXS (Chapter 4). A shoulder feature in the \(Q\) range 0.05 Å\(^{-1}\) - 0.08 Å\(^{-1}\) is not prominent. The control specimen displayed several Bragg peaks, the most visible of which was at 0.13 Å\(^{-1}\). In addition to the peak
and shoulder features previously observed by Cu-Kα SAXS, peaks were located at approximately 0.38 and 0.40 Å⁻¹ and are shown in Figure 6.2a.

Control spleen

As shown in Figure 6.2d, a shoulder feature was not prominent in the SAXS data from control spleen tissue. Several structure peaks with similar positions and intensities to the control liver along with a peak at ~0.35 Å⁻¹ could be observed in the spleen tissue.

Dietary iron loaded liver

The shoulder feature revealed in Chapter 4 was prominent in the synchrotron data from dietary iron loaded liver shown in Figure 6.2b. Peaks similar to those observed in the control liver and spleen tissue could be observed for some liver specimens subjected to dietary iron loading.

Dietary iron loaded spleen

The SAXS from dietary iron loaded spleen tissue displayed a prominent shoulder in the region 0.05 Å⁻¹ - 0.08 Å⁻¹ (see Figure 6.2e and Figure 6.3e). The change in slope in this region is sharper than observed in the dietary liver tissue and the shoulder almost resolves a local maximum. Again, structure peaks were present in the spleen tissue.

Transfusional iron loaded liver

As shown in Figure 6.2c, the transfusional iron loaded liver displayed similar scattering to the dietary iron loaded liver with a shoulder and structure peaks.

Transfusional iron loaded spleen

A sharp shoulder feature similar to that observed in the SAXS from the dietary spleen is prominent in the transfusional spleen data (Figure 6.2f and Figure 6.3f). A slight shoulder in the Q-range 0.15 to 0.20 Å⁻¹ may be a second harmonic of the prominent shoulder at lower Q. Bragg peaks were not prominent in the Q-range 0.02 to 0.45 Å⁻¹.
6.3.3 Anomalous small angle x-ray scattering

The energy dependence of typical features in the SAXS from control and iron loaded rat tissue are shown in Figure 6.4.

Figure 6.4: Energy dependence of SAXS features: dietary iron loaded liver with tissue iron concentration 65.5 mg.g⁻¹ dry weight a) shoulder feature acquired with incident beam energy in the key b) ratio of net peak area fitted to shoulder to its net peak area at 6.900 keV c) Net peak area fitted to all four features in the data including the shoulder from b (Q-positions labelled); control liver with iron concentration 2.0 mg.g⁻¹ dry weight d) Bragg peak acquired with incident beam energy in the key e) ratio of net peak area fitted to Bragg peak to its net peak area at 6.900 keV f) Net peak area fitted to all six Bragg peaks in the data including the peak from e (Q-positions labelled).
Shoulder feature

An example shoulder feature at 0.05 Å⁻¹ - 0.08 Å⁻¹ acquired at a range of incident beam energies is shown in Figure 6.4a. The scattering intensity at the shoulder reduces with increasing incident beam energy. As shown for all incident beam energies in Figure 6.4b, there is a systematic reduction of the shoulder feature to less than half its original intensity passing through the iron K-edge. The background scattering is high from data collected at 7.120 and 7.125 keV and is noticeable as an increase in intensity as shown in Figure 6.4b. Use of these data above the absorption edge is impractical due to high specimen absorbencies and background fluorescent intensity. As shown in Figure 6.4c, the shoulder was the only feature to display energy dependence on the approach to the iron K-edge in the dietary iron loaded liver sample.

The shoulder feature was present in all iron loaded tissue samples, all of which showed a similar energy dependence indicating that the scattering was iron-dependent.

Peak intensity with energy

An example of the Bragg peak scattering from control liver tissue at multiple incident x-ray energies are shown in Figure 6.4d. The net peak area of the Bragg peak in the SAXS data remains relatively unchanged for incident beam energies approaching the iron K-edge (Figure 6.4e). There is a reduction in area at the iron K-edge owing to sudden increase in sample absorption. As shown in Figure 6.4f, none of the Bragg peaks displayed energy dependence approaching the iron K-edge.

None of the scattering peaks displayed energy dependence indicating that they are not attributable to iron rich structures.

6.3.4 SAXS with tissue iron concentration

Since ASAXS revealed that the shoulder feature was iron dependent, its intensity was compared with tissue iron concentration as shown in Figure 6.5. The intensity of the shoulder measured at 0.065 Å⁻¹ increased with iron loading across each of the six sample series.
6.4 Discussion

6.4.1 Anomalous dispersion

The scattering factor of an atom is reduced by the real anomalous dispersion factor, $f'$, for incident x-ray energies approaching an absorption edge in the atom (see Equation 2.26). The steady increase in sample transmission observed as the incident beam energy increases from 6.900 to 7.100 keV is consistent with a reduction in the scattering length of iron approaching the iron K-edge. The sudden drop in transmission over incident beam energies 7.120 – 7.130 keV is consistent with the sharp increase in the imaginary term, $f''$, and the absorption of x-rays with energies equivalent to the iron K-edge. The observed transmission behaviour (see Figure 6.1) in this energy range is therefore due to the iron content of the tissue which is of sufficient concentration to observe anomalous dispersion and perform ASAXS.

6.4.2 Anomalous small angle x-ray scattering

Iron dependent shoulder feature

Anomalous dispersion near the iron K-edge serves to reduce the scattering contrast of iron oxide particles with their less dense tissue matrix. The systematic reduction in scattering intensity of the shoulder feature in the region 0.05 Å⁻¹ – 0.08 Å⁻¹ with energy (Figure 6.4 a, b & c) is consistent with complex contrast variation approaching the iron
K-edge. As shown in Figure 6.4b, the shoulder feature reduces to less than half its original intensity. This significant reduction in scattering confirms that the shoulder feature is due to iron-rich structures in the tissue.

The intensity of the shoulder feature increases with level of iron loading (Figure 6.5) indicating that the iron rich structures are the result of deposition of iron with iron loading. As such, scattering in the shoulder region \(0.05 \, \text{Å}^{-1} < Q < 0.08 \, \text{Å}^{-1}\) could potentially be used to estimate the bulk tissue iron concentration.

The shoulder feature in the scattering from iron loaded spleen tissue appears to be sharper than in the iron loaded tissue. This would suggest a greater degree of order in the iron-rich structures of the spleen tissue.

From TEM (see Chapter 3) we know that iron oxide deposits in the tissue as nanoscale spheres. Furthermore, radially averaged FFT of TEM images of clusters showed a peak at 0.07 Å\(^{-1}\) when regions of order were present in the tissue. Since the FFT peak at 0.07 Å\(^{-1}\) coincides with the shoulder at 0.05 – 0.08 Å\(^{-1}\), the shoulder may be related to these clusters. In order to gain quantitative information regarding the size and clustering of these deposits from SAXS, modelling is required. Fitting the SAXS data with a model of spherical nanoparticles is discussed in Chapters 7 and 8.

Iron independent peaks

The Bragg peaks first identified by Cu-K\(_x\) SAXS \(Q\) values 0.103, 0.128, 0.136, 0.235 and 0.260 Å\(^{-1}\) and those identified by the broader \(Q\)-range of the synchrotron SAXS \(Q\) values 0.35, 0.38 and 0.40 Å\(^{-1}\) showed no energy dependence. Therefore, the peaks were not iron related and most likely due to organic structure in the tissues.

Bragg peaks have been observed in the scattering profiles from various biological tissues as reviewed in Chapter 4. It is not known what structure in the rat tissue gives rise to Bragg peaks in the small angle scattering data. As suggested in Chapter 4, it is possible that the structure may be due to fat tissue which displays crystallinity [175]. Rat liver contains of the order or 10% fat [176, 177]. Removal of lipids by Soxhlet extraction [187] followed by SAXS analysis of the remaining tissue may help identify if the structure is from fat. It is interesting to note that the Bragg peaks are more prevalent in the control tissue than iron loaded where lipid peroxidation is a result of iron toxicity [77]. If the peaks can be demonstrated to be attributable to fat, a study of the peak positions and area may reveal changes accompanying iron loading. Further, if the peaks are from fat, small angle scattering may present a suitable technique for the study of
fatty liver disease, which is the most common liver disease in the western world [188, 189].

If the peaks are not attributable to fat, a study of the peaks with iron loading may reveal other structural change accompanying iron loading in the tissue. Since we are interested in characterisation of the iron deposits, non-iron structure will not be discussed further.

6.5 Conclusions

The flux of the incident beam and speed of data collection on the synchrotron source has enabled SAXS data collection for all control and iron loaded sample series.

Iron dependent features in the SAXS data have been identified by anomalous dispersion since those features decrease in intensity as the incident beam energy approaches the iron K-edge. The shoulder in SAXS data at $0.05 \text{ Å}^{-1} - 0.08 \text{ Å}^{-1}$ is iron dependent and its intensity increases with iron loading. Bragg peaks in the SAXS data remain intense as iron contrast is reduced and are therefore most likely to be due organic structure in the tissues rather than ordering of iron oxide particles.
7 ASAXS Data Modelling Strategy: Unified Scattering Function

7.1 Introduction

In the previous chapter we established that anomalous small angle x-ray scattering (ASAXS) can distinguish between iron dependent and non-iron related features in the scattering data from iron loaded mammalian tissue. Having demonstrated that the shoulder at 0.05 – 0.08 Å⁻¹ is due to iron in the iron loaded rat tissue we turn to modelling the data to extract quantitative information regarding the nature of the iron scattering bodies in the rat tissue. We are interested in the form and structure of the scattering bodies we know from transmission electron microscopy (TEM) to be nano-scale, spherical and unevenly distributed (Chapter 3). As discussed in Chapter 1, the diameter and clustering behaviour of iron oxide particles are related to their tissue environment and are of particular interest since they are likely to have implications for the toxicity of deposits.

The IRENA package [144] of SAXS analysis macros was introduced in Chapter 1 and enables modelling of Beaucage's theoretical Unified scattering function [145] to experimental data. Multiple levels, each containing Guinier regions and associated power laws can be fitted to the data. Correlation may also be included to describe scattering from clustered scattering bodies. The Unified scattering function given in Equation 2.25 is

\[ I(Q) = G \exp \left( -\frac{Q^2 R_g^2}{3} \right) + B \left\{ \frac{\text{erf} \left( \frac{Q R_g}{\sqrt{6}} \right)^3}{Q^p} \right\} \]

Equation 7.1

where \( R_g \) is the radius of gyration and \( P \) is a constant. The exponential prefactor \( G \) is given by
\[ G = n^2 N_p I_e \]

Equation 7.2

where \( n_e \) is the number of electrons in a particle, \( N_p \) the number of particles and \( I_e \) is the scattering factor for a single electron. The constants \( B \) and \( P \) define the intensity and exponent of the power law term and depend on the type and regime of scattering. For the case of power law scattering associated with the Guinier knee \( B = 2\pi N_p \rho_e^2 S_p I_e \) where \( \rho_e \) is the electron density of a particle and \( S_p \) the surface area of a particle.

The IRENA model outputs the prefactor for the Guinier term, \( G \), which is proportional to the number of scattering particles (Equation 7.2). The Guinier term is also described by the radius of gyration \( R_g \) from which the radius of a spherical particle \( R_P \) can be determined by \( \sqrt[3]{\frac{3}{5}} R_g \). Spatial correlation of spheres can be incorporated into the model with the parameters \( Pack \) and \( ETA \). \( Pack \) provides an estimate of the strength of the correlation and is given by \( Pack = \frac{8}{V_H} \) where \( V_H \) is the volume of hard spheres in the occupied volume \( V_O \). Cubic close packed spheres occupy 74% of the unit cell so for a face-centred cubic (FCC) arrangement of particles the maximum \( Pack \) value is 5.9. The minimum \( Pack \) value of 0 would imply no correlation. \( ETA \) gives the distance between planes of packed spheres.

