Magnetic resonance study of the spider Nephila edulis and its liquid dragline silk

Author(s):
Hronsky, Monika

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Magnetic resonance study of the spider *Nephila edulis* and its liquid dragline silk

A dissertation submitted to the

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presented by

MONIKA HRONSKY
Dipl. Natw. ETH
born on the 31th August 1974
citizen of Volketswil, ZH

accepted on the recommendation of

Prof. Dr. Beat H. Meier, examiner
Prof. Dr. Peter Bösiger, Prof. Dr. Markus Rudin, co-examiner

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Abstract

This thesis concerns the investigation of the native liquid dragline silk of the spider *Nephila edulis* by Nuclear Magnetic Resonance (NMR). It leads from a description of in-house breeding of spiders and the harvesting of silk samples, through NMR–spectroscopic analysis of native and immobilised silk dope to in–vivo studies of the anatomy and physiology of the spider and its silk production by Magnetic Resonance Imaging (MRI).

Spider silk is spun from a liquid, but very viscous and highly concentrated, aqueous protein solution to virtually insoluble fibers at low pressure and ambient temperature. A detailed molecular model both of the liquid silk and the spinning process may be the key to the biomimetic production of natural or engineered silk. While great progress has been made towards a full characterisation of the structure and properties of the solid fibre, liquid silk dope and the intermediate phases formed during spinning remain poorly understood. Because NMR spectroscopy offers a wealth of methods to study the structure and function of biomacromolecules on both molecular and macroscopic levels, MR techniques were applied to study the native liquid silk within the lumen of the wide sac of the major ampulla (m.a.) silk gland.

The analysis of native silk dope at room temperature by slow magic–angle sample spinning (MAS) was shown to lead to a considerably enhanced resolution compared to previously described spectra, and thus allowed an almost complete assignment of the $^{13}$C and the $^1$H spectra. This reduction in linewidth was ascribed to the averaging of susceptibility effects arising from the inhomogeneous sample distribution within the rotor, rather than to residual dipolar couplings or chemical–shielding anisotropy (CSA).
Abstract

The absence of CSA and dipolar couplings, together with the chemical-shift information obtained from these spectra, demonstrates conclusively that the native silk protein is dynamically disordered throughout the molecule, in the sense that each amino acid of a given type is subject on average to an identical environment, and that the viscous silk dope forms an isotropic liquid phase.

To study possible local ordering among the silk molecules in the absence of dynamic averaging, native silk dope was immobilised using a shock-freezing technique. Double-quantum spectroscopy (DOQSY) experiments performed at low temperature (8 K) together with an isotopic labelling strategy gave access to the backbone torsion angles ($\phi$, $\psi$) of glycine-glycine pairs. The distribution of dihedral angles among these pairs indicated that glycine-rich domains within the immobilised silk dope do not contribute to a well-defined structure, and thus support the results from the NMR spectroscopic work performed on native liquid spider silk at room temperature.

Promising studies were performed by applying MRI, a noninvasive, in-vivo tomography, to Nephila edulis. Unique, three-dimensional images of high spatial resolution were obtained within a relatively short timeframe, providing hitherto unavailable insight into spider phenotyping. Both static experiments, revealing anatomical and physiological details, and real-time imaging of the spider during silking were performed. The application of an aqueous contrast agent permitted to highlight the intestine and other organs involved in digestion and food storage and to monitor its dispersion within the body. These experiments yield valuable information unavailable from classical (bio)chemical and biological techniques, and provide a new basis for a wealth experiments to answer relevant biological questions concerning digestion, silk production and the mechanism of venom ejection in spiders.

Further, the experiments monitoring in real time the changes within the m.a. gland which occur during the silking process required a motor-drive system capable of operating in the magnetic field of the NMR spectrometer. A diametrally-magnetised DC electrical motor was modified for this purpose, essentially by replacement of most metallic parts, while the magnetic field of the spectrometer took the place of the static permanent magnet. The response of the motor was characterised in full with a view to its further application in time-dependent MR studies of the silking process or positioning, reorientation and spinning of NMR samples inside the bore of the magnet.
Zusammenfassung


Spinnenseide wird aus einer wässerigen und dennoch recht zähflüssigen, hoch konzentrierten Proteinlösung zu praktisch unlöslichen Fäden gesponnen. Dabei benötigt das Tier weder hohe Temperaturen noch extremen Druck, wie dies zur Herstellung künstlicher Polymere nötig ist. Um den natürlichen Produktionsprozess von Seide technisch nachahmen zu können, ist detailliertes Wissen auf molekularer Ebene über die flüssige Seide und den Spinnvorgang von zentraler Bedeutung. Obwohl bereits viel über die Struktur sowie die chemischen und physikalischen Eigenschaften der vom Tier gesponnenen Seidenfäden bekannt ist, blieben die Flüssigkeit und ihre Zwischenformen, wie sie während des Spinnvorgangs auftreten, sowie der Spinnvorgang selbst, nur sehr schlecht verstanden. Die NMR Spektroskopie bietet eine Vielzahl an Methoden zur strukturellen und funktionellen Charakterisierung biologischer Makromoleküle, sowohl auf molekularer wie auch auf makroskopischer Ebene. Mit Hilfe einiger dieser Methoden wurden die native flüssige Seide innerhalb des Lumens der Vorratskammer der major ampulla (m.a.) Drüse untersucht.

Um allfällige lokale Ordnung innerhalb der Proteine zu beobachten, welche bei Raumtemperatur auf Grund dynamischer Ausmittlung hier nicht erfasst werden konnte, wurde die native flüssige Seide imobilisiert. Dies geschah mittels schockartigem Einfrieren in flüssigem Stickstoff. Die daran bei 8K durchgeführten Doppelquanten Experimente (Double-quantum spectroscopy, DOQSY) lieferten zusammen mit der geeigneten Isotopenmarkierung Informationen über die Diederwinkel ($\phi$, $\psi$) von Glycin–Glycin Paaren. Die statistische Verteilung dieser Winkel weist darauf hin, dass die glycinreichen Domänen der imobilisierten Seidenproteine zu keiner klar definierten Struktur beitragen. Somit stützen diese Resultate die Ergebnisse der vorgängigen Messungen bei Raumtemperatur.

Experimente zur Beantwortung wichtiger biologischer Fragen im Zusammenhang mit Verdauung, Seidenproduktion und Giftausstoss der Spinnen.

1 Introduction

The spectrum of thoughts, feelings and reactions associated with the word spider is broad: most people are frightened or nauseated by these hairy, eight-legged animals without being able to explain the reason for such negative feelings. Many others think of cultural icons such as the movies Spiderman, where a superhero with spider-like power protects the people, Arachnophobia, where impenetrable curtains of webs are capable of capturing small birds, or Tarantula, a 1955 horror-thriller starring a giant, killer tarantula. Others still think about Greek mythology, where the weaver Arachne turned into a spider, or the spider-shaped ice-field on the North face of the Eiger [Fig. 1.1].

A small minority of people think about spiders as producers of a silk with extraordinary mechanical properties. Spider silk combines contrasting characteristics of other man-made materials, namely the elasticity of rubber [1, 2], the strength of steel and the toughness of Kevlar, while still being as lightweight as nylon [3–5]. Furthermore, spider silk is biocompatible [6] and is spun from an aqueous solution which avoids all the toxic solvents used in preparing conventional polymers.

Spinning silk from liquid dope to virtually insoluble fibers, as mastered by many insects and spiders, is still a poorly understood process. The gene sequence of silk has been described [7, 8] and considerable progress has been made quite recently towards a full characterisation of the spinning process [9–11], several authors suggesting a number of partially folded intermediate proteins, some of which form liquid-crystalline phases. Nevertheless, a detailed molecular model for either liquid silk dope or its changes during the spinning process are still missing. Because the extraordinary properties of solid spider silk lie not only in its protein constituents but also in its manner of processing, such a
Introduction

Figure 1.1: Spiders in popular culture: common associations with the word "spider" include films (Tarantula (1955), Arachnophobia (1990), Spiderman (2002)), sticky webs to be cleaned away, Greek mythology in which the weaver Arachne is turned into a spider and the infamous ice-field on the upper North Face of the Eiger in the shape of a spider. Images reproduced with permission (see picture credits in App. D).

molecular–level understanding may be the key to the biomimetic production of natural or engineered silk. At present, however, technical processes remain unable to produce that which is so easily manufactured and controlled by the spider. Better knowledge both of liquid silk dope and of the spider’s spinning process is a prerequisite for commercial production of man–made spider silk.