IRENA also yields the prefactor and exponent for the power law term, \( B \) and \( P \).

Multiple levels containing Guinier and power law terms may be included in the model. From the area under the fitted curve, IRENA estimates the invariant attributable to each level of the model. For particles of small volume fraction in a matrix, the scattering invariant \( Q_{INVARIANT} \) is proportional to the scattering contrast and volume fraction and does not depend on structure (see Chapter 2).

This Chapter assesses the use of the IRENA unified scattering function to quantitatively analyse the scattering data from control and iron loaded mammalian tissue.
7.2 Methods

ASAXS data from iron loaded rat tissue (as described in Chapters 5 and 6) were analysed using the Unified scattering function by way of the IRENA package. Having determined that the shoulder region at 0.05 – 0.08 Å\(^{-1}\) was attributable to iron (Chapter 6), the \(Q\) region around the shoulder was chosen for quantitative analysis. From TEM we know that the iron oxide deposits are in the form of iron oxide spherical nanoparticles so a model of spherical scattering bodies was chosen to fit to the data. A subset of samples and data were chosen to investigate the package, develop a robust modelling procedure and assess its reliability in modelling scattering from iron oxide nanoparticles in tissue.

7.2.1 Sample and data selection

Rat samples

Pelletised freeze-dried ground tissue samples from the dietary iron loaded liver series were chosen for the development of the modelling strategy. This series was chosen on the basis of the large number of samples available in order to minimise the impact of biological variation. The liver samples were chosen over the spleen owing to their higher iron concentration and scattering signal strength.

Camera length and \(Q\)-range

The medium camera on 15-ID-D at APS sat at approximately 1.9 m and produced a \(Q\)-range 0.008 – 0.12 Å\(^{-1}\) usable above the noise and detector floor. This camera length was selected as most suitable for studying the region around the shoulder feature of interest and its data were chosen for modelling. An example dataset collected with incident beam energies 6.900 keV and 7.116 keV is shown in Figure 7.1a.
Figure 7.1 a) SAXS from highly dietary iron loaded rat liver (65.5 mg Fe.g⁻¹ dry weight) acquired well below the iron K-edge at 6.900 keV and approaching the edge at 7.116 keV. The differential SAXS data generated by subtracting the data near the edge from the data well below the edge are also shown. b) Differential SAXS data magnified to show signal to noise ratio from three dietary iron loaded liver samples with tissue iron concentration in the key (mg.g⁻¹ dry weight).

Energies

Data were collected with up to 16 incident beam energies in the range 6.900 keV to 7.122 keV.

Differential SAXS data were generated by subtracting data collected with an incident energy near the iron K-edge from the data collected well below the edge. As discussed in the previous chapter, any difference is due to the change in scattering contrast for iron so the resulting differential SAXS represents iron specific scattering data. Differential SAXS data were generated by subtracting data collected at 7.116 keV from data collected at 6.900 keV. These energies were chosen to increase the signal to noise ratio as far as possible before onset of sample fluorescence. Differential SAXS data for three iron loaded rat liver samples are shown in Figure 7.1b.

7.2.2 Selection of model

Single energy SAXS and differential SAXS were both tested for suitability to model. Several models ranging from simple to complex were tested to fit the data.

Single Energy SAXS

Single energy SAXS were acquired with incident beam energy 6.900 keV to achieve the greatest contrast between iron and the tissue. Scattering data from the 14 dietary iron
loaded rat liver samples were modelled. Single energy data have the advantage of better signal to noise ratio than differential SAXS data. The disadvantage of single energy SAXS is the contribution of tissue scattering which is ill-defined and difficult to model. Several constraints were required in order to achieve a physically meaningful fit to the data. In order to achieve meaningful fitting parameters the following procedure was adhered to:

1) Level 1- Fit a power law between $0.11086 \text{Å}^{-1} < Q < 0.11849 \text{Å}^{-1}$. The power law parameters were held, forcing the power law to be the only contributor to the scattering curve in this range. This constraint contributes some error to the fit since the error function (see Equation 7.1) reduces the contribution of the power law to 74 – 79 % in this $Q$-range.

2) Level 1 – Include a Guinier knee with spatial correlation over the range $0.05 \text{Å}^{-1} < Q < 0.11849 \text{Å}^{-1}$. Hold all level 1 parameters.

3) Level 2 – Fit a power law between $0.0201 \text{Å}^{-1} < Q < 0.0277 \text{Å}^{-1}$

4) Both levels – expand range of fit to $0.0201 \text{Å}^{-1} < Q < 0.11849 \text{Å}^{-1}$. Hold level 1 power law and $R_g$ parameters and fit over the expanded range.

**Differential SAXS**

Differential SAXS data from each of the 14 dietary iron loaded rat liver tissues were modelled. A two level model in the IRENA package was chosen to fit the data. Level one contained a Guinier knee which could incorporate spatial correlation between particles and level two contained a low $Q$ power law component. This two level model was chosen as it was simple, robust and required few to no constraints to produce a good fit with meaningful fitting parameters.

A power law associated with the Guinier knee was not included in the model as a reliable fit was achieved without it. Inclusion of a power law associated with the Guinier knee made the model more unstable and required many constraints in order to achieve a reliable fit.
Model parameters

The parameters of the IRENA model of the Unified scattering function are summarised in Table 7.1.

Table 7.1: Parameters given for each level of the IRENA model to the Unified scattering function

<table>
<thead>
<tr>
<th>Term</th>
<th>Parameter</th>
<th>Units</th>
<th>Formula</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinier with spatial correlation</td>
<td>$G$</td>
<td>A</td>
<td>$G = n^2 N p I_e$</td>
<td>Prefactor for Guinier term</td>
</tr>
<tr>
<td></td>
<td>$R_g$</td>
<td>A</td>
<td>$R_g = \sqrt[3]{3} R_p$</td>
<td>Radius of gyration</td>
</tr>
<tr>
<td></td>
<td>ETA</td>
<td>A</td>
<td>Pack $= \frac{8 V_H}{V_Q}$</td>
<td>Distance between layers of packed spheres</td>
</tr>
<tr>
<td>Power law</td>
<td>$B$</td>
<td></td>
<td>$B = 2\pi N p S_p^2 I_e$</td>
<td>Constant prefactor for power law term</td>
</tr>
<tr>
<td></td>
<td>$P$</td>
<td></td>
<td></td>
<td>Exponent of power law</td>
</tr>
<tr>
<td>$Q_{\text{IN Variant}}$</td>
<td>cm$^{-1}$Å$^{3}$</td>
<td></td>
<td></td>
<td>Integral of scattering curve attributable to level</td>
</tr>
</tbody>
</table>

Meaningful fit

A fit was deemed meaningful if the $R_g$ was in a realistic range for ferritin and haemosiderin deposits (i.e. between 10 and 50 Å) and satisfied the criteria:

$$\sqrt{\frac{5}{3}} 2R_g = R_p \leq \frac{\text{ETA}}{\sqrt{2}}$$

Equation 7.3

7.2.3 Testing the model

A number of tests were performed to assess the reliability of results from modelling the differential SAXS data.

Energy dependence

One highly loaded rat liver sample (21.5 months dietary iron loading) was chosen for further investigation to determine any dependence on energy chosen to generate differential SAXS curves. Multiple differential SAXS curves were generated by subtraction of the scattering data collected at nine different incident beam energies from...
the scattering data collected with incident beam energy of 6900 eV as shown in Figure 7.2. These energies were chosen to increase the signal to noise ratio as far as possible before onset of sample fluorescence.

Figure 7.2: SAXS data were acquired for each dietary iron loaded rat liver sample shown in the grid. Differential SAXS data were generated by subtracting the data collected at the energies in the grid from 6.900 keV. All rat liver samples were studied using the differential SAXS data obtained by subtracting 7.116 keV from 6.900 keV (dark shading). The light shading represents the data chosen to investigate the dependence of modelling on the energy pair used to generate differential SAXS data.

**High Q Power law omission**

The impact of disregarding the power law known to be associated with the Guinier knee was assessed by inspection of the error function (erf) in Equation 7.1 for typical values of radius of gyration.

**Low Q Power law contribution**

The contribution of the power law at low $Q$ was investigated by constraining the power law parameters and allowing the parameters of the Guinier knee to vary freely in the fit.

**$R_g$ compensation**

To test for any change in fitted $R_g$ in order to compensate for and mask an actual change in any other parameter, $R_g$ was constrained and all other parameters were allowed to vary.
7.2.4 Model independent tests

Model independent tests were performed for comparison with the results of modelling the differential SAXS data.

**Guinier analysis**

To provide a model-independent assessment of particle size, Guinier plots of the differential SAXS data were analysed and the radius of gyration obtained from the slope. This method is only valid for a region of the Guinier plot where $Q_{\text{mix}}R_g \leq 1$. Since Guinier analysis is only applicable for dilute solutions of monodisperse scatterers, any clustering and points of high local iron concentration in the tissue will limit the validity of results.

**Visual inspection**

Radially averaged differential SAXS data were studied by visual inspection for any observable significant difference in Guinier knee position in $Q$ space.

7.3 Results

7.3.1 Single energy SAXS

The single energy data showed good signal to noise but were difficult to model without multiple constraints and strictly followed modelling procedure. The shoulder feature could not be fitted with a simple Guinier knee corresponding to dilute monodisperse spheres. It was necessary to include spatial correlation between the particles in the model in order to obtain satisfactory fits to the data.

An example fit obtained from modelling the single energy SAXS from a highly dietary iron loaded liver is shown in Figure 7.3a.

The radii of gyration obtained from modelling the single energy data from the 14 dietary iron loaded rat liver samples are shown in Figure 7.4. The radius of gyration obtained by curve fitting decreased from approximately 30 Å to 24 Å with increasing tissue iron concentration.
Figure 7.3: Unified scattering function fit to data from dietary iron loaded rat liver sample (21.5 months, 65.5 mg Fe.g⁻¹ dry weight). Error bars on scattered intensity are shown with the Unified fit and residuals between the model and the data a) Single energy (6.900 keV) SAXS fit achieved with multiple constraints on level 1 and level 2 parameters b) differential SAXS (6.900 – 7.116 keV) fitted with a two level model incorporating spatial correlation between particles and a low $Q$ Power law with no constraints.

Figure 7.4: Radius of gyration obtained from Unified scattering function. Results from modelling single energy SAXS (6.900 keV) and differential SAXS (6.900 – 7.116 keV) are shown against a) tissue iron concentration and b) duration of iron loading
7.3.2 Differential SAXS

The differential SAXS data could be fitted without constraining any of the fitting parameters. Again, the shoulder feature could not be fitted with a simple Guinier knee and it was necessary to include spatial correlation between the particles in the model.

An example fit obtained from Unified modelling the differential SAXS from a highly dietary iron loaded liver is shown in Figure 7.3b.

The radii of gyration obtained from modelling the differential SAXS data from the 14 dietary iron loaded rat liver samples are shown in Figure 7.4. The radius of gyration obtained by curve fitting decreased from 30 Å to 23 Å with increasing iron concentration. The reduction in $R_g$ of about 20% was very similar to that obtained from the single energy SAXS data.

**Selection of model**

The differential data were chosen in favour of the single energy data for further modelling because of the comparative simplicity of the iron specific scattering pattern and absence of constraints on fitting procedure. The remainder of the level one and level two fitted parameters from modelling differential SAXS data collected from the dietary iron loaded rat liver tissue samples are shown with tissue iron concentration in Figure 7.5.

$G$ values showed a weak positive correlation with iron concentration. Curve fitting showed $ETA$ values scattered around $83 \pm 3$ Å which appeared to increase with iron concentration whilst $Pack$ values remained scattered around 1 to 2. The invariant for the level 1 Guinier term increased with increasing tissue iron concentration.

No significant trends were observed in the level two power law parameters, $P$, $B$ or $Q_{INVARIANT}$, for the 14 samples. The $Q_{INVARIANT}$ values for the power law were some 10 orders of magnitude less than for the Guinier term.
Figure 7.5: IRENA parameters obtained from modelling dietary iron loaded liver series differential SAXS data: a), b), c), d) and e) parameters of the Level 1 (●) Guinier term with spatial correlation; f), g) and h) parameters of the Level 2 (○) power law term.
7.3.3 Testing the model

**Energy dependence**

The fitted parameters for Unified modelling of the differential SAXS from one dietary iron loaded liver (21.5 months) generated from the nine pairs of incident beam energies are shown in Figure 7.6. The parameters are plotted against the incident beam energy of the data subtracted from the 6.900 keV data used to generate the differential SAXS curve.

$G$ showed a sharp increase of approximately 400% following the shape of the real part of the anomalous dispersion factor, $f'$, as the subtracted incident beam energy approached the iron K-edge. The significant reduction in iron scattering contrast near the edge resulted in an increase in the differential SAXS signal strength. The radius of gyration obtained by curve fitting showed an apparent increase of about 10% from 22 to 25 Å as the differential SAXS signal strength increased. Similarly, there was an apparent decrease of about 5% in the $ETA$ fitted to the differential SAXS data from the iron loaded rat liver sample. The $Pack$ parameter appeared stable over the various energies used and showed no systematic energy dependence. The invariant for the correlated Guinier term of the Unified fit displayed the same energy dependence as $G$ owing to the variation in $f'$ near the iron K-edge.

The power law parameters, $B$, $P$ and $Q_{\text{INVERT}}$ showed no significant energy dependence. The contribution of the power law to the scattering curve remained very small as indicated by the invariant.
Figure 7.6: IRENA parameters obtained from modelling differential SAXS data from one dietary iron loaded liver (21.5 months). The parameters are plotted against the incident beam energy subtracted from 6.900 used to generate the differential SAXS data: a), b), c), d) and e) parameters of the Level 1 (•) Guinier term with spatial correlation; f), g) and h) parameters of the Level 2 (o) power law term.
**High Q Power law omission**

The error function applied to the power law term associated with the Guinier knee is shown for typical values of $R_g$ in Figure 7.7. The error function significantly reduces the contribution of the power law in the $Q$ range of modelling, $0.008 - 0.118$ Å$^{-1}$. The power law can only be modelled with confidence using $Q$ values beyond 0.12 Å$^{-1}$ which are not available on the medium camera length used.

![Figure 7.7: Power law weighting function $erf$ of the power law scattering associated with the Guinier knee for typical values of $R_g$ (given in the key).](image)

**$R_g$ G compensation**

The $G$ value did not increase as significantly with iron concentration as had been anticipated (see Figure 7.5b) since either the particle size or the number of particles must increase to accommodate increasing tissue iron. It was decided to investigate whether the decrease in $R_g$ with iron concentration was masking an actual increase in $G$. Firstly, $R_g$ was constrained to the average $R_g$ obtained from free fitting (see Figure 7.5a) of 25.8 Å. $R_g$ was also constrained to the lowest value obtained by free fitting, 23.3 Å. Data obtained from all 14 dietary iron loaded rat liver samples could be successfully fitted with the constrained $R_g$.

As shown in Figure 7.8, when $R_g$ was constrained, $G$ rose more sharply and with stronger correlation with iron concentration than the free fit.
Figure 7.8: Comparison of $G$ parameter from constrained and free fits to the dietary iron loaded liver differential SAXS. Constraining $R_g$ to 25.8 Å or 23.3 Å resulted in a steeper slope and stronger correlation of $G$ with tissue iron concentration than for free fit.

**Low Q Power law contribution**

To further investigate the nature of the decreasing trend in $R_g$ with iron concentration it the level 2 power law was tested for any systematic effect on $R_g$. In order to test the contribution of the power law to the fit, the data were fitted with the power law constrained and the correlated Guinier allowed to vary. The power law was constrained to the parameters ($P=1.38315$ and $B=0.029889$) which was the average of the power law parameters obtained from the free fit (Figure 7.5).

A meaningful fit was obtained for 11 of the 14 dietary iron loaded liver samples when the power law was constrained. As shown in Figure 7.9, a decreasing trend in $R_g$ with increasing iron concentration or age was still present however weaker than when power law was allowed to vary.
7.3.4 Model independent tests

*Guinier analysis: model independent fitting*

To further test the decreasing trend in $R_g$ with iron concentration, model independent Guinier analysis was performed. Guinier regions where $R_g Q \leq 1$ could be found in the radially averaged scattering data from 10 of the 14 iron loaded tissue samples. The radius of gyration by Guinier analysis is shown in Figure 7.10. The radii of gyration are much smaller by the Guinier method and no significant decrease with iron loading can be observed. Guinier analysis is only valid for dilute systems and since we know that the data could not be fitted without accounting for interparticle interference, the results are of limited validity.
Visual comparison

Differential SAXS curves from the most highly iron loaded and the least loaded were compared to test if the difference in radius of gyration from modelling could be confirmed independently. On visual inspection, a decrease in $R_g$ with increased iron concentration could not be observed as a shift in the position of the Guinier feature.

7.4 Discussion

7.4.1 Selection of model

The single energy SAXS showed a superior signal to noise ratio over the differential SAXS data. However, the signal is less clearly understood and difficult to model since the tissue structure is unknown. The single energy data required many constraints and strictly followed procedure in order to obtain meaningful results. The differential SAXS data shows reduced signal to noise but the scattering pattern was simple to model without constraints. It is interesting to note the similarity in radius of gyration obtained by each method as shown in Figure 7.4. Despite constraints, the single energy data yielded radii of gyration decreasing from 30 – 24 Å in good agreement with the range of radii from differential SAXS, 30 - 23 Å.

On the basis of simplicity of scattering and absence of constraints, the differential SAXS was selected as the preferred data for analysis with the IRENA Unified scattering function. Further discussions pertain to results obtained from the preferred differential SAXS data modelling.

7.4.2 The model of clustered spherical nanoparticles

Spatial correlation required - indicative of clustering

Dispersed and clustered electron dense nanoparticles contribute to the scattering form factor resulting in the "Guinier knee" or "shoulder" in the scattering curves. Only clustered nanoparticles contribute to the spatial correlation parameters $ETA$ and $Pack$ obtained from fitting the Unified scattering function to the data. To obtain a reasonable fit of the Unified scattering function to the differential SAXS data from the iron loaded tissues the model required a structure factor owing to spatial correlation of spheres. Clustering of particles, such as within siderosomes, is therefore likely to be extensive throughout the iron loaded tissue.
Realistic fitting parameters – suitable model

Fitting of the differential scattering data for the dietary iron loaded tissue yielded a radius of gyration in the range 23 Å – 30 Å equivalent to a diameter range of 5.9 nm – 7.8 nm for spherical particles. This particle size is slightly larger than the dispersed core sizes measured in the iron loaded tissues by TEM of 5.27 ± 0.83 nm (Chapter 3) although is within the maximum possible ferritin core size of 8 nm [4]. Cytosolic ferritin cores are typically smaller than the ferritin cores in siderosomes [75, 86]. SAXS data are obtained from all iron oxide cores in the tissue and the inclusion of siderosomal ferritin is likely to increase the average size from that observed for cytosolic ferritin by TEM.

Modelling the differential SAXS data from the iron loaded tissue with a structure factor suggests effective $d$-spacings of planes of packed iron oxide particles of 8.0 - 8.6 nm. For FCC close packing as observed by TEM (Chapter 3) an $ETA$ of 8.0 - 8.6 nm would indicate a unit cell of 16 - 17 nm, which compares favourably with the unit cell estimated by TEM of 17 nm. Dry ferritin crystals have been observed with FCC unit cell 15.8 nm [65] whereas the unit cell for preparations of wet isoferritin crystals is swollen at 18.4 nm [101]. The unit cell of 16 – 17 nm observed in the current ASAXS study is well within previously observed unit cell sizes for ferritin and indicates realistic fitting parameters.

7.4.3 Testing the model

Energy dependence

Several trends were revealed in the parameters of the unified model fitted to the differential scattering for the energy dependent study of one dietary iron loaded rat liver tissue (Figure 7.6).

The trends in $G$ and $Q_{INVARIANT}$ for the correlated Guinier term of the Unified fit were as expected owing to the variation in $f'$ near the iron K-edge. Energy dependence in the $ETA$ term varying from 86 – 82 Å was unexpected given that there was no actual change in packing between the datasets. This trend is likely to be an artefact of curve fitting and the most likely result is that when the differential SAXS signal strength is the greatest approaching the iron K-edge.

$R_g$ was also shown to increase as the signal strength increased although there is no actual change in particle size. Similarly, Stuhrmann [104] has observed that the radius
of gyration increases by 4 % at the iron K-edge. The variation in $R_g$ in this study is much greater, ranging from about 22.5 to 25.5 Å. The wide variation observed in this study is then likely to be an artefact of curve fitting and the most likely result is that when the differential SAXS signal strength is the greatest. This corresponds to a most likely radius of gyration of 22.5 Å, equivalent to a spherical diameter of 5.8 nm.

*High $Q$ Power law omission*

As shown in Figure 7.7, the power law known to be associated with the Guinier knee does not reach full intensity until beyond the $Q$ range of data collected on the medium camera length. Exclusion of the power law tail was therefore deemed appropriate at the $Q$ range used for modelling.

*R$_g$ G compensation*

Given the greater slope and strength of correlation between $G$ and iron concentration when $R_g$ is constrained (Figure 7.8), it is suggested that $R_g$ may reduce with iron concentration to part compensate for an actual increase in $G$ value. To further explore the possibility that the $R_g$ does not vary between samples of different iron concentration it would be preferable to simultaneously fit all data using the one $R_g$ rather than taking the average or lowest value and constrain.

*Low $Q$ Power law contribution*

When the low $Q$ power law was constrained, a decreasing trend in $R_g$ with iron concentration could still be observed (Figure 7.9). As such, it can be determined that variation in the power law is not the sole contributor to the decreasing trend in $R_g$ with iron loading.

**7.4.4 Model independent tests**

Model independent tests by the Guinier method and visual inspection were unable to confirm a decreasing trend in $R_g$ with iron concentration.

**7.4.5 Trends with iron loading**

Several trends in the parameters of the unified model fitted to the differential scattering data were observed with increasing degree of iron loading. The $Q_{INVAR}$ gives a measure of the amount of scattering material in the beam path. It was found to increase steadily with iron concentration. The fitted parameters indicate a decrease in radius of
gyration as iron concentration increases. The structure factors suggest that the particles may become slightly more widely spaced with duration of iron loading.

Several tests were performed on the modelling to explore the validity of these trends. The results are summarised as either supporting that the trends observed are a real effect of iron loading or an artefact of curve fitting.

**Real effect of iron loading**

We know from the energy dependence study that changing scattering signal strength can affect the results of curve fitting. It was found that the intensity of the shoulder in the differential SAXS data decreased as incident beam energies further from the iron K-edge were used. It was found that the parameters derived from fitting these differential SAXS datasets yielded an increasing trend in radius of gyration with increasing relative intensity of shoulder feature. However, with increasing signal strength due to increasing iron loading the fitted radius decreased. These trends are in opposing directions, thus supporting the point of view that the observed decrease in particle size with increased duration of iron loading may be real rather than an artefact of increasing signal strength.

The decrease in particle size from 7.8 nm to 5.9 nm with increasing iron loading could not be observed by TEM (Chapter 3). TEM observations did not indicate any correlation between duration of iron loading and size of dispersed particles. However, TEM was unable to yield particle sizes in the clusters and hence any trend in the nanoparticulate sizes in the clusters would be obscured.