To provide a brief summary of these opening remarks, the spider stores its silk as a liquid but spins it to a solid fibre. The structure of this thread has already been investigated in detail, and there exist various models purporting to explain it. In contrast, very little is known about the liquid: what is the protein (are the proteins), and how does it
1.1 Silk

1.1.1 What is silk?

Silk is a natural protein fibre produced by a number of animal species, including caterpillars, spiders and mussels, for a variety of purposes. Silk proteins are produced and stored as an aqueous solution within specific glands. Alanine and glycine make up to 50% of the amino acids used in the silk protein [12]. Many types of silk show a remarkably repetitive primary structure [7,8,13-17]. The solid, partially crystalline and water-insoluble fibre is spun by the animal from this liquid dope by gradually increasing the concentration of the solution and by applying elongational stress. The spinning process leads to a thread with a diameter of 1—50 μm, thinner than a human hair (ca. 100 μm).

Although many species of silk-spinning worms and insects are known, to date only the silk spun by the larvae of silk moths, in form of threads which form a cocoon, is of commercial use [18]. Silk woven into textiles is obtained primarily from the cocoon of the domesticated mulberry silkworm larva, Bombyx mori, by a process known as sericulture, during which the larvae are killed [19,20]. Other forms of domestic silk include that produced by the tussah worm in India and China, which feeds on oaks and spins a coarser, flatter, yellower filament than Bombyx mori. Wild silks produced by a number of undomesticated silkworms differ in one major respect from the domesticated varieties: the cocoons, which are gathered in the wild, have usually already been chewed through by the pupa, and thus the single thread of which the cocoon is made has been cut into shorter lengths and is of no industrial use. In addition to the use of silk as a luxury fibre for clothing and other handicrafts, it has also found applications in items such as parachutes, bicycle tyres and surgical ligatures and non-absorbable sutures [21–25]. Medical doctors
have used silk to make prosthetic abdominal arteries in dogs [26]. Early bulletproof vests were also made from silk in the era of gunpowder weapons (prior to World War I) [27].

Figure 1.2: Images from silk production. The cocoon spun by the larvae of the moth *Bombix mori* is made of one silk thread. This thread is unwound from the cocoon during the sericulture process and is gathered as raw silk bundles. Images reproduced with permission (see picture credits in App. D).

The properties and appearance of different silks vary strongly according to the biological function for which they were produced: some silks are strong, some are brittle and others are extremely elastic. This difference in properties is found not to be determined simply by the primary protein structure. Many silks with very similar amino-acid sequences show entirely different mechanical properties, while other silks differing strongly in their primary structure can have almost identical behaviour [28, 29].

Silk from the silkworm *Bombyx mori* has many properties which contribute to its reputation as a luxury fibre [30]. Silk fabrics may absorb up to one third of their own
weight in water without feeling wet to the touch, and will remain warm despite their lightness. Although the density of silk is low, it is one of the strongest natural fibres known, and is at the same time far more elastic than either cotton or linen. Its strength is primarily tensile, meaning that it can withstand extreme pulling forces without breaking — indeed its toughness is comparable to Kevlar or steel. This combination of low density, high elasticity and strength leads to a very high energy requirement to break a unit of mass. A report from Arizona in 1887 with regard to the toughness of silk refers to a medical doctor who had noticed that a silk handkerchief was undamaged when its owner was shot in a duel [31]. This was the basis for the first bulletproof vests made from silk in the era of gunpowder weapons [27].

Silk is thus a fascinating material with outstanding properties, but it does possess in addition certain negative attributes. It is a poor conductor of electricity, which is the origin of the reputation of silk fabrics for “static cling” in dry atmospheric conditions. It becomes brittle with age and exposure to sunlight, and is sensitive to the salts in human perspiration. It is harmed by high alkalinity, and by acidic or oily soils.

1.1.2 History of silk

Silk was first used in early China, around 5000 BC [32]. Although initially reserved for the emperors of China, its use spread gradually throughout Chinese culture, both socially and geographically. From these origins, silken garments began to arrive in many regions of Asia, and because of its texture and lustre, silk rapidly became a popular luxury fabric in the many areas accessible to Chinese merchants. Silk cloth refracts incoming light at different angles, lending the material the shimmering appearance for which it was prized. Because the emperors of China kept the knowledge of sericulture secret from other nations, China maintained a monopoly on its production. The high demand for the fabric made silk one of the staple items of international trade prior to industrialisation and contributed to the existence of the network of routes now known as “the Silk Road” [Fig. 1.3]. Contrary to common assumption, the Silk Road was not established solely for the purpose of trading in silk; many other commodities were also traded, from gold and ivory to exotic animals and plants. However, of all the precious goods crossing Central Asia, silk was perhaps the most remarkable for the people of the Occident. As this new material
became very popular in Rome for its soft texture and attractive appearance, the Romans sent their own agents to obtain silk at prices lower than those set by Asian merchants. For this reason the trade route to the Orient was seen by the Romans primarily as a route for silk rather than for the many other goods which were traded [33]. In fact the term “Silk Road” originates not from the Romans, but was coined by the nineteenth-century German scholar Ferdinand von Richthofen [34].

In spite of their secrecy, the Chinese were destined to lose their monopoly on silk production, and silk reached the West through a number of different channels [35]. Sericulture was established in Korea around 200 BC, with the arrival of waves of Chinese immigrants. By the 4th century AD, sericulture had travelled westward and the cultivation of the silkworm was established in India. It is said that in 440AD when a Chinese princess married the king of Khotan, an oasis north of the Tibetan plain, she smuggled both silkworm cocoons and the seeds of the mulberry tree on which they feed in her voluminous hairpiece. Silk spread further west by similar ploys. Around 550AD, two Persian monks visiting Khotan hid silkworm cocoons in their hollow bamboo staves, subsequently delivering the foundations of silk cultivation to the Byzantine emperor Justinian I. The Byzantines were as secretive as the earlier Chinese, and a silk industry was established in the Middle East which undercut the market for ordinary-grade Chinese silk. However, high-quality silk textiles, woven in China especially for the Middle Eastern market, continued to bring high prices in the West, and trade along the Silk Road therefore flourished as ever [36].

Only in the 13th century, at the time of the Second Crusades, did silk production begin in Italy. Italian silk was so popular in Europe that François I invited Italian silkmakers to France to found a French silk industry, particularly in Lyon. By the 15th century, France and Italy were the leading manufacturers of silk in Europe. Religious persecution later caused large groups of skilled Flemish and French weavers to flee to England, and an industrial complex for silk weaving developed at Spitalfields in London around 1620. Silk cultivation was introduced to the American colonies at this time by James I of England, but no indigenous silk industry, comparable to those in Europe or Asia, was established. The advent of global ocean travel at this time, and the accompanying development of worldwide trade routes ensured that silk from existing production sources became widely available. Thus this status quo was largely maintained until the World Wars of the 20th century interrupted the silk trade from Japan and silk prices increased...
Figure 1.3: The “Silk Road” is the name given to the ancient route(s) followed by silk traders from China through Central Asia and the Middle East to the Mediterranean trading ports (ending primarily, in different eras, in Rome and Venice). Inserted images indicate typical clothing from the Liao (916-1125 AD) and Ch'ing (mid-19th century) Dynasties. Images reproduced with permission (see picture credits in App. D).

dramatically. The search of western, and particularly US, industries for substitute fibres led to the development of nylon and synthetic silks [37].

After World War II, silk leadership in Europe swung from Lyon, France, back to Como in Italy. Silk became a speciality product as new synthetic fibres captured many former silk markets, and Japan became the world’s major supplier of raw silk. In the late 1970s Chinese production was increased dramatically, and China has again become the world’s leading producer of silk. World silk production has approximately doubled during the last 30 years in spite of its replacement in some uses by synthetic fibres. At the time of writing, China and India arc the two main producers, together manufacturing more than 60% of annual world production (China 54%, India 14%, Japan 11%) [38].
1.1.3 Spider silk

The importance of spider silk is reported in a story from approximately 3060 years ago, where changes in the course of history were ascribed to it. David was being pursued by King Saul when he hid in a cave near Jerusalem. A spider made its web across the opening. When Saul saw the web, he called his men away, saying that it was useless to search the cave because the web showed that no one could have entered. Thus was David's life saved, and he lived on to become King of Israel [39]. King Robert the Bruce of Scotland, similarly hiding in a cave from English pursuers, was motivated by the persistence of a spider he watched weaving its thread to continue his struggle, eventually defeating King Edward of England in battle [40].