The decrease in particle size with increasing iron loading was observed in the results of modelling differential SAXS with the level two power law constrained and the power law is therefore not causing an artificial trend. The scattering data from iron loaded samples were analysed in random order so as not to influence the fitting parameters.

Decreasing particle size with duration of iron loading is consistent with the observed saturation of ferritin synthesis and increase in deposition of haemosiderin [19]. Haemosiderin typically displays smaller particle sizes than ferritin with cores ranging from 0.8 – 7 nm [10]. A decrease in the ratio of ferritin to haemosiderin would result in a decreasing average particle size. The degradation of iron oxide particles within the low pH lysosomal environment may also cause a decrease in the mean core size of iron deposits owing to partial dissolution.
Artefact of fitting procedure

A visual comparison of the scattering curves from high and low iron loaded tissue was unable to confirm the presence of a significant decrease in particle size with iron loading. Model independent analysis by the Guinier method was unable to confirm a trend in $R_g$ with iron concentration since Guinier analysis is unreliable in cases of clustered particles as in the rat liver tissue. Similarly, the correlation function in IRENA is designed for low concentrations of scatterers so there will be an increasing amount of error on the method as the iron concentration increases.

In a separate study, magnetic energy barrier distribution measurements following a previously published method [190] were used to assess ferrihydrite nanoparticle size distributions in the same dietary iron loaded rat tissues. No trend in particle size distribution was observed with duration of iron loading [191]. These data are contained in Figure A1 of the Appendix. In light of the magnetic measurements, the trend observed in the scattering data observed in this study may be an artefact of the fitting procedure.

If the trend is an artefact of the fitting procedure then the most reliable fitted parameters are likely to be when the signal strength is greatest. It is worth noting that the particle sizes derived from the SAXS data tend towards the values for the dispersed particles measured using TEM. Fitted parameters for the most highly loaded liver sacrificed at 21 months places the average particle diameter at 6.3 nm with a FCC unit cell of 16.8 nm.

Preferred interpretation

The examination of the model and independent tests as described above found some support yet considerable doubt as to the validity of a decrease in $R_g$ with iron concentration above the uncertainty in the model. On balance, we prefer the hypothesis that the radius of gyration of iron oxide deposits does not change with degree of iron loading, in keeping with findings from TEM (Chapter 3) and magnetic energy barrier results (Appendix). The implications are two fold; 1) care needs to be exercised when interpreting small angle scattering data since curve fitting may introduce artefacts, 2) the particle size and therefore molar toxicity of iron remains constant with iron loading in the dietary iron loaded rat liver series.
7.5 Conclusions

In summary, ASAXS data can be used to assess iron loading in rat liver tissue and yield particle sizes close to those observed by other techniques without the need for \textit{a priori} constraints on data fitting parameters. Both single energy and differential SAXS data can be fitted to yield similar and meaningful results. The differential SAXS is preferred owing to the simplicity of scattering and absence of constraints during fitting.

The differential SAXS data from dietary iron loaded rat tissue can be fitted reliably with a model of interfering spherical nanoparticles. The modelling parameters indicated particle sizes of approximately 5.9 nm – 7.8 nm and FCC unit cell of 16.0 – 17.2 nm. It is likely the lower values in the range of particles sizes are most reliable, that is, a diameter of 5.9 nm. The model and parameters are consistent with iron oxide particle sizes and close packing behaviour observed by TEM presented in Chapter 3 and the literature reviewed in Chapter 1.

Apparent information derived from the data suggesting subtle trends in iron oxide particle size and spacings with duration of iron loading need to be treated with caution since they are inconsistent with measurements made using magnetic energy barrier distribution measurements. Examination of the results of IRENA modelling in this chapter was unable to confirm the validity of a decrease in $R_g$ with iron concentration above the error in the model. Modelling may introduce fitting artefacts and such trends will be treated with caution when considered in the following chapter. It is hypothesized that in the case of dietary iron loaded rat liver, iron oxide particle size and hence molar toxicity of iron deposits does not change with iron loading.
8 Control and Iron Loaded Rat Tissue: Iron Oxide Particle Size and Clustering Behaviour by Anomalous Small Angle X-Ray Scattering

8.1 Introduction

There has been much attention in the literature regarding the size of the mineral cores of ferritin and haemosiderin deposited in various biological organisms. Particle sizes measured by electron microscopy for both iron storage compounds, in various mammalian tissues in a variety of normal and diseased states, cytosolic and lysosomal were summarised in Chapter 1. The limitations of microscopy techniques in quantifying particle sizes and the need for an objective, bulk method of analysis were also discussed.

Having developed the ability to assess particle sizes in the bulk using anomalous small angle x-ray scattering (ASAXS) (Chapter 7) the current study aims to elucidate iron oxide particle sizes in our series of iron loaded rat samples. Furthermore, whilst clustering of deposits is discussed qualitatively in the literature, a method for quantifying clustering behaviour has been lacking. The use of ASAXS in the current study presents a suitable technique for assessing the clustering of particles in the iron loaded rat tissue samples.

In this extensive ASAXS study we are able to quantify iron oxide particle sizes and clustering behaviour in bulk iron loaded tissue samples. Tissue specificity is explored by comparison of x-ray scattering from liver and spleen. By studying samples removed from rats sacrificed at various ages, the influence of duration and degree of iron loading are examined. The effect of iron loading pathway on the deposition of iron is explored by investigation of tissues loaded by dietary supplementation and by parenteral transfusion of red blood cells. In addition, any change in particle size or clustering following cessation of iron loading is investigated.
8.2 Materials and methods

8.2.1 Rat tissue samples
Pellets of freeze-dried ground tissue samples were prepared as described in Chapter 4. Liver and spleen tissues from the control, dietary iron loaded and transfusional iron loaded rat tissue series described in Chapter 2 were studied.

8.2.2 ASAXS data acquisition
Scattering data were acquired on the medium camera length of 15-ID-D at APS as described in Chapter 5. In all cases, differential SAXS was generated by subtracting the data collected with an incident beam energy of 7116 eV from data collected at 6900 eV.

8.2.3 IRENA Unified scattering function
The differential SAXS data were modelled with the simplest model possible using the IRENA Unified scattering function according to the strategy described in Chapter 7.

Control liver
The scattering data from control liver were fitted with a two level model. The first level was a Guinier knee without spatial correlation and the second level a power law.
The fits were performed over the $Q$-range 0.008 - 0.118 Å$^{-1}$.

Control spleen
The scattering data from control spleen were fitted with a two level model. The first level was a Guinier knee, with spatial correlation where necessary and the second level a power law. The fits were performed over the $Q$-range 0.008 - 0.118 Å$^{-1}$.

Dietary iron loaded liver
The scattering data from dietary iron loaded liver were fitted with a two level model. The first level was a Guinier knee incorporating spatial correlation and the second level a power law. The fits were performed over the $Q$-range 0.008 - 0.118 Å$^{-1}$.

Dietary iron loaded spleen
The scattering data from dietary iron loaded spleen were fitted with a two level model. The first level was a Guinier knee incorporating spatial correlation where required and
the second level a power law. The fits were performed over the $Q$-range 0.0133 - 0.118 Å$^{-1}$ and in cases of very low signal to noise on the low $Q$ data points, the lower limit was increased to 0.02 Å$^{-1}$.

**Transfusional iron loaded liver**

The two youngest transfusional iron loaded liver samples were fitted with a one level model of a Guinier knee without spatial correlation over the $Q$-range 0.02 - 0.112 Å$^{-1}$. For the more highly loaded transfusional liver with better signal to noise a second level containing a power law was included and spatial correlation was incorporated. The $Q$-range was 0.008 - 0.118 Å$^{-1}$ for the two level models.

**Transfusional iron loaded spleen**

The scattering data from transfusional iron loaded spleen were fitted with a two level model. The first level was a Guinier knee incorporating spatial correlation and the second level a power law. The fits were performed over the $Q$-range 0.008 - 0.118 Å$^{-1}$.

### 8.3 Results

#### 8.3.1 Scattering data

ASAXS data were collected from the liver and spleen of all six control, 18 dietary iron loaded and eight transfusional iron loaded rats. Scattering showed no signs of asymmetry so the data were radially averaged for analysis. Typical one dimensional SAXS data from control and iron loaded tissues are shown in Figure 8.1.

A shoulder at 0.05 - 0.08 Å$^{-1}$ can be observed in the scattering data from the iron loaded rat tissue. As discussed in Chapter 6, the shoulder feature reduces in intensity as the incident beam increases towards the iron K-edge indicating that the shoulder is related to iron contrast in the sample. Peaks were present in the scattering data outside the $Q$-range shown in Figure 8.1. As discussed in Chapter 6, the peaks were not iron related so were not examined any further.

Differential SAXS curves, generated by subtracting the data collected with incident beam energy of 7116 eV from the data collected at 6900 eV, are also shown in Figure 8.1 and enlarged in Figure 8.2. The resulting iron specific scattering data from the pelletised tissue displayed a shoulder at 0.05 - 0.08 Å$^{-1}$ and were otherwise featureless in the modelled $Q$-range 0.01 - 0.1 Å$^{-1}$.  

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Figure 8.1: Example single energy SAXS and differential SAXS from each rat tissue series, tissue iron concentration given in mg.g\(^{-1}\) dry weight. Single energy SAXS taken with an incident beam energy of 6900 eV well below the iron k-edge and 7116 eV approaching the edge. Differential SAXS generated by subtracting 7116 eV intensity data from 6900 eV data (6.900 - 7.116 keV) with error bars shown in light green.
Figure 8.2: Example differential SAXS data with error bars from control, dietary iron loaded and transfusional iron loaded rat tissue samples. Tissue iron concentrations are given in the legend in mg.g⁻¹ dry weight.
Control liver

A shoulder of weak intensity can be resolved in the differential SAXS data from the control liver tissue. As discussed in Chapter 5, iron concentrations of only a few mg.g\(^{-1}\) dry weight are near the limit of detection.

Control spleen

A shoulder feature can be clearly resolved in the differential SAXS data from the control spleen tissue. The scattering intensity is greater than from the control liver series. The shoulder feature tends to increase in intensity with iron loading and a maximum at finite \(Q\) can be observed in the most highly loaded control spleen.

Dietary iron loaded liver

The shoulder feature is prominent in the differential SAXS data from the dietary iron loaded livers and it tends to increase in intensity with iron concentration. The differential SAXS data are of the order of ten times the intensity of the control scattering data. In some cases, for example 65.5 mg.g\(^{-1}\) tissue iron concentration in Figure 8.2c, the intensity at low \(Q\) is depressed resulting in a sharpening of the shoulder feature.

Dietary iron loaded spleen

A shoulder feature can be clearly resolved in the differential SAXS data from the dietary iron loaded spleen tissue. The scattering intensity is generally less than from the dietary iron loaded liver series but greater than the control animals. The data tend to exhibit a sharper shoulder feature than the dietary iron loaded liver or control animals, for example compare the spleen data of Figure 8.1d with the dietary iron loaded liver of Figure 8.1c. Several of the data display a maximum at finite \(Q\), for example the 34.4 mg.g\(^{-1}\) tissue iron concentration from Figure 8.2d.

Transfusional iron loaded liver

A smooth shoulder feature can be clearly resolved in the differential SAXS data from the transfusional iron loaded liver. The scattering is considerably less intense than the liver receiving iron loading by the dietary route (see Figure 8.1e and Figure 8.2e).
Transfusional iron loaded spleen

A sharp shoulder feature can be clearly resolved in the differential SAXS data from the transfusional iron loaded spleen. Most of the shoulder features display maxima at finite $Q$. The scattering is generally more intense than any other series of rat samples (see Figure 8.1f and Figure 8.2f).

### 8.3.2 Curve fitting

Examples of the IRENA unified scattering function modelled to the differential SAXS data from each sample series are shown in Figure 8.3. The parameters from modelling the differential SAXS data for each series are shown in Table 8.1.

<table>
<thead>
<tr>
<th>Rat series</th>
<th>n</th>
<th>$R_g$ ± SD (Å)</th>
<th>Diameter ± SD (nm)</th>
<th>$G$</th>
<th>ETA (Å)</th>
<th>Pack</th>
<th>$Q_{INVARIANT}$ (cm$^{-1}$Å$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control liver</td>
<td>6</td>
<td>31 ± 5</td>
<td>7.9 ± 1.2</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>1.1 x 10$^{-4}$</td>
</tr>
<tr>
<td>Control spleen</td>
<td>6</td>
<td>30 ± 3</td>
<td>7.8 ± 0.8</td>
<td>5.4</td>
<td>79</td>
<td>0.98</td>
<td>4.3 x 10$^{-4}$</td>
</tr>
<tr>
<td>Dietary liver</td>
<td>18</td>
<td>26 ± 2</td>
<td>6.7 ± 0.5</td>
<td>8.8</td>
<td>84</td>
<td>1.5</td>
<td>12 x 10$^{-4}$</td>
</tr>
<tr>
<td>Dietary spleen</td>
<td>15</td>
<td>28 ± 2</td>
<td>7.2 ± 0.6</td>
<td>6.7</td>
<td>86</td>
<td>1.5</td>
<td>7.6 x 10$^{-4}$</td>
</tr>
<tr>
<td>Transfusional liver</td>
<td>8</td>
<td>31 ± 2</td>
<td>8.0 ± 0.6</td>
<td>4.9</td>
<td>79</td>
<td>1.5</td>
<td>3.8 x 10$^{-4}$</td>
</tr>
<tr>
<td>Transfusional spleen</td>
<td>8</td>
<td>25 ± 2</td>
<td>6.5 ± 0.5</td>
<td>11</td>
<td>86</td>
<td>2.2</td>
<td>17 x 10$^{-4}$</td>
</tr>
</tbody>
</table>

Table 8.1: IRENA Unified scattering function parameters for the Level 1 Guinier term – average values from modelling the differential SAXS data for each series with n samples.

Spherical particle diameter calculated from radius of gyration shown in italics.
Figure 8.3 Example IRENA Unified Scattering Function fits to the differential SAXS data for each series, tissue iron concentration given in mg.g\(^{-1}\) dry weight. The dashed line is the fitted curve, error bars on the differential SAXS data are shown whilst the data points are omitted for clarity.
Control liver

Meaningful fits were achieved for all six of the control liver tissues, an example of which is shown in Figure 8.3a. Spatial correlation between particles was not required in the model. Modelling parameters for the differential scattering data from the control liver are given in Figure 8.4. There was some variation in the radius of gyration and the mean (± SD) was 31 ± 5 Å equivalent to a particle diameter of 7.9 ± 1.2 nm for spherical particles. The average $G$ value was 1.2 and the scattering invariant for the Guinier feature was $1.1 \times 10^{-4}$ cm$^{-1}$Å$^{-3}$.

Figure 8.4: Differential SAXS modelling parameters against tissue iron concentration for the six samples of the control liver series. IRENA Unified Scattering Function parameters for the Guinier term (without spatial correlation) a) radius of gyration b) $G$ exponential prefactor c) scattering invariant
Control spleen

Meaningful fits were achieved for all six of the control spleen tissues, an example of which is shown in Figure 8.3b. The two youngest rats and the fifth rat in the series did not require spatial correlation on the Guinier term. A satisfactory fit could be reached without spatial correlation for the other three control spleen samples. However, correlation improved the fit for these samples and so was included in the modelling. Modelling parameters for the differential scattering data from the control spleens are given in Figure 8.5.

The mean (± SD) radius of gyration was 30 ± 3 Å, equivalent to a particle diameter of 7.8 ± 0.8 nm for spherical particles. Modelling returned a considerably higher $G$ and $Q_{\text{INVARIANT}}$ for the control spleen compared with liver and tended to increase with iron concentration. The average $G$ value was 5.4 and the scattering invariant was $4.3 \times 10^{-4}$ cm$^{-1}$Å$^{-3}$.

The average $ETA$ was 7.9 nm and the packing factor, $Pack$, which increased steadily with iron concentration averaged to 0.98.
Figure 8.5: Differential SAXS modelling parameters against tissue iron concentration for the six samples of the control spleen series. IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) $G$ exponential prefactor c) $ETA$ distance between layers of packed spheres d) $Pack$ packing factor e) scattering invariant. Note only three of the more highly loaded samples required spatial correlation described by the $ETA$ and $Pack$ parameters.
Dietary iron loaded liver

Meaningful fits were achieved for all 14 dietary iron loaded liver tissue samples, as described in Chapter 7, and for the 4 samples allowed to unload. Spatial correlation between particles was required in each case to achieve satisfactory fits to the data. Example fits to the data are shown in Figure 7.3b and Figure 8.3c. The model parameters were given in Figure 7.5 and are summarised including unloaders in Table 8.1 and Figure 8.6.

The radius of gyration obtained by curve fitting decreased from 30 Å to 23 Å with increasing iron concentration. Upon cessation of iron supplementation there is a reversal of the trend in $R_g$ and the mean diameter from modelling appears to increase as the tissue unloads with iron. The mean ± SD radius of gyration was 26 ± 2 Å corresponding to a spherical particle diameter of 6.7 ± 0.5 nm.

Modelling returned higher $G$ values than for the control animals with an average at 8.8. $ETA$ averaged at 84 Å and $Pack$ values were scattered though generally higher than for the control animals averaging at 1.5. The invariant shows a steady increase in scattering from the iron loaded tissue with increasing tissue iron concentration.
Figure 8.6: Differential SAXS modelling parameters against tissue iron concentration for the dietary iron loaded liver series. 14 samples experienced iron loading (•) and four samples were allowed to unload following cessation of iron supplementation (○). IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) G exponential prefactor c) ETA distance between layers of packed spheres d) Pack packing factor e) scattering invariant.
Dietary iron loaded spleen

SAXS data were not available for the spleen of two dietary iron loaded animals. A meaningful fit could not be obtained for the youngest spleen of the series with a tissue iron concentration of only 3 mg.g\(^{-1}\) dry weight due to poor signal to noise. Meaningful fits were achieved for the remaining 11 dietary iron loaded animals and 4 animals allowed to unload following cessation of dietary iron supplementation.

Three of the spleen samples did not require spatial correlation to achieve a satisfactory fit to the differential SAXS data. The remaining 12 samples required the incorporation of interparticle interference in a satisfactory model.

An example fit to the data is shown in Figure 8.3d and the model parameters are summarised in Table 8.1 and Figure 8.7.

The radius of gyration obtained by curve fitting ranged from 24 Å to 32 Å with an average value of 28 ± 2 Å. The corresponding mean spherical diameter is 7.2 ± 0.6 nm.

Modelling returned higher \(G\) values than for the control animals with an average at 6.7, slightly lower than the dietary iron loaded liver tissue. For the 12 samples requiring spatial correlation the \(ETA\) values were varied but averaged at 86 Å. \(Pack\) values were again scattered and averaged at 1.5, the same as returned from modelling the dietary liver data. As seen in the dietary iron loaded liver data, the invariant showed a steady increase with splenic tissue iron concentration.
Figure 8.7: Differential SAXS modelling parameters against tissue iron concentration for the dietary iron loaded spleen series. 11 samples underwent iron loading (●) and four samples were allowed to unload following cessation of iron supplementation (○). IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) $G$ exponential prefactor c) $ETA$ distance between layers of packed spheres d) Pack packing factor e) scattering invariant.
Transfusional iron loaded liver

Meaningful fits to the differential SAXS data were obtained from all eight transfusional iron loaded liver samples, including the two samples allowed to unload following cessation of packed red blood cell transfusions.

The two samples with the lowest iron concentrations did not require spatial correlation. However, the remaining six required the incorporation of interparticle interference for a satisfactory model. An example fit to the data is shown in Figure 8.3e and the model parameters are summarised in Table 8.1 and Figure 8.8.

The radius of gyration obtained by curve fitting ranged from 29 Å to 35 Å with a mean (± SD) of 31 ± 2 Å. The corresponding mean spherical diameter is 8.0 ± 0.6 nm. $G$ values increased steadily with iron concentration (Figure 8.8b) and were comparable with values obtained from modelling control spleen and lower than from the dietary iron loaded animals.

For the six samples requiring spatial correlation the $ETA$ and $Pack$ values were consistently close to the average values at 79 Å and 1.5 respectively.

As seen in the dietary iron loaded data, the invariant showed a steady increase with tissue iron concentration.

The data in Figure 8.8 showed less scatter than the dietary iron loaded series ($n=18$) and we note that there are fewer data for the transfusional iron loaded liver ($n=8$).
Figure 8.8: Differential SAXS modelling parameters against tissue iron concentration for the eight transfusional iron loaded liver samples. Six samples underwent iron loading (●) and two samples were allowed to unload following cessation of iron transfusions (○). IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) \( G \) exponential prefactor c) \( ETA \) distance between layers of packed spheres d) \( Pack \) packing factor e) scattering invariant.
**Transfusional iron loaded spleen**

Meaningful fits to the differential SAXS data were obtained from all eight transfusional iron loaded spleen samples, including the two samples allowed to unload following cessation of packed red blood cell transfusions.

The sample with the lowest iron concentration did not require spatial correlation. However, the remaining seven required the incorporation of interparticle interference for a satisfactory model. An example fit to the data is shown in Figure 8.3f and the model parameters are summarised in Table 8.1 and Figure 8.9.

The mean radius of gyration obtained by curve fitting was $25 \pm 2$ Å, the corresponding mean spherical diameter being $6.5 \pm 0.5$ nm. There was little change in the radius of gyration returned from curve fitting for samples with a high degree of iron loading, beyond $30 \text{ mg.g}^{-1}$ dry weight (see Figure 8.9a).

$G$ values increased with iron concentration and were greater than values obtained from modelling all other rat tissue series.

For the seven samples requiring spatial correlation the $ETA$ and $Pack$ values were very consistently close to the average values of $86$ Å and $2.2$ respectively. The $Pack$ value was greater than observed in the other rat tissue series. The scattering invariant showed a steady increase with tissue iron concentration.
Figure 8.9: Differential SAXS modelling parameters against tissue iron concentration for the eight transfusional iron loaded spleen samples. Six samples underwent iron loading (●) and two samples were allowed to unload following cessation of iron transfusions (○). IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) $G$ exponential prefactor c) $ETA$ distance between layers of packed spheres d) $Pack$ packing factor e) scattering invariant.
8.3.3 Summary of differential SAXS curve fitting parameters

The IRENA Unified Scattering Function parameters fitted for each of the six series of rat tissue samples are shown for comparison in Figure 8.10.

Figure 8.10: Differential SAXS modelling parameters against tissue iron concentration for all six series of rat tissue samples (as given in the legend). IRENA Unified Scattering Function parameters for the Guinier term a) radius of gyration b) $G$ exponential prefactor c) $ETA$ distance between layers of packed spheres d) $Pack$ packing factor e) scattering invariant.
**Radius of gyration**

As shown in Figure 8.10a, the radius of gyration returned from IRENA curve fitting is scattered at low iron concentrations and appears to approach lower values at higher tissue iron concentrations. For iron concentrations in excess of 40 mg.g\(^{-1}\) dry weight, the mean radius of gyration was 25 ± 1 Å.

**G value**

The G value increased with tissue iron concentration for all series of rat tissues (see Figure 8.10b).

**ETA**

The distance between packed layers of spherical nanoparticles is also scattered at low tissue iron concentrations and tends towards more consistent values as the tissue iron concentration increase as shown in Figure 8.10c. The average ETA for tissue iron concentrations in excess of 40 mg.g\(^{-1}\) dry weight is 86 ± 3 Å.