In addition to these old myths, more prosaic known applications of spider silk include Polynesian fishermen using the thread of the golden orb-web weaver Nephila as fishing line, and some tribes in New Guinea using webs as hats to protect their heads from rain [41].

In 1709 the Frenchman Bon de Saint Hilaire demonstrated the possibility of making fabric from spider silk cocoons, and some socks and gloves were produced [42]. A study of the economic yield from this industry revealed that it was unlikely ever to become profitable, as it was calculated that 1.3 million spider cocoons would be needed to produce one kilogramme of silk [43]. In 1777 a device for "forcible spider silking" was proposed by R.M. Termeyer [44].

As shown in Fig. 1.4, most spiders have a whole battery of silks which they employ, each optimised for a particular task such as web construction, defence, capturing of prey or building cocoons containing the egg sac [45–48]. These different types of silk, each varying in amino-acid composition and structure, are produced and stored within different glands, all located in the abdomen of the spider [49–53]. In addition to this repertoire of silk types, the rate of production, mechanical properties and diameter of the individual silk threads can be controlled by the spider during the spinning process itself [28, 51]. A total of 7 different types of silk gland is known, although in general individual spider species use only 5 to 6 of these, and often fewer: in brief, not all spiders produce all possible types of silk.
1.1 Silk

Spiders put the ability to spin silk to the most spectacular variety of uses [51]. They form draglines to catch themselves if they fall. Small, and especially young, spiders spin a "parachute" thread which enables them to be carried by the wind, sometimes for hundreds of kilometres. Males use silk in transferring sperm to the palpal organ, and females make cocoons with it. Spiders, using their silk for capturing prey, have in the course of the last 400 000 000 years developed a wide diversity of hunting strategies. The simplest method is to drape a disordered mesh of threads across a space in which the prey becomes entangled. Some such "entangle strategies" involve throwing the web, previously constructed between the forelegs of the spider, over a passing insect. Another common trapping method is the use of pitfalls: the spider hides in a tunnel which can be closed with a door, and when the prey walks over a signal thread the door is opened and the spider grabs it. One particularly special catching technique is to throw threads with adhesive bubbles on their end over the victim, after first attracting it with decoy pheromones. Beside all of these different uses of specialist silks the most general and familiar one is the construction of orb webs to trap insects. These webs, suspended above the ground, are highly sophisticated structures which can stop an insect, often flying at high velocity, without collapsing [3,54].

Figure 1.4: Schematic representation of the seven different spider silk glands producing different types of silk for specific functional usage. Reproduced with permission [51] (see picture credits in App. D).
The spinning of silk itself is a complex process, involving the combination of sticky and nonsticky strands, and is controlled by the spinnerets at the tip of the abdomen. A spider usually has three pairs of spinnerets (anterior, median and posterior), corresponding phylogenetically to modified extremities [55]. Early spiders had four pairs of spinnerets, but among extant species this ancient trait is retained only in some primitive examples. A small number of spiders have in the evolutionary process reduced their spinneret count to only one pair. During spinning, the spinnerets move independently and work together in a highly coordinated manner. Each silk gland leads to one specific spinneret, which opens to the outside in the form of a tiny tap [56]. Silk secreted in the lumen of the gland is extruded via the glandular duct by an increase of the spider’s abdominal haemolymph pressure and/or by active extraction of silk from the spinnerets using the hind legs. The irreversible transition from the liquid silk dope to the solid thread is completely unrelated to simple exposure to air but is a complex process combining proton pumps [57], divergent/convergent geometry of the duct lumen [58], active water retention [59], or in more detail a control process for water–protein interactions and shear forces [9]. A repair mechanism specifically for threads broken accidentally during the spinning process has even been postulated [60].

Although considerable progress towards the characterisation of the spinning process has been reported [9–11,47], and a number of partially folded intermediates, some of them forming liquid–crystalline phases, have been postulated, the spinning of spider silk remains a poorly understood process and a detailed molecular model is lacking.

Silks used for building orb webs, labelled by their gland of origin, are major ampulla silk for the radial threads, minor ampulla silk for the auxiliary spirals in the hub of the web and flagelli form silk for the sticky spirals [Fig. 1.4]. The spider silk which has received the most investigation is that from the major ampulla (m.a.) gland, because of its impressive mechanical properties [45,61]. This silk is used by the spider not only for web construction, but also as a dragline – the safety line left behind continuously as the spider moves around, and anchored at intervals by silk from the piriform glands.

Spider dragline silk consists predominantly of silk proteins (spidroins) whose composition exceeds 60% alanine and glycine only [62,63]; these residues are organised in highly repetitive segments traditionally divided into “amorphous” and “crystalline” domains [64,65]. Solid–state NMR and X–ray studies have shown that the alanine–rich
regions form predominantly $\beta$-sheets [64–70], while the glycine residues are found only partially in $\beta$-sheets but contribute largely to helical structures showing a 3-fold symmetry [70].

The silk dope stored in specialised glands [52, 71], is a concentrated solution, over 30% of the mass being protein [72, 73]. The molecular structure of silk dope, and of possible intermediate forms appearing during the transition from the soluble form to the solid thread along the silk production pathway, remains essentially unknown and awaits any kind of detailed characterisation [60, 74, 75]. A structure has been postulated for liquid spider silk dope [73] on the basis of NMR spectroscopy, namely the “dynamic loose helical structure” proposed by analogy with the “silk I” structure suggested for solid silk from the domesticated silkworm Bombyx mori [76, 77].

Results from CD spectroscopy suggest a stable secondary structure poor in $\alpha$-helices and $\beta$-sheets for the silk dope at the upstream end of the m.a. gland of the spider Nephila clavipes, known as the “A-zone” [60], while at the downstream end (“B-zone”) $\beta$-sheet structures were reported [75]. No classical secondary structure was found in silk dope from the silkworm Bombyx mori [78, 79], but for the silk dope of the wild silkworm Samia cynthia ricini $\alpha$-helical structures were detected [78–82].

1.2 Spiders

Spiders are found all over the planet: from warm to cold, sunny to shaded or dry to wet regions, they have conquered almost every possible habitat. Spiders began to evolve approximately 380 000 000 years ago [83]. The lack of wings forced these animals to develop new hunting strategies: capturing their prey with silk and killing it with a venomous bite. When the supercontinent Gondwanaland broke up around 150 000 000 years ago, spiders were isolated on the separate continents, where they evolved independently into new, but related, groups. In the modern era there exist over 38 000 different spider species.
1.2.1 Spider diversity

Spiders are often confused with insects, although they are easy to distinguish, because spiders have 4 pairs of legs while insects have only 3 pairs. The systematic ordering of animals according to their appearance and habits into a hierarchy of groups and subgroups is called taxonomical classification [84,85]. Spiders, Aranea, are classified within the kingdom Animalia (all animals) into the division Arthropoda (hard exoskeleton), subdivision Chelicerata (presence of a fang portion) and class Arachnida (4 pairs of legs). Arachnids have the head and thorax combined (cephalothorax), with simple eyes, jaws adapted for tearing or piercing prey, a pair of pedipalps and eight walking legs. Spiders are the only Arachnids which have special glands in their abdomen to produce silk. The scientific classification of spiders is shown in Fig. 1.5. Because some 105 families of spiders are known, plus approximately 10 which are extinct, this diagram provides only a summary overview of the existing species. The advent of DNA analysis has made the positioning of each animal more precise than before, and several genus or species have been, and are expected still to be, rearranged.

Two suborders among the order Araneae are widely but not universally recognised: Mesothelae and Opisthotothelae. Mesothelae are the most ancestral spiders known. They have a segmented abdomen with eight spinnerets placed centrally on its lower surface and dagger–like, downward–striking fangs. Opisthotothelae includes all other spiders. They have the spinnerets placed at the end of the abdomen, a far more efficient position for silk spinning. These spiders are divided into two infraorders: Mygalomorphae and Araneomorphae. Mygalomorphae retain the dagger–like fangs while Araneomorphae, the vast majority of spiders, have pincer–like fangs which are more efficient for biting and manipulating prey.

Over 100 families of Araneomorphae are known. They are distinguished primarily by their techniques for hunting or capturing prey, by their body shape and by their different web types. The spiders studied within this work belong to the family Tetragnathidae. It is a small family (containing 55 geni) and includes both diurnal and nocturnal species, weaving suspended, sticky, wheel–shaped orb webs with an open hub and few, wide–set radii and spirals. The webs have no signal line and no retreat. This orb–weaving family was previously included in the family Araneidae but was separated due to certain
Figure 1.5: Spider taxonomy: a summary indicating the relationship of spiders (Araneae) with other Arachnidae. Because the infraorder of Araneomorphae contains over 100 different families, the family of Tetragnathidae over 50 different genera and the genus nephila 27 species, only a small section is shown. The scientific classification of Nephila edulis is marked with grey. Data taken from Ref. [184].
morphological differences and to the way in which they capture and consume their prey. These spiders have an elongated and slender body, ranging in length from 5 – 30 mm, with long legs. The legs are thin and tend to point fore and aft when the spider is at rest on a twig or in its web. The chelicerae are very large and sometimes of rather bizarre shape, but these spiders pose no serious threat to man as they are reluctant to bite. Symptoms are usually negligible or mild local pain, numbness and swelling although occasional nausea and dizziness can occur after a bite.

The largest and most impressive genus of the family Tetragnathidae is Nephila, or golden silk orb-weaver, named after the golden tint of their silk. 27 species belong to this genus. A Nephila web is usually supported between two trees and can span enormous spaces, metres wide, and usually at least 1.5 m from the ground. Nephila is a diurnal spider remaining in its web both day and night. Protection from bird attacks is effected by the presence of a ‘barrier network’ of threads on one or both sides of the orb web. Golden orb-weavers are large spiders with a long oval abdomen. Adult males and females differ enormously in size and weight: females are large, with body lengths of 15 – 30 mm, while males are approximately 5 mm across and have only one thousandth of the female’s weight. Juvenile spiders are equal in size for male and female, and are distinguished by their leg hairs: the second and fourth pairs of legs on the female have a brush of bristles on the tibia, while the third pair shows no brush.

The species used in the study is called Edulis and originates from the Sydney region in Australia. This species exhibits striking sexual dimorphism, which is when the male and female have physical characteristics so different that they appear to be different species [Fig. 1.6]. An important characteristic of the female is the presence of black brushes along the legs. The web is remarkably strong and has a characteristic yellow colour, as does the fluffy egg sac which tends to be left in the tree the spider was using for support. In many parts of the Darling Downs, a farming region in southern Queensland, this species is present in very large numbers, especially throughout the warmer months of the year. It is common for a single dead tree to have as many as 30 individual golden orb-weaver webs attached to it.
1.2 Spiders

1.2.2 Spider anatomy

This subsection provides a brief overview of the anatomy of the spider, as an aid both to the understanding of the sample-preparation procedures described in Ch. 2.3 and 2.4, and to the interpretation of the images obtained in Ch. 6. More detailed anatomical information may be found in Refs. [86-88].

The spider body consists of two primary divisions: an anterior part known as the prosoma (or cephalothorax) and a posterior part named the opistoma (or abdomen). These are connected by a small tube, the pedicel [Fig. 1.7]. The prosoma is formed of chitin, a hardened material, and contains the head, breast and legs. Its functions are predominantly food uptake, nervous integration and locomotion. The head is composed of several eyes, mouth, jaws and feelers. The majority of spiders have eight eyes, but some have only six and very few species can have as many as twelve, or none at all. The mouth consists
of two parts: the lips (labium) under the mouth opening and two maxillae, one on each side of the labia. Each of the two jaws compromises a large basal part (chelicera) and a fang. The fangs function as a claw and have an opening near their tip for the poison. The feelers, pedipalps (or simply palps), arise as an appendage just in front of the legs, and in adult males they are modified for the transfer of semen.

Figure 1.7: Anatomy of a spider: external appearance in lateral (a) and ventral (b) views. Figures courtesy of Rainer F. Foelix (see picture credits in App. D).

The soft abdomen contains most of the organs and fulfils the vegetative tasks such as respiration, reproduction, digestion, silk production and excretion. Respiration is provided by a lung slit and a breathing pore (spiracle), both located on the underside of the abdomen and leading to the respiratory organs. The sclerotised structure between the lung slits is associated with the reproductive opening of adult females, the epigynum. The epigastric fold separates the anterior from the posterior part of the ventral abdomen. Silk is
1.2 Spiders

Spun by the **spinnerets**, fingerlike abdominal appendages at the tip of the ventral abdomen. The **anal tubercle**, a small projection close to the spinnerets, contains the anal opening.

Figure 1.8 provides a schematic representation of the internal organs of the prosoma and opistoma. Inside the opistoma are the central nervous system, a pair of **poison glands**, the first part of the intestinal tract (**midgut diverticulum**), a **sucking stomach** and an extensive musculature (**pharynx muscle**). The central nervous system collects information from the sensory organs and controls the behaviour of the spider. Apart from a small number of primitive species, and the **Uloboridae** family, all spiders possess a pair of poison glands. The poison is extruded through a duct which terminates shortly before the tip of the chelicera. The alimentary canal begins with the sucking stomach, continues through the entire body and ends in the excretory system. A singular characteristic of spiders is the branched midgut which spreads into the entire prosoma and often extends into the coxa, the segments of the legs nearest to the body. The individual midgut branches constitute the midgut diverticulum.

Figure 1.8: **Anatomy of a spider: longitudinal section of the prosoma (left) and opistoma (right).** Only female spiders show ovaries. Images courtesy of Rainer F. Foelix (see picture credits in App. D).

The organs located in the opistoma are the **heart**, **trachea** and **book lungs** for respiration, several silk glands, **ovaries** in female spiders and the second part of the intestine, which ends at the **stercoral pocket** for excretion. The heart is located at the front, upper
side of the abdomen and usually beats with a frequency of \(30 - 70 \text{ beats/minute}\), reaching a maximum of \(200 \text{ beats/minute}\) when the spider is tense or exhausted. The book lungs are an air-filled cavity, containing stacks of blood-filled leaves to provide a maximal surface for aeration, with an opening on the underside of the abdomen. Air is carried around the body through tubes known as trachea, which open at a breathing pore on the underside of the abdomen, the spiracles. Nearly all of the digestive processes take place in the midgut. The broken-down digestive products are absorbed into the body cavity largely in the midgut, and in sac-like structures called the caeca (singular caecum). The caeca can proliferate to varying degrees, expanding to surround other organ systems such as ovaries and silk glands and supplying these with necessary nutrients. In well-fed spiders the bulk of the abdomen consists of caecal fluids, and this is the main reason many spider species can spend long periods of time without food. Malpighian tubuli are special excretory organs designed to dispose of nitrogenous waste, and thus have a function similar to human kidneys. Because spiders cannot afford to lose water as humans do, instead of urine they produce uric acid, which is a near-solid. The malpighian tubuli drain into the stercoral pocket, which is attached to the midgut, to combine the uric acid waste from the “kidneys” with solid waste from the digestive tract, and this combined waste product exits through the anus.

1.3 Thesis outline

To summarise the preceding sections, solid spider dragline silk, which is spun from a viscous liquid stored within specific glands and is used in draglines and web support elements such as radii, is well known for its extraordinary mechanical properties \[45, 61\]. Nonetheless, a detailed understanding of the spinning process is lacking. Further, the liquid silk precursor is remarkable not only because it is the starting material for a spinning process which, in contrast to any known synthetic procedures occurs at ambient temperature, low pressure and with water as solvent \[74, 89\], but also because the spider succeeds in maintaining this highly concentrated (>30%) and stress-sensitive viscous silk dope in a liquid state, avoiding the formation of insoluble structures.
Nuclear magnetic resonance (NMR, Ch. 3) offers a wealth of methods to investigate, on the molecular as well as on the macroscopic level, the structure and function of biological macromolecules [90]. The aim of this thesis is to study the structure of liquid silk stored within the wide sac of the major ampulla gland of the spider *Nephila edulis*, primarily by the use of two different NMR methods (Ch. 4 and 5). A third method, magnetic resonance imaging (MRI), was applied to perform *in–vivo* spectroscopy on the spider, leading to a promising method for the study of anatomical details in real time (Ch. 6).