**Pack**

Pack values show no clear correlation with tissue iron concentration (see Figure 8.10d) and were generally around 1 to 2.

**Invariant**

The scattering invariant demonstrates a strong positive correlation with tissue iron concentration. The linear correlation is shown in Figure 8.10e. These data show striking similarity to the intensity of the shoulder measured at 0.065 Å\(^{-1}\) with iron concentration in Chapter 6 (Figure 6.5).

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**8.4 Discussion**

**Control liver**

Since the differential scattering from the control liver samples could be fitted without a structure factor, clustering of deposits is unlikely to be extensive in these tissues. Most of the iron in the control liver tissues is likely to be in the form of cytosolic ferritin consistent with the observation that very few clusters were observed in the TEM images of these specimens. These observations are also consistent with findings that lysosomes
become iron laden only following saturation of ferritin synthesis with iron loading [192].

The parameters obtained from fits of the Unified scattering function to the differential SAXS data are scattered, likely due to the low tissue iron concentration near the limit of detection by this method. Fitting of the differential data for the control liver tissues indicated a mean (± SD) radius of gyration of 31 ± 5 Å equivalent to a mean particle diameter of 7.9 ± 1.2 nm for spherical particles. This particle size is larger than the dispersed core sizes measured in the control liver tissues by TEM (5.48 ± SD 0.78 nm) although is comparable with the maximum possible ferritin core size of 8 nm. In an early study of ferritin by SAXS, Kleinwächter also acknowledges the average diameter of 7.6 nm by SAXS is greater than diameters observed by TEM [66].

**Control spleen**

Since the differential scattering from the two youngest control spleen samples could be fitted satisfactorily without a structure factor, clustering of deposits is unlikely to be extensive in these tissues. As the rats aged and spleen iron concentration increased, packing became more significant as evidenced by the improved fit when interparticle interference was taken into account. Though minimal, the clustering is therefore more prevalent for the control spleen than for the control liver. The clustering may be a result of the deposition of aggregates of haemosiderin since higher tissue iron concentrations were observed in the spleen. The observation of clustering in the spleen is also consistent with the greater proclivity for lysosomal uptake of ferritin of some spleen cells compared with cells of other organs [87]. Dense packing of particles depresses the intensity of the scattering curve at low Q and in high enough concentrations the scattering curve will exhibit a local maximum [137]. One control spleen showed a maximum at finite Q and modelling yielded the highest Pack value of 1.2.

The average *ETA* of 7.9 nm indicates close packing which is consistent with a FCC unit cell of 15.8 nm comparable with that observed for air dried preparations of apoferritin crystals [65].

The scattering intensity is considerably greater for the control spleen than for the control liver tissue owing to the higher tissue iron concentrations achieved in the control spleen. The higher tissue iron concentration is reflected in the average $G$ and $Q_{INvariant}$ for the spleen (5.4 and $4.3 \times 10^{-4}$ cm$^{-1}$Å$^{-3}$) when compared with the liver from the same animals (1.2 and $1.1 \times 10^{-4}$ cm$^{-1}$Å$^{-3}$).
The higher tissue iron concentration and signal to noise in the spleen scattering data likely contributes to less uncertainty in the radius of gyration yielded from IRENA fitting when compared with the liver. The mean radius of gyration for the spleen tissue of 30 ± 3 Å, equivalent to a mean particle diameter of 7.8 ± 0.8 nm, was comparable with that modelled for the control liver tissue (diameter 7.9 ± 1.2 nm). This particle diameter is larger than the dispersed core sizes measured in the control spleen tissues by TEM (5.40 ± 1.15) although is comparable with the maximum possible ferritin core size of 8 nm.

**Dietary iron loaded liver**

The differential scattering data are considerably more intense for the dietary iron loaded liver than for the control animals. This observation is consistent with the higher tissue iron concentrations achieved through iron loading. The shoulder feature is more prominent and some dietary iron loaded liver samples display a sharp change in slope around the 0.05 – 0.08 Å⁻¹ region. Depression of the intensity at low $Q$ and a sharpening of the shoulder feature indicates a greater degree of dense packing of nanoparticles in the dietary iron loaded livers than in the control animals.

To obtain a satisfactory fit of the Unified scattering function to the differential SAXS data for the iron loaded tissues, a structure factor was required in the model. Clustering of particles, such as within siderosomes, is therefore likely to be extensive throughout the dietary iron loaded liver tissue.

The radius of gyration ranged from 23 Å – 30 Å equivalent to a diameter range of 6.0 nm – 8.2 nm for spheres. These particles sizes are smaller than those measured by SAXS for the control liver tissue. Similarly, electron diffraction and Mössbauer spectral studies of ferritin and haemosiderin cores have shown smaller cores than for cytosolic ferritin [73, 98].

$G$ values were considerably higher for the dietary iron loaded liver than for the control animals, consistent with the higher degree of iron loading observed in the dietary series. An increase in $G$ rather than $R_g$ in the dietary iron loaded liver when compared to the control animals indicates an increase in the number rather than size of iron oxide nanoparticles with iron loading.

Modelling the differential SAXS data from the iron loaded tissue with a structure factor yielded effective $d$-spacings for iron oxide particles of 7.5 nm – 9.3 nm. Such interparticle differences are smaller than the 12 nm ferritin shell and indicate that close
packing, consistent with observations by TEM indicating a packing arrangement approximating FCC in iron loaded tissue (Chapter 3).

ETA values were very similar between the dietary iron loaded liver and control series while the Pack values were generally higher for the iron loaded animals. These observations indicate that in the dietary iron loaded liver, the packing distance is not reduced, but the occupancy of the packed clusters is greater to accommodate the iron burden in the tissue. The higher Pack values for the dietary iron loaded liver supports the qualitative observation of dense packing from the sharpening of the shoulder feature in these animals.

Several trends in the parameters of the unified model fitted to the differential scattering data were observed with increasing degree of iron loading. The invariant gives a measure of the amount of scattering material in the beam path, independent of the structure of material. It was found to increase steadily with iron concentration. As shown in Figure 8.6e, a reduced invariant is observed for the samples allowed to unload following cessation of iron loading. The scattering invariant therefore gives a measure of degree of iron loading in the tissue.

The fitted parameters indicate a decrease in radius of gyration as iron concentration increases. Trends in fitted parameters were examined in Chapter 7 where it was discussed that the validity of such trends remains unclear. However, decreasing particle size with duration of iron loading is consistent with the observed saturation of ferritin synthesis and deposition of smaller haemosiderin cores during iron loading [19, 98]. The degradation of iron oxide particles within the low pH lysosomal environment also is likely to result in a decrease in the mean core size of iron deposits owing to partial dissolution.

Further, Figure 8.6a shows an increase in particle size following cessation of iron loading. An increase in average particle size is consistent with the preferential mobilisation of iron shown in vitro from ferrihydrite haemosiderin cores smaller than ferritin cores [73, 74].

**Dietary iron loaded spleen**

Dietary supplementation of iron results in a higher degree of iron loading in the liver than in the spleen of the same animals (Figure 2.1). This difference in iron loading of the two organs is reflected in the lower scattering intensity observed in the differential SAXS data from the dietary iron loaded spleen tissues. Qualitatively, the radially
averaged scattering data show a sharper shoulder feature in the spleen than in the liver. This observation suggests considerable dense packing of iron oxide nanoparticles in the spleen, despite tissue iron concentrations lower than the liver. Ultrastructural observations of the spleen support this hypothesis as the spleen is known to rapidly sequester iron from the cytosol into lysosomes of the spleen [87]. Clustering of nanoparticles is likely to be extensive in the dietary iron loaded spleen tissue since most data required a structure factor to obtain a satisfactory fit.

The average radius of gyration for the dietary iron loaded spleen series was $28 \pm 2$ Å comparable with the liver from the same animal. Similarly, no significant difference was observed for the different organs of the same animal by TEM (Chapter 3). The radius of gyration corresponds to an average spherical diameter of $7.2 \pm 0.6$ nm which is slightly larger than the diameter of cytosolic ferritin measured by TEM in Chapter 3 of $5.51 \pm 0.75$ nm. There was no clear trend with iron loading as seen in the dietary iron loaded liver series however the range of iron concentrations achieved in the spleen was considerably smaller.

$G$ values were greater than for the control animals but less than the dietary iron loaded liver, consistent with the degree of iron loading in the tissues. The variation in $G$ values suggests that the number of iron particles rather than size of iron particles change with different degrees of iron loading.

For those samples in which dense packing was accounted for in the model, the packing distance was $8.6$ nm. Particles are packed at a similar distance as in the dietary iron loaded liver, which showed an $ETA$ of $8.4$ nm.

$Pack$ values for the dietary iron loaded spleen were very similar to those obtained from modeling the differential SAXS data from the liver of the same animals despite considerably higher hepatic iron concentrations. Qualitative observations of the shape of the scattering shoulder suggest a high degree of dense packing in the spleen. In combination these results may indicate that the proportion of iron occupied in clusters to free iron is higher in the spleen than the liver.

The scattering invariant was generally lower in the spleen than the liver consistent with the lower degree of iron loading obtained in the spleen by the dietary route. As shown in the liver, the scattering invariant showed a positive correlation with tissue iron concentration and reduced for the four animals allowed to unload following cessation of iron loading.
Transfusional iron loaded liver

The low scattering intensity from the transfusional iron loaded liver reflects the low degree of iron loading achieved in the liver following transfusions of packed red blood cells (Figure 2.1).

Qualitatively the shoulder feature was prominent but did not resolve to a clear maximum at finite $Q$ for any of the transfusional iron loaded liver samples. However, clustering of nanoparticles is likely to be significant since spatial correlation was required to produce a satisfactory fit to most of the data.

The average radius of gyration for the transfusional iron loaded liver series was $31 \pm 2$ Å comparable with tissue with similar degrees of iron loading such as control spleen. The average value was slightly higher than observed in the liver of animals loaded with iron by way of the dietary route. However, the higher values returned for the radius of gyration may be an artefact of the fitting procedure when modelling data from samples with low iron concentration as discussed in the previous chapter. In the previous chapter we hypothesised that there is more uncertainty on the radius of gyration returned from modelling data with low iron concentrations. Despite the low iron concentration, there is a clear decline of $R_g$ values with increasing iron loading suggesting that the uncertainty may exhibit as systematic rather than random errors in the modelling procedure.

$G$ values demonstrated a steady increase with iron concentration as the number of iron oxide nanoparticles increased with iron loading but did not reach the $G$ values seen in more highly loaded tissues from the dietary iron loaded series.

For the six samples in which dense packing was accounted for in the model, the mean interparticle packing distance was 7.9 nm, slightly larger than the average particle mineral core diameter for the same six samples (7.7 nm). Particles are packed at a similar distance as in the dietary iron loaded tissue with similar occupancy ($Pack = 1.5$) despite the lack of local maxima in the transfusional iron loaded liver differential SAXS data.

The scattering invariant was generally lower in the transfusional iron loaded liver than the more highly loaded tissues by the dietary path. As shown in Figure 8.8e, the scattering invariant showed a strong correlation with tissue iron concentration and reduced for the two animals allowed to unload following cessation of iron loading.
Transfusional iron loaded spleen

The high intensity of scatter from transfusional iron loaded spleen is consistent with the high degree of iron loading achieved in this tissue series (Figure 2.1). Qualitatively the shoulder feature was sharp and resolved to a clear maximum at finite $Q$ for most of the transfusional iron loaded spleen samples indicating a high degree of interparticle interference. Interestingly, ordered clustered deposits were simple to locate by TEM in the transfusional iron loaded spleen tissue (see Chapter 3).

The average radius of gyration for the dietary iron loaded spleen series was $25 \pm 2$ Å, comparable with dietary iron loaded liver tissue with similarly high tissue iron concentration. There was little variation in the radius of gyration obtained for the transfusional spleen series, which may reflect a greater certainty on fitted particle size with high iron concentrations proposed in the previous chapter.