1.3.1 Chapter contents

Chapter 2 explains the in–house breeding of the spider *Nephila edulis* and the preparation of the samples used for the three experiments described in the chapters 4 to 6.

Chapter 3 presents an overview of the principles of NMR and of the specific techniques and equipment employed to perform the experiments both on liquid silk dope (Ch. 4 and 5) and for *in–vivo* spectroscopy (Ch. 6).

Chapter 4 describes the analysis by NMR spectroscopy of native silk dope from the spider *Nephila edulis* at room temperature. Slow magic–angle sample spinning (MAS, Ch. 3.2.2) is employed to obtain high–resolution $^1$H and $^{13}$C NMR spectra. The almost complete assignment of spectral peaks to specific amino acids is shown to indicate a random–coil arrangement of the protein molecules and an isotropic liquid phase for the native silk dope.

Chapter 5 investigates possible local ordering among the silk proteins within the native liquid dope in the absence of the dynamic conformational averaging occurring at room temperature. Silk dope was immersed abruptly in liquid nitrogen in the hope of trapping the molecules in their specific conformations at the moment of shock–freezing, i.e. assuming that folding/unfolding processes were not triggered by the shock–freezing process. Solid–state NMR spectroscopy (DOQSY, Ch. 3.2.3) at low temperatures (8 K) in combination with an isotopic labelling strategy (Ch. 2.3.1) gave access to backbone torsion–angle pairs in the immobilised protein molecules. The distribution of these torsion angles is found to suggest that glycine–rich domains within immobilised silk dope do not contribute to a well–defined structure, adopting instead a random–coil conformation.
Chapter 6 displays magnetic–resonance images of live spiders. High–quality, full–body, in–vivo images were obtained not only of *Nephila edulis* spiders, but also of their intestine and silking processes in real time. Localised spectroscopy was performed within the three zones of the m.a. gland.

A summary of the results from the individual chapters, the primary conclusions they provide and the possibilities they offer for further research are presented in Chapter 7.

A list of abbreviations is found in Appendix A. Additional details concerning spider breeding, specifically with regard to the food supply required at different stages of the lifecycle of *Nephila edulis*, have been placed in Appendix B. The experimental characterisation of the diametrally–magnetised DC electrical motor specifically modified to operate in the bore of a 400 MHz NMR spectrometer (Ch. 3.3.3) is presented in Appendix C. The behaviour of the motor in fields from 0.4 T to 9.4 T is displayed, and its influence on the homogeneous field of the spectrometer measured in detail. A list of credits for reproduced images is provided in Appendix D.
2 From spider cocoons to NMR samples

2.1 Introduction

The characterisation of spider silk begins with a reliable and reproducible supply of samples. Because of their temperature–sensitivity, the transport of even the most suitable spider species is a complex process. Some of the experiments conducted in the course of these investigations also required isotopically labelled silk samples, obtained by specific feeding protocols. As a result of these factors it was advantageous to prepare all of the samples used in these studies from spiders bred in–house. This chapter reports on the procedures adopted for the breeding of spiders and for the harvesting of silk samples.

2.2 Breeding the spider *Nephila edulis*

2.2.1 Spider cages

Before discussing the care and feeding of spiders, the artificial environments optimised for the different stages of their life cycle are introduced. Because spiders have a preference for high vantage points, tall cages were used for cocoons and spiderlings. At a later stage the animals were separated because of cannibalism, and were placed in boxes. Mature spiders were moved to cages containing frames suitable for the isotopic labelling of their silk (Ch. 2.3).
The tall cages used for the cocoons and spiderlings are shown in Fig. 2.1(a). The walls of these cages were made of plexiglass and supported by four aluminium brackets. The top and bottom were made of aluminium, with the top panel containing a hole, closed by a plastic disc, for feeding with fruit flies. The front wall was a sliding door which opened upwards. A porous tissue was fixed across the small slit between the top and the sliding door to prevent fruit flies or spiderlings from escaping. Wooden sticks functioning as artificial trees were placed in the cage to help the spiderlings to construct their fine webs, and were used to fix the cocoons with needles [Fig. 2.1(a)]. A water box placed inside the cage maintained a suitable level of humidity.

Figure 2.1: Three types of spider cage adapted for the different stages of the life cycle. Panel (a) shows the tall plexiglass cages for breeding spiderlings until they are capable of building proper orb webs (middle enlargement). A spider cocoon fixed with needles to wooden sticks placed inside a tall cage is seen in the rightmost enlargement. (b) Boxes for juvenile spiders containing one or two boughs and a soil floor. (c) Frame-shaped cages separated by thin plexiglass disks for adult spiders, which are fed $^{13}$C-labelled amino acids with a syringe (lower right enlargement).
2.2 Breeding the spider Nephila edulis

Older, and hence larger, spiders were separated from their colony and moved to smaller boxes [Fig. 2.1(b)]. These grey boxes have a window made of plexiglass in the front door. There is an additional rectangular opening covered with either tissue or a metallic mesh for fresh air. At the bottom was a tray filled with wet soil to maintain the required humid climate. The walls were roughened and some twigs placed inside to provide the spider with enough possibilities to fix its web.

The square frames [Fig. 2.1(c)] used for mature female spiders during the period of labelling their silk with $^{13}$C were also constructed from plexiglass. The inner walls of these frames were roughened either by scratching with a knife or by fixing sandpaper to them. This procedure, and the presence of a single wooden stick, enable the spider to make a well-constructed web within the frame. Individual frames were separated by square plexiglass sheets covered by a thin film of vaseline. The discs prevented the spiders from moving to adjacent frames, and the vaseline prevented web-building on the partition wall.

2.2.2 Growth and feeding

The cocoon is made of silk and has the form of an ellipsoid containing hundreds of fertilized eggs. Cocoons were fixed with needles to the wooden sticks [Fig. 2.1(a)] inside the tall plexiglass cages [Fig. 2.1(a)]. 2 – 3 weeks after cocoon spinning, spiderlings of around 2 mm in size hatch and emerge to scramble nervously in and around the loose threads of the cocoon. After some days they begin to produce thin threads to build a disordered network with the cocoon in its centre. This small, meshed thread construction helps to remove the old skin of the spiderlings for their first 2 – 3 moults [87].

During this initial period of life the spiderlings are nourished from their egg sac [87]. However, on reaching some 5 mm in size the spiderlings begin to explore their environment and to build small orb webs with a diameter of order 15 cm to catch their first prey. The diet of Nephila edulis depends on the age and size of the spiders, and here was composed from fruit flies, house flies and mealworms, the supply of which is described in detail in App. B. In this preliminary stage of their life cycle the juvenile spiders still use their webs collectively, and were fed 3 to 5 times weekly with small fruit flies for the next 3 weeks. Their diet was changed to larger house flies as soon as they
reached half the size of their prey. At this stage the spiders now construct larger webs with diameters of up to 40 cm, and treat everything entering the web as prey. It becomes necessary to separate the spiders into more than one cage, as otherwise cannibalism and fights for the available space would lead to a rapid decrease in the number of spiders. The warm and humid climate favoured by *Nepila edulis* was created here by a reservoir of water inside the cage, weekly sprays of water with the same type of spray bottle as used for plants, and the plexiglass construction which prevents heat loss.

Juvenile spiders reaching a size of approximately 10 mm, building proper orb webs and strictly defending the territory they occupy, were isolated in smaller boxes [Fig. 2.1(b)]. Here the required humidity was supplied by the wet soil floor and weekly water sprays. Feeding with house or blow flies was easier than in the tall cages as the fly larvae could be placed inside the cage in a small jar when ready to pupate and hatch, as opposed to having to handle agitated adult flies.

To maintain the spider colony it was necessary to create conditions favouring mating. This means either bringing the males close to the female’s web or placing them in the same box. Fertilised females were not used for further labelling of silk samples until their eggs had been laid.

### 2.3 Liquid and immobilised silk samples

#### 2.3.1 Isotopic labelling

The experiments on native (Ch. 4) and immobilised (Ch. 5) liquid spider dragline silk required isotopically labelled silk samples (Ch. 3). The labelling procedure began by moving mature female spiders from their grey boxes [Fig. 2.1(b)] onto square plexiglass frames [Fig. 2.1(c)] to be fed with an aqueous amino-acid solution (85 mg/ml). The amino-acid composition of this solution was chosen to match that of m.a. silk, given in Tab. 2.1, and has been determined for solid dragline silk using standard amino-acid analysis [91,92]. The specific $^{13}$C labelling of particular amino acids in the feeding solutions required for separate experiments is explained in Ch. 4 and 5.
2.3 Liquid and immobilised silk samples

<table>
<thead>
<tr>
<th>amino acid</th>
<th>amount (mol%)</th>
<th>amino acid</th>
<th>amount (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>32(1)</td>
<td>Lys</td>
<td>0.3(1)</td>
</tr>
<tr>
<td>Arg</td>
<td>2.1(2)</td>
<td>Met</td>
<td>0.2(2)</td>
</tr>
<tr>
<td>Asp/Asn</td>
<td>1.8(5)</td>
<td>Phe</td>
<td>0.3(3)</td>
</tr>
<tr>
<td>Gly</td>
<td>39(4)</td>
<td>Pro</td>
<td>4(1)</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>10(1)</td>
<td>Ser</td>
<td>3.6(5)</td>
</tr>
<tr>
<td>His</td>
<td>0(0)</td>
<td>Thr</td>
<td>0.6(1)</td>
</tr>
<tr>
<td>Ile</td>
<td>0.6(1)</td>
<td>Tyr</td>
<td>3(1)</td>
</tr>
<tr>
<td>Leu</td>
<td>2.7(3)</td>
<td>Val</td>
<td>0.7(2)</td>
</tr>
</tbody>
</table>

Table 2.1: Amino-acid composition (mol%) of the solid dragline silk of Nephila edulis as determined by standard amino-acid analysis. Values are quoted with standard deviations obtained from the Student t-distribution.

Freshly moved spiders usually build a complete web inside the frame within 1 – 3 days. While waiting in the comfort and security of this web, the spiders are easy to feed by syringe with isotopically labelled amino-acid solution. With spider and syringe separated by the spider’s web, the needle with a droplet of solution was moved towards the spider’s mouth and soaked up by the spider. Feeding the spider twice a day with a total of up to 20 droplets over a period of approximately 2.5 weeks leads to a 3 – 10% enrichment of the liquid m.a. silk with $^{13}$C in the amino acid of choice. In the metabolic processes of the spider, certain amino acids are broken down and the resulting parts reconstituted differently within the silk protein. This leads to a “scrambling” of the $^{13}$C isotopes in the labelled feeding solution and the other amino-acid constituents of the measured sample. The level of isotopic scrambling was not measured explicitly in these studies, but from the results presented in Ch. 4 and 5 can be estimated to be on the order of 10%, a rather low value in agreement with earlier findings [67,93].

Because it was necessary to empty the m.a. gland of unlabelled silk before labelled material could accumulate, the spider was silked twice during the first week of the labelling period [Fig. 2.2(a)] [94]. For this procedure the spider was trapped in a plastic mug and anaesthetised with carbon dioxide until its movements became very slow. Because this species is highly sensitive to carbon dioxide, great care is required concerning its exposure to the gas.
Figure 2.2: Silking a spider. (a) A motor inside the grey tower drives the reel to spool the silk. The motor speed is controlled by the black power supply, which allows five motors to be driven simultaneously. Panel (b) shows the spider on its back, fixed by needles and a net onto a styrofoam plate. (c) Magnified view of spinnerets: the arrow indicates the two silk threads drawn from the two m.a. glands.

The anaesthetised spider, lying on its back, was then fixed with several needles and a close-meshed net onto a styrofoam plate [Fig. 2.2(b)]. The needle placement was optimised to prevent movements along the body axis while the net kept the legs stretched away from the body to hinder torsional movement. In this position the silk could be spooled onto a reel through a hole in the net allowing access to the spinnerets at the tip of the abdomen [Fig. 2.2(b)]. These in turn were also fixed by two crossed needles to
2.3 *Liquid and immobilised silk samples*

prevent movements which might cut the thread while spooling. The silk thread from the m.a. glands, which is spun at the posterior spinnerets [56] [Fig. 2.2(c)], was caught with the aid of thin, right-angled tweezers and drawn by hand onto a reel in the form of a plate to be spooled at a speed of 55 ±1 mm/s by a motor.

2.3.2 *Dissection*

The experiments conducted on native (Ch. 4) and immobilized (Ch. 5) silk dope required dissection of the wide sac of the m.a. gland [Fig. 2.3]. The female spider was killed instantly by cutting the thorax with scissors while it remained in its web. The legs and thorax were removed and the abdomen placed in a petri dish. The upper and lower lips of the epigynum were separated using sharp tweezers to create a fissure along the sternum, and the skin peeled away. Schartau and Leidersher's spider Ringer solution (pH = 7.5) [95] was used to prevent the inner parts from drying, and loose parts such as eggs were washed away with a syringe. The two, mostly gold-coloured m.a. glands and their associated structures were separated with tweezers from the abdomen. The duct and tail segment were removed with a scalpel and the remaining wide sac of the m.a. gland dissected [60].

For the experiments on native liquid silk dope described in Ch. 4, the finished sample was obtained by drawing the wide sac into a MAS rotor using a syringe. For the measurements on denatured silk dope, one to two wide sacs were placed overnight in approximately 50 ml of 7.7 M urea containing 0.05 % (w/v) of NaN₃. The sacs were ruptured by 5 cycles of sonication (in an ultrasonic bath) and vortexing. The sample was subsequently clarified by centrifuging (10 min, 9000 g) and the resulting supernatant was loaded into the MAS rotor.

For the investigation of immobilised silk dope described in Ch. 5 the m.a. gland was shock-frozen by immersion in liquid nitrogen and moved to a prepared sample container, a thin glass tube 3 mm in diameter, 1 to 1.5 cm in length and sealed at one end with a stopper made from Teflon tape. To prevent the sample from defrosting the glass tube was embedded in a styrofoam block while the sample was moved into it. The tube was cooled briefly in liquid nitrogen before closing the other end with a second Teflon stopper. It was necessary to wear a mask during the process of sample packing to prevent exhaled water...
Figure 2.3: Dissecting the wide sac of the m.a. gland of the spider *Nephila edulis*. 
(a) Ventral view of thorax and abdomen with the head pointing upwards. 
(b) Peeling the skin sideways from the epigynum to open the view of the ovary and m.a. glands. 
(c) Two yellow m.a. glands (arrows) and associated structures separated from abdomen. 
(d) Wide sac of the m.a. gland used for the experiments on native liquid silk (Ch. 4) and immobilised liquid silk dope (Ch. 5).

vapour from condensing and freezing either on the m.a. gland or on the glass tube, as this would preclude both accurate positioning and adjustment of the sample.

The immobilised silk samples were stored in a liquid–nitrogen dewar until measurement. The 3 mm glass tube was inserted into an Eppendorf tube which itself was placed into a slightly larger glass container. For both vessels a layer of Teflon tape placed between the previously punctured lids functioned as a pressure membrane.
2.4 In–vivo samples

All of the magnetic–resonance images presented in Ch. 6 were obtained using live spiders. A prerequisite in this process was to keep the spider still to prevent blurring caused by unwanted movements. This immobilisation was achieved by fixing the spider on its front with a piece of elastic medical gauze sewn onto a thin styrofoam plate [Fig. 2.4(a)]. Prior anaesthesia with CO₂ relaxed the spider and assisted in placing the body and legs appropriately: the abdomen was placed in the centre of the 1.0x1.5x7.0 cm styrofoam plate while the 4 front and 4 hind legs pointed away from the body and along the axis of the plate. The abdomen, legs and gauze were first fixed by pins in a position optimising the comfort of the animal and the available space, and then sewn in place to eliminate the metallic needles from the MRI sample. The fragile styrofoam plate was sintered in the heat of a lighter flame to prevent its disintegration after being perforated with needles. The completed construction of immobilised spider and holder was placed in the resonator of the probe head and fixed with transparent tape [Fig. 2.4(b)].

A similar configuration was used to obtain the “real–time silking” images presented in Ch. 6.3, where the spider was silked while its silk glands were monitored. For these experiments it was necessary to change the orientation of the spider’s abdomen on the styrofoam, namely by placing the animal on its back, to allow the required access to the spinnerets. The styrofoam together with the spider on it was taped into a half–shell containing the reel to spool the silk thread [Fig. 2.5]. The half–shell was made of polyetheretherketone (PEEK), and was inserted into the resonator of the probe head. A DC electrical motor, modified to run in magnetic fields (Ch. 3.3.3), was placed below the NMR magnet to drive the reel to spooling the silk thread.