$G$ values were consistently high in the transfusional iron loaded spleen series and the average of 11 was greater than for any other series. This observation indicates that there are a greater number of iron oxide nanoparticles in this series, consistent with the highest tissue iron concentrations seen in the transfusional iron loaded spleen.

For the seven samples in which dense packing was accounted for in the model, the interparticle packing distance was 8.6 nm, larger than the average particle diameter (6.5 nm). The $Pack$ value (2.2) was greater than for any other series which was indicated qualitatively by the prevalence of local maxima in the differential SAXS data. This high degree of occupancy explains the ease of locating clusters with a high degree of order by TEM. The $Pack$ values are higher than from the dietary iron loaded livers which achieved similar iron concentrations.

The scattering invariant was higher than for any other rat tissue series, consistent with the higher degree of iron loading achieved in the transfusional iron loaded spleen. As shown in Figure 8.10e, the scattering invariant showed a strong correlation with tissue iron concentration and reduced for the two animals allowed to unload following cessation of iron loading.
8.5 Summary

8.5.1 Quantification of iron concentration and diameter and clustering of iron oxide particles

**Tissue iron concentration**

The scattering invariant provides a measure of the amount of scattering material in the tissue, independent of shape or clustering behaviour. The invariant increased steadily with iron loading for each rat tissue series and the correlation between invariant and tissue iron concentration is clear in Figure 8.10e. The invariant provides the best measure of the amount of iron in each rat tissue sample of the modelled parameters and iron concentration could be estimated in mg.g⁻¹ dry weight from the relationship:

\[
Fe_{\text{conc}}[\text{mg.g}^{-1}\text{dryweight}] = \frac{Q_{\text{INVARIANT}}[\text{cm}^{-1}\text{Å}^{-3}]- 1.2 \times 10^4}{2.5 \times 10^{-5}}
\]

**Iron oxide particle size**

As has been discussed, the modelled radius of gyration has a tendency to decrease with tissue iron concentration. It is our preferred interpretation that the decrease with iron loading is an artefact of the fitting procedure owing to the weaker signal at low iron concentrations. The most likely radius of gyration for iron oxide nanoparticles in rat tissue is then 25 ± 1 Å, the mean (± SD) measured from tissues with an iron concentration in excess of 40 mg.g⁻¹ dry weight. This radius of gyration corresponds to a spherical diameter of 6.4 ± 0.3 nm which is slightly larger than cytosolic ferritin diameters measured by TEM (5.27 ± 0.83 nm) and consistent with the literature summarised in Table 1.1. As shown in Table 1.1, cytosolic ferritin cores are smaller than lysosomal ferritin so it is likely that a larger particle size would be found with the inclusion of all ferritin cores in the tissue.

**Clustering behaviour**

The ETA value, which provides an estimate of the distance between layers of packed spherical nanoparticles, is varied at low iron concentrations and tends towards more consistent values with iron loading (Figure 8.10c). The variation at low iron concentrations may be due to greater biological variability with low iron concentrations or may be an artefact of the fitting procedure owing to the weaker signal at low iron
concentrations. In any case, we suggest the ETA would be best indicated by the average measured from tissues with an iron concentration in excess of 40 mg·g⁻¹ dry weight, 86 ± 3 Å. Effective d-spacings of 8.6 ± 0.3 nm are smaller than the ferritin protein shell of 12 nm indicating the particles are closely packed. Such packing distances are supported by TEM observations of approximately FCC clustering behaviour (Chapter 3). The estimated packing distances of 8.6 ± 0.3 nm would indicate a FCC unit cell of 17 nm, the same as measured from fast Fourier transforms of TEM images. The unit cell lies within those observed for FCC crystals of ferritin by x-ray diffraction, that is 15.8 nm for air dried [65] and 18.4 nm for wet preparations [101]. The difference from the wet crystal unit cell may be attributable to dehydration during freeze-dried preparation of samples.

8.5.2 Duration & degree of iron loading

The fitted parameters against degree of iron loading are summarised for the six sample series in Figure 8.10. In light of TEM results reported in Chapter 3 and magnetic measurements referred to in the previous chapter, the decreasing trend in $R_g$ with iron concentration is likely an artefact of the fitting procedure. This interpretation indicates that there is no appreciable change in particle size with degree or duration of iron loading. The number of iron oxide nanoparticles deposited in the tissue increases with degree and duration of iron loading to account for the higher tissue iron concentrations observed in the older animals. It is known that oxidative stress induces synthesis of ferritin [193]. The present study would suggest that ferritin shells do not appear partially filled and that synthesis of the shell, or formation of the core, is an all-or-nothing response.

There is no significant difference in effective d-spacings from ETA values between the samples. Although the results are scattered with iron loading, the distances approach more consistent values with increasing tissue iron concentration. Pack values do not demonstrate any global correlation with degree of iron loading. It is therefore unlikely that clusters of particles compress or become more occupied in order to accommodate increasing iron concentration. Alternatively, the number or size of clusters must increase with iron loading.

Scattering invariant shows a strong correlation with degree of iron loading and provides a good indication of the tissue iron burden. The scattering invariant with iron concentration shows striking similarity to the intensity measured at 0.065 Å⁻¹ with iron
concentration in Figure 6.5. Albeit crude, measuring the absolute intensity of scattering at $Q = 0.065 \ \text{Å}^{-1}$ from rat tissue would provide a good indication of tissue iron concentration.

8.5.3 Tissue specificity

The differences between the organs discussed throughout Section 8.4 are attributable to iron concentration differences between the tissues. Differences in radius of gyration are likely due to the different signal strength in each case and as such there is no clear tissue specificity in the size of iron oxide deposits in rat tissue.

G values which reflect the number of iron oxide particles, clearly increase with tissue iron concentration (Figure 8.10) and vary from liver to spleen on account of the different iron loadings in the organs. Variation in the estimated packing distance, ETA, again is more dominated by iron concentration and no clear difference between the liver and spleen is observed. Differences in the invariant between the liver and spleen, as tabulated in Table 8.1 are a result of differing iron concentrations between the series.

For the control series, the liver and spleen display different clustering behaviour. Since interparticle correlation improves the fit to the spleen differential SAXS data, the iron is contained in clusters in the control spleen. Dispersed iron is the dominant form of iron in the liver from the same control animals. It should be remembered that the spleen contains some 5 times the iron concentration of the liver, and the difference may simply be a result of the higher splenic iron burden.

It is worth noting that the $Pack$ values obtained for the transfusional iron loaded spleen are generally greater than for the dietary iron loaded liver despite similar tissue iron concentrations. For tissue iron concentrations in excess of 40 mg.g$^{-1}$ dry weight the transfusional spleen returns $Pack$ values of 2.4 ± 2 compared to dietary liver $Pack$ values of 1.4 ± 0.6. In addition, the dietary spleen achieved similarly occupancy to the liver from the same animal, despite considerably lower splenic iron concentrations. The higher occupancy observed for the spleen may be a consequence of the distribution of iron in particular cell types in the spleen. Only the red pulp is available for iron storage [86] so the iron concentration in these cell types is likely to be in excess of the average tissue iron concentration. Occupancy may increase in order to accommodate the high local iron concentration in the red pulp. In addition, the clustering behaviour may differ in the liver and spleen as a consequence of the different proportions of macrophages. Macrophages such as Kupffer cells in the liver and reticuloendothelial cells in the
spleen serve to remove debris or foreign material from the circulation and sequester the material into lysosomes. The liver contains only about 15% Kupffer cells capable of clustering whereas the primary function of the spleen is sequestering debris in its many reticuloendothelial cells. Increased occupancy in the spleen may then be a consequence of the prevalence of lysosomes with the ability to segregate iron into clusters.

8.5.4 Iron loading pathway

The control, dietary and transfusional iron loading pathways result in the accumulation of different iron burdens in their respective tissues as shown in Figure 2.2. As a result, differences in the concentration dependent parameters $G$ and $Q_{invariant}$ occur between the series.

Differences in particle size between the control, dietary or transfusional routes were observed from the fitting results. Larger radii of gyration were returned for control fits than for the dietary and transfusional series. Larger particle size is consistent with a greater proportion of full ferritin cores to the generally smaller haemosiderin cores in the control animals. However, in a separate study magnetic energy barrier distribution measurements failed to reveal any change in particle size with iron loading (Figure A1, Appendix). On balance, we prefer the interpretation that differences between the control and iron loaded series occur owing to the different iron scattering signal strength.

Clustering does not occur to an appreciable extent in the control series given that all data could be fitted without spatial correlation. Correlation improved the fit for the control spleen, but cluster occupancy, estimated by the $Pack$ value, was still lower than for the dietary and transfusional series.

Inspection of the transfusional data in Figure 8.8 and Figure 8.9 show more uniformity in the fitted parameters than for the control or dietary loaded series (Figure 8.4, Figure 8.5, Figure 8.6 and Figure 8.7). The processing of dietary iron is thought to be more rapid and uncontrolled than the processing of transfusional iron by the reticuloendothelial system. It has been proposed that the differing mechanisms may result in a more disordered nature of iron deposition by dietary routes [94]. It is possible that the uniformity in the model parameters with iron loading observed for the transfusional series is attributable to less biological variability and more order in processes of dealing with iron loading from the transfusional pathway. However, it
should be noted that the strength of correlation may be a consequence of the fewer samples in the transfused iron loaded series.

8.5.5 Cessation of iron loading

Following the cessation of iron loading in the dietary and transfusional series, the invariant and $G$ reduce with the iron concentration. There is a reversal of the decreasing trend of $R_g$ with iron concentration and fitting begins to return slightly higher values for the particle size. An increase in average particle size would be consistent with preferential mobilisation from haemosiderin cores, smaller than ferritin cores [73-75]. However, we prefer the interpretation that the increase in radii is an artefact as a result of the decreasing signal strength. As such, the results suggest that the number of particles, rather than size of particles, reduce as the tissue unloads.

No significant differences in clustering were observed from the spatial correlation parameters, $ETA$ and $Pack$ following cessation of iron loading. This would serve to suggest that the size (or number) of clusters reduces as the tissue unloads, rather than the occupancy of clusters.
8.6 Conclusions

Small angle x-ray scattering can be used to assess the degree of iron loading, the iron oxide particle size and clustering behaviour in bulk iron loaded mammalian tissue.

Results from the most highly loaded rat liver and spleen tissue indicate that the most likely particle diameter for spherical iron oxide deposits is 6.4 ± 0.3 nm. The distance between planes of packed spheres is 8.6 ± 0.3 nm.

There is no appreciable difference in particle size with iron concentration. As such, the number rather than size of iron oxide deposits increases with increasing tissue iron burden.

There is no significant difference in the nature of clustering behaviour with degree of iron loading for the iron loaded tissue samples. The packing distance and occupancy in clusters remains unchanged with increasing tissue iron concentration. Therefore, the number or size of micron scale clusters must increase to accommodate the increasing iron storage requirements of heavily iron laden tissue.

There are no significant differences between the liver and spleen of the same animals that could not be accounted for by the difference in iron concentration between the tissues. Nor was there any significant difference between tissues from the dietary or transfusional series which could be attributed to the iron loading pathway.

Cessation of iron loading resulted in a decrease in the number of iron oxide nanoparticles deposited in the tissue rather than a change in particle size or clustering behaviour.

The iron oxide particle size and clustering behaviour remained remarkably similar under the wide variety of iron loading conditions in this study. As such, the primary determinant of the size and packing distance of iron oxide cores in rat liver and spleen tissue is most likely the ferritin protein shell.
Conclusions and Further Work

9.1 Final conclusions

The particle size and clustering behaviour of iron oxide particles in iron loaded mammalian tissue have been investigated. With a view to quantitatively characterising particle size and clustering in situ in bulk tissue samples, transmission electron microscopy (TEM) and small angle x-ray scattering (SAXS) techniques were employed. The liver and spleen of control, dietary iron loaded and transfusional iron loaded rat sample series were studied to examine the physical form of iron cores deposited under a variety of iron loading conditions.