The contrast agent MultiHance [97], containing gadobenate dimeglumine, was used for the experiments monitoring the intestine. Spiders were fed directly before fixation with 15 drops of an aqueous 1 % (V/V) solution.
2. From spider cocoons to NMR samples

Figure 2.4: Series of images showing preparation of spiders for MRI experiments and their positioning in the resonator of the probe head. (a) Fixing anaesthetised spider with pins and elastic medical gauze. (b) Spider sewn into position with legs orientated along longitudinal axis of styrofoam. (c) Construction placed in resonator with area of interest indicted by dotted line and fixed with transparent tape. Panel (d) shows the complete probe head which was used for all MRI experiments.

2.5 Discussion

In–house breeding of spiders involves a number of compromises in the space, food and locations available to the spiders relative to their natural environment. This section outlines some of the experimental consequences of the breeding procedures described in this chapter. The primary constraint for such a territorial species was a lack of space, which meant that the animals had to be cultured in several cages according to their age and size. While this ensured full control over the diet of mature female spiders for isotopic labelling, it also brought a number of disadvantages. A space shortage increases the propensity of
Figure 2.5: Silking a spider [94] during MRI experiments [96]. The upper panel shows a schematic representation of the apparatus pictured in the lower panel. The silk thread (a) from the spider (b), placed in a half-shell (c) at the upper end of the probe head (d), is spooled by the motor (e) onto the reel (f).

individual spiders to fight, resulting in injury or death. It also restricted the number of appropriate locations for cocoon construction, resulting in further unnatural behaviour with unknown consequences for silk quality.

The small net size of the whole (divided) spider colony meant that all animals were supported, contrary to the natural selection process which would produce mostly large and healthy animals, presumably with superior silk. The reproduction rate in a small colony is also low: male and female numbers were not always equal due to fluctuations in the females’ life cycles, and these frequent periods of imbalance facilitated inbreeding. A further natural effect of a small reproduction rate is cannibalistic behaviour, and incautious males were prone to being eaten on approaching the females before mating.

In this constrained environment disease was a major concern, as serious diseases would propagate rapidly and affect the whole colony. Illness, and general weakness or lethargic behaviour, were observed more often than expected in nature. Care was required not to exchange the feeding needles of the individual spiders to prevent transmission of
potential infections, and a sterile needle was used every time to soak up the labelling solution from the stock.

A final consequence of the unnatural environment of the spiders, again with unknown consequences for the quality and representative nature of their silk, was unpredictable behaviour due to their stressed disposition. Some spiders would run away from the approaching needle, disrupting their feeding pattern. Some spiders living in frames for isotopic labelling would eat their own web and thus become difficult to feed. Some spiders, after being labelled for up to 2.5 weeks to the point of being ready for dissection or imaging, would make a cocoon, using all their isotopically labelled amino acids to produce cocoon silk instead of dragline silk. The results shown in Ch. 4 to 6 were obtained using silk from spiders showing a minimum number of these potential problems.

As a closing note concerning the silking process, it was found to be easier to catch the correct silk thread from the spinnerets if the spider was allowed to run around on the table before anaesthesia. Unless they are sitting in the safety of their webs, spiders always have a small piece of dragline pointing out of their spinnerets ready for use as a safety line. Some spiders could be silked for up to 8 h. The silk gland can genuinely be emptied, as the spider will produce more silk very quickly if there is a web where it can be fed. It is therefore important not to destroy the spider’s web when catching it for silking.
3 Nuclear Magnetic Resonance: Theory and Instrumentation

This chapter introduces the fundamental principles of nuclear magnetic resonance (NMR), as applied in this thesis to the study of spider silk, and describes the NMR-related experimental techniques and apparatus employed in Chs. 4, 5 and 6.

3.1 Introduction to NMR

Nuclear magnetic resonance (NMR) is a physical phenomenon based upon the magnetic properties of the atomic nucleus, and first described independently by Felix Bloch and Edward Mills Purcell in 1946 [98–100]. Nuclei possessing a spin, which is the atomic analogue of a magnetic moment, experience a resonant precession when exposed to both a static magnetic field and a second, oscillating field. From this nuclear resonance it is possible to extract information relevant to a wide range of physical phenomena and materials properties. The versatility of NMR ensures its widespread use throughout the sciences.

NMR spectroscopy is the application of the NMR phenomenon to study the physical, chemical and biological properties of matter. While simple, one-dimensional techniques are used routinely by chemists to study the molecular structures of small, organic compounds, sophisticated two- and three-dimensional techniques can be applied to determine structures, correlations and dynamics in complex systems of large molecules [101]. These
properties have made NMR spectroscopy an invaluable tool in the understanding of protein and nucleic acid structure, dynamics and function [90, 102, 103]. NMR is not, however, limited to classical, solution-state experiments, and the investigation of media with no or little mobility (crystals, powders, large-membrane vesicles, molecular aggregates) is possible with specific solid-state NMR techniques. A particularly appealing application of the simplest type of NMR spectroscopy is magnetic resonance imaging (MRI): MRI is a tomographic and volume-imaging technique used mostly to visualise the insides of living organisms. MRI length- and timescales give it an essential role in human medicine, in detecting pathological or other physiological alterations of the nature or function of living tissues.

For periodic, crystalline structures, NMR spectroscopy does not compete with (X-ray and neutron) diffraction techniques, but for powdered, amorphous and dynamic systems it is a method of choice. A brief and selective insight into the wealth of methods it offers to study these types of system, on both the molecular and the macroscopic levels, will be presented in the following section. Because liquid spider dragline silk is neither an isotropic liquid nor a well-defined solid, but rather a viscous dope of macromolecules, NMR spectroscopy is uniquely suited to its investigation. MRI techniques are particularly advantageous for studying the anatomy and silk production of spiders, not merely because they preserve the live samples but because these can be imaged in their entirety in real space, and during biological processes in real time.

3.2 NMR Theory

This section outlines the basic principles required for an understanding of the spectroscopic techniques applied in this thesis. Extensive and descriptive introductions may be found in a number of textbooks [96, 104], while Refs. [105] and [103] provide more detailed and mathematical explanations. A survey of important concepts in NMR is given in Ref. [106].
3.2 NMR Theory

3.2.1 Principles of NMR

Protons ($^1\text{H}$) and the carbon isotope $^{13}\text{C}$, the nuclei of interest within this study, each possess a nuclear spin quantum number of $I=\frac{1}{2}$. A nucleus of spin $I$ has $2I+1$ degenerate states, by which is meant states of equal energy. In an applied magnetic field these energy levels are split by the Zeeman interaction and the equilibrium population of the energy levels is described by the Boltzmann distribution. Nuclei may then be excited between the available energy levels by radiation of a frequency matching the level separation: the energy difference between the lower and the higher level of the excitation process is the resonance frequency. The signal detected in NMR spectroscopy occurs at this frequency, and thus appears with an intensity proportional to the population difference between the two energy states involved.

The behaviour of nuclei investigated by NMR spectroscopy can be understood in a tangible manner using a semiclassical description. The angular momentum of a particle with spin is a vector whose orientation can have any direction in space. The response to a magnetic field $\vec{B}_0$ applied to such a spin magnetic moment is a precession, i.e. a movement of the vector around the $\vec{B}_0$-axis on a cone maintaining a constant angle to $\vec{B}_0$. If an ensemble of equal nuclei is placed in a magnetic field, the isotropy of the angular momentum is broken and a macroscopic nuclear magnetic moment, coinciding with the direction of the applied field, develops. This net magnetic moment is the nuclear magnetisation. If the thermal equilibrium of nuclear orientations is disturbed by an electromagnetic pulse (at radio frequencies (r.f.) for the energy scales relevant to NMR), this is equivalent to a rotation of the magnetisation away from its orientation parallel to $\vec{B}_0$, creating a transverse magnetisation. This tilted magnetisation is subject to a force which causes precession around the $\vec{B}_0$-axis, and the oscillating electric current induced in a detection coil by this precession, the free-induction decay (FID), corresponds to the detected NMR signal.