Iron oxide cores of the iron storage compounds ferritin and haemosiderin were imaged in unstained ultrathin sections of rat tissue by TEM. Three types of clustering behaviour could be identified from the radially averaged fast Fourier transform (FFT) of TEM images. Particles were dispersed, clustered together, or clustered together in close packed arrays. Measurement of dispersed cytosolic ferritin cores yielded spheres with a mean (± SD) particle size of 5.27 ± 0.83 nm. The iron cores from different samples were remarkably similarly sized despite a wide range of iron loaded states. No global correlation was found between diameter and tissue iron concentration or organ type. The effective d-spacings of arrays of clustered particles were measured from the radially averaged FFT of digital images to be 9.0 ± 0.5 nm. No significant difference was found between the different iron loading states. The d-spacings and locations of spots in the FFT were consistent with ferritin protein shells of 12 nm comprising a face centred cubic (FCC) unit cell of dimension 17 nm.

The mean particle size and effective d-spacing of clustering of iron oxide cores has been investigated in the bulk by SAXS. The preferred method of sample preparation for bulk tissue samples was freeze-drying, followed by grinding to a fine powder and compacting to a pellet. The sample pellets were homogeneous and representative of the bulk. Scattering from iron oxide deposits was compounded by tissue structure and complex contrast variation near the iron K-edge was required to elucidate the origin of features.
Beamline 15-ID-D at the Advanced Photon Source was investigated for suitability to perform anomalous SAXS (ASAXS) near the iron K-edge for the first time. Accurate sample transmission data is required at all energies and the transmission detector was found to contribute the greatest uncertainty to scattering intensity by ASAXS. On the strength of these results, the transmission detector arm has been replaced which will benefit future experiments on the beamline. Iron concentrations in excess of 2 mg.g\textsuperscript{-1} dry weight were significant enough to observe ASAXS despite the limitations of the transmission detector.

Modelling of the iron specific scattering data with the Unified scattering function yielded mean particle size and quantified clustering behaviour of spherical iron oxide nanoparticles in the bulk tissue samples. As such, ASAXS from pellets of freeze-dried ground tissue is a suitable technique for investigating iron oxide deposits \textit{in situ} in bulk specimens of iron loaded mammalian tissue. Particle size and clustering behaviour of ferritin and haemosiderin \textit{in situ} in bulk specimens are reported quantitatively for the first time.

ASAXS from the most highly loaded rat liver and spleen tissue indicate that the most likely particle diameter for spherical iron oxide deposits is (mean ± SD) 6.4 ± 0.3 nm. This measurement compares favourably with TEM data which are limited to measuring small (~200) numbers of cytosolic ferritin. A decreasing trend in radius of gyration with iron concentration was attributed to artefacts of fitting increasing signal strength. As such, no appreciable difference in particle size with iron concentration, iron loading pathway or organ type could be identified. The scattering invariant which reflects the iron volume fraction displays a linear increase with iron concentration. As such, the number rather than size of iron oxide nanoparticles increases with iron loading to accommodate the iron burden.

The scattering data indicated spatial interference between particles of the iron loaded tissue whereas spatial interference was less significant for the control tissue. Since spatial correlation was identified for clusters with ordered regions by FFT of TEM images, these data indicate that close packed arrays of iron oxide particles are extensive in iron loaded but not control tissue. The most likely distance between planes of packed spheres from the iron specific scattering data was of 8.6 ± 0.3 nm. Again, no significant change in packing distance could be observed with iron loading. The effective $d$-spacings observed by ASAXS are consistent with TEM data and a FCC unit cell of dimension 17 nm. The unit cell dimension and occupancy revealed no significant
change with iron loading, therefore the number or size of micron scale clusters of iron laden cellular organelles must increase to accommodate the increasing storage requirements of heavily iron loaded tissue.

The sample volume investigated by ASAXS in this study was some 9 orders of magnitude greater than by TEM. The mean (± SD) diameter of spherical iron oxide particles was similar by each method at 5.27 ± 0.83 nm for TEM and 6.4 ± 0.3 nm for the ASAXS bulk method. The effective d-spacings in clustered deposits were objectively measured at 9.0 ± 0.5 nm from FFT of TEM images which compares well with 8.6 ± 0.3 nm obtained by modelling ASAXS data.

Putatively, a wide number of biological and environmental factors can influence the physical form of iron oxide deposits. These observations have chiefly been made by TEM of iron oxide particles extracted from control and iron loaded tissues. Subjectivity and small sample volumes are complications of measurement by TEM. Furthermore, the process of isolation may bias the measured core size. The iron oxide particle size and clustering behaviour remained remarkably similar under the wide variety of iron loading conditions in this study. As such, the primary determinant of the size and packing distance of iron oxide cores is most likely the ferritin protein shell.

9.2 Further work

Rat tissue structure

Several structure peaks were identified in the x-ray scattering data from rat tissue in this study. Since the peaks were not attributable to iron structure, further investigation of the peaks was beyond the scope of this study. It was suggested that the peaks may be due to regular ordering of fatty tissue. Lipid extraction followed by SAXS was suggested to determine if the peaks are indeed due to fat. If the peaks are fat related, the SAXS method may present a suitable technique to investigate fatty liver disease, the most common liver disorder in the western world.

Preliminary analysis did not show any relationship between iron loading and the positions or width of tissue structure peaks in this study. However, in principle it is possible to identify changes in tissue structure accompanying iron overload. Limited fibrosis and cirrhotic change have been observed in animal models for the human iron overload conditions. With the exception of the baboon, animal models have not been
able to emulate the structural change known to affect the human tissues [87, 194]. A study of iron loaded human tissues by SAXS may contribute to our understanding of tissue structural change in the disease state.

**SAXS for diagnosis of disease**

The small angle x-ray scattering invariant showed a strong correlation with tissue iron concentration in this study and could be used to assess iron burden in the tissue. The technique is non-destructive but does not offer any other significant advantages over chemical analysis of biopsy samples in the diagnosis of iron overload or fatty liver in humans. Recently, non-invasive techniques for assessing the total body iron burden have been developed to replace biopsy. In particular, magnetic resonance imaging has attracted much attention [22, 195-199]. The development of SAXS apparatus capable of *in vivo* measurements would be of great interest as another non-invasive diagnostic tool. Progress has been made in this regard with several groups investigating diagnosis of breast cancer by SAXS [164-167].

**Iron loading systems**

Small angle x-ray scattering could be used to study iron oxide particle size and clustering behaviour in other organs or species. Preliminary SAXS data have been acquired from some hypotransferrinaemic mice, a proposed animal model for haemochromatosis [118, 200]. Some scattering data have also been collected for Belgrade rats that underwent an iron deficient diet which may be of interest to study the underlying tissue structure in the absence of significant iron stores (see Figure A2, Appendix).

Some studies have reported larger particle sizes for iron oxide deposition in the iron loaded heart [71, 90]. Measurement of the particle sizes in the bulk by SAXS would be of interest in this tissue. In addition, observation of a difference by ASAXS may give further indication of the resolution afforded by this technique.

**Preferential mobilisation of iron from smaller particles**

The literature indicates that with chelation therapy, iron is preferentially mobilised from smaller iron oxide cores [74]. Only a small number of rats in the current study were allowed to unload following cessation of iron loading so the ASAXS data were unable to confirm these observations. Further study of iron depleted tissue would be of interest.
Ultra small angle x-ray scattering

Ultra small angle x-ray scattering (USAXS) probes fluctuations in electron density on nanometre to micron length scales. With USAXS one may expand on the current study to investigate the size and number of clusters deposited in the iron loaded tissues. The current study indicated that the size or number of clusters must increase with iron loading since the particle size and cluster packing do not change. Fusion of micron scale iron laden siderosomes to larger clusters has been observed [201]. The distribution of iron on the micron length scale is organ specific and has been observed by optical microscopy to change with iron loading [116]. Preliminary USAXS data have been collected (see Figure A3, Appendix) and may contribute to the quantification and understanding of any differences in the number and size of clusters with iron loading, or between organs.

X-Radiography

Optical and electron microscopy have both identified an uneven distribution of iron across the iron loaded tissue. Iron deposition is conspicuous in periportal and pericanalicular areas of the liver with fewer deposits in the lobular tissue [86]. Further differences in clustering are observed from different cells of the liver. With hepatic iron loading, siderosomal deposition becomes prominent in Kupffer cells which form clumps around the liver [116]. Splenic deposition is generally limited to the reticuloendothelial cells of the red pulp with very few dispersed particles located in the white pulp [86].

X-Radiography may present an opportunity to study the variation of iron deposition across sections of the tissue. Magnified radiographs were obtained on a mammography unit and are shown in Figure A4 of the Appendix. The intensity of the radiographs appears to increase and become more mottled with iron loading. Preliminary texture analysis indicated a more even distribution in the liver. In the iron loaded spleen, the iron deposits are clearly mottled on the scale of red pulp to white pulp variations. Further, the spleen appears to show a “crust” of poor iron deposition around the organ surface. Texture analysis of the radiographs may present an opportunity to quantify the variation in tissue iron on the micro to millimetre length scale in the tissue.

Scanning SAXS

Scanning SAXS involves collecting SAXS data at various positions across a section of a sample to gain position resolved information regarding the structure of the material.
The resolution is generally defined by the beam size so can reveal changes in structure over distances of a few 100 nm. The technique has been used extensively to study local variation of bone composition and structure of wood [156, 159, 202-204] and offers potential in the study of iron overload.

To complement the radiography, scanning SAXS would provide useful information regarding the uneven distribution of iron across sections of iron loaded tissue. There was some indication in this study to support a local increase in unit cell occupancy in the spleen since tissue concentrations in the red pulp are expected to exceed that of the whole tissue. The current study indicates that in bulk samples there are no differences in particle size and clustering behaviour from organ to organ within a rat, or between animals with different tissue iron loading. With scanning SAXS, local variation within the tissue may be quantified. Some scanning ASAXS data have been obtained and a 2-D map of the distribution of iron oxide nanoparticles in an iron loaded spleen has been generated (Figure A4, Appendix).

9.3 Closing remark

It is envisaged that small angle scattering techniques and quantitative transmission electron microscopy will continue to play an important role in the investigation of iron oxide deposits in iron overload diseases.
References


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Cookson, D SAXS / WAXS: Camera Setup.


Figure A1: Iron oxide particle diameter (mode) in dietary iron loaded rat liver tissue obtained from magnetic energy barrier distribution measurements. The full width half maximum (FWHM) of the energy barrier distribution is also shown for each rat sample (from [191]).
Figure A2: Radially averaged SAXS from pellets of freeze-dried ground Belgrade rat liver and spleen.

Figure A3: Preliminary USAXS from control and iron loaded rat liver and spleen tissue. The shoulder feature at 0.05 – 0.08 Å⁻¹ is visible. Abbreviations: control (CR), dietary iron loaded (R), transfusional iron loaded (TR), liver (Li), spleen (SP).
Figure A4: X-radiographs of 1 mm thick sections of wax embedded liver and spleen tissue from control and iron loaded rats. Intensity has been inverted such that dark regions are electron dense. The scale bar is 1 cm. For each rat the liver section is on the left and spleen on the right. The transfusional iron loaded spleens show a particularly uneven distribution of iron and a "crust" of iron appears around the perimeter. Samples marked with an asterisk were allowed to unload following cessation of iron loading.

Figure A5: Images of a 1 mm thick section of spleen tissue from a transfusional iron loaded rat sacrificed at 12 months a) x-radiograph b) 2-Dimensional scanning SAXS map from a 2 mm square region of the sample. The scattering intensity is proportional to electron density fluctuations over the nanometer range. One pixel in the SAXS map corresponds to a 100 x 100 μm square.