There exist two primary damping mechanisms by which the magnetisation is returned to its most favourable orientation parallel to $\vec{B}_0$, causing the detectable signal to vanish. The spin–lattice or longitudinal relaxation ($T_1$) involves energy exchange with the environment and depends on both the field strength $B_0$ and the internal motion of the molecules. $T_1$ refers to the mean time required for an individual nucleus to return
to its equilibrium state, and determines the time lapse before the sample can be probed again. The origin of the spin–spin or transverse relaxation ($T_2$) lies in the slow decay of the transverse magnetisation arising because of the breaking of exact synchrony between the precessing nuclear magnets. The time constant $T_2$ describes this gradual loss of phase coherence, as reflected in the homogeneous decay of the precessing macroscopic nuclear magnetisation, and thus of the observed NMR signal. In the NMR spectrum, which is in fact the Fourier transform of the FID signal, $T_2$ defines the width of the NMR response: nuclei with a longe intrinsic $T_2$ yield a sharp signal, and conversely. The constants $T_1$ and $T_2$ are closely related to molecular motion, and in liquids to the size of the molecules under investigation.

Nuclei of the same isotopic species do not always precess at exactly the same frequency because the local magnetic field in a physical system differs for each nucleus. This arises because local electronic effects shield each nucleus from the main external field to a different extent. The effect of such frequency differences due to nuclear shielding is known as the chemical shift, and can cause the appearance of many peaks in a NMR spectrum. The chemical shift is a function of the local environment of the nucleus and is measured relative to a reference compound. The shift information is a probe of chemical structure by nuclear magnetic resonance.

Spatial anisotropy is the property of being directionally dependent, or having different characteristics in different directions. Local magnetic fields induced by electrons in a molecule tend to vary across the entire molecular framework in such a way that nuclei in distinct molecular environments usually experience unique and anisotropic local fields. This effect is called chemical–shielding anisotropy (CSA) and is a local property of each nuclear site. Identical molecules or nuclei experience different local environments for different orientations, which leads to signal–broadening in the NMR spectrum. This effect is strong in media with little or no molecular mobility (such as crystals, powders, large membrane vesicles or molecular aggregates), where anisotropic interactions are not averaged by Brownian motion as they are in liquids or other mobile phases. The most important method for obtaining high–resolution spectra in solid–state NMR is sample spinning around a special “magic angle” (MAS), at frequencies up to 70 kHz (Ch. 3.2.2).

Two further effects related to weaker interactions in nuclear spin systems cause characteristic splittings of individual signals in the NMR spectrum and can be used to gain
structural information. The indirect spin–spin coupling ("$$J$$–coupling") and the magnetic
dipole–dipole coupling are strongest for neighbouring nuclei, causing splittings on the
order of 0 – 300 Hz and 0 – 100 kHz, respectively. The internuclear coupling mediated
through a small number of chemical bonds is referred to as spin–spin coupling and
contains information concerning both the direct chemical bonds and the dihedral angles
in chain–like molecular structures. Dipole–dipole couplings, by contrast, are mediated
through space. These interactions are a function of internuclear separation $$r$$ and of the
orientation of the internuclear vector. The dipole–dipole coupling has a decay of the
form $$\frac{1}{r^6}$$.

3.2.2 Magic–Angle Sample Spinning (MAS)

Magic–angle spinning (MAS) is a technique widely used to perform experiments in solid-
state NMR spectroscopy for the narrowing of broad lines, and therefore increasing the
resolution for better identification and analysis of the spectrum [107]. The sample is
spun at an angle of $$\theta_m \approx 54.7^\circ$$ with respect to the direction of the magnetic field $$\vec{B}_0$$ at
frequencies up to 70 kHz.

In solids or media with restricted molecular motion dipolar couplings and chemical–
shielding anisotropy are removed by sufficiently fast spinning at $$\theta_m$$, leaving only the
isotropic chemical shift and the $$J$$–coupling in the spectrum. In liquids the anisotropic
interactions are time–averaged to zero because of the rapid molecular motion that occurs.
It is this orientational averaging in solution which is mimicked by MAS of a solid sample.

The spinning of the sample is achieved via a gas–turbine mechanism. The sample
containers are cylindrical rotors, axially symmetric about the axis of rotation. Samples
are packed into the rotors and these are then sealed with a single– or double–end cap.
One of the caps is shaped in the form of a turbine to perform the spinning. The rotors
exist in a variety of diameters (outside diameter generally 2.0 – 15 mm) and are usually
spun on air or nitrogen gas. The higher is the spinning frequency, the smaller must be
the diameter, as surface speeds must be limited to below the speed of sound because of
the possibility of centripetal forces causing the spinner to explode. The materials used
for the rotors and caps depend on the application required. Rotors are usually made from
ceramics (zirconium oxide, silicon nitride), while cap materials may be Kel-F, Vespel, zirconia or boron nitride.

### 3.2.3 Double–Quantum Single–Quantum Spectroscopy (DOQSY)

The DOQSY technique was originally devised to obtain the torsion angles in polymeric chain systems, and was applied to pair of sites separated by only one or two bonds along the chain [108, 109]. However, the same type of experiment can also be applied for the simultaneous determination of both torsion angles ($\phi$, $\psi$) in a protein, which is a chain system of amino–acid residues [110, 111]. In a protein where the carbon atoms of the carbonyl groups of two neighbouring amino acids are $^{13}$C–labelled [Fig. 3.1], a DOQSY experiment yields a signal which is dependent on both dihedral angles. In a non-crystalline but immobile protein system, as investigated in Ch. 5 the method is used to determine a backbone torsion–angle distribution [112].

![Figure 3.1: Schematic representation of a dipeptide indicating the backbone torsion-angle pair ($\phi$, $\psi$) of neighbouring amino acids (red, blue). The grey arrow represents the orientation (major axes) of the chemical–shift tensor, and $\phi$ and $\psi$ determine the relative orientation of the two chemical–shift tensors.](image)

A chemical–shift tensor describes the response of the nucleus to the direction of the applied magnetic field $\vec{B}_0$, and is in general anisotropic. The DOQSY experiment measures the correlation between the directions of chemical–shift tensors at two neighbouring NMR–active sites, and requires the excitation and detection of a “double–quantum” (DQ)
state involving both carbon atoms of the individual carbonyl groups simultaneously. The excitation and detection steps are effected by the application of a specific r.f. pulse sequence, of which further details are presented in Ch. 5. It yields a two-dimensional spectrum in which the first spectral dimension reflects the sum of the chemical shifts of the dipolar-coupled sites and the second, “direct” dimension contains the individual chemical shifts. The conformational information is obtained from the correlation of these two dimensions.

### 3.2.4 Magnetic Resonance Imaging (MRI)

In addition to this chapter and the introductory literature recommended above, Refs. [113–115] provide specific introductions to MRI.

In medical applications MRI provides a non-invasive, *in-vivo* imaging technique yielding anatomical and physiological information in three dimensions at high spatial resolution and typically within a rather short timeframe (from some minutes to a few hours). It is an attractive method as it uses magnetic fields and r.f. irradiation, as opposed to the ionising radiation used by many other imaging methodologies, such as positron emission tomography (PET) or X-rays (either as traditional still plates or by computer tomography). A key advantage of MRI over competing imaging methods is a high spatial resolution (up to $\mu$m) combined with high contrast, enabling adjacent tissues of very similar constitution to be distinguished. In addition, the use of chelates containing gadolinium as paramagnetic contrast agents causes certain tissues and fluids to appear extremely bright on $T_1$-weighted images, allowing e.g. high-resolution angiography.

MRI refers to the use of proton NMR, as described in Ch. 3.2.1, to image proton density or the relaxation times $T_1$ and $T_2$, in a large sample. The proton density, $T_1$ and $T_2$ are the three parameters which determine the brightness and contrast of MRI images. The essential concept of MRI is that the resonance frequency is made position-dependent, such that the different frequencies detected correspond to a spatial position rather than to a chemical shift. This is achieved by using an inhomogenous external magnetic field. Most commonly-used magnetic-field gradients are those where the amplitude varies linearly with position. To obtain an image of a three-dimensional object, all three dimensions must be en- and decoded independently. The strength of the magnetic-field gradient and
the transmission frequency determine the position of the image "slice". Slice thickness then is controlled by the bandwidth of the transmitter pulse and the spatial position within the slice is determined by applying additional field gradients perpendicular to the initial magnetic field.

3.3 Instrumentation

This section describes the instrumentation used for the experiments performed in the course of this thesis. A brief and elementary introduction to the NMR spectrometers and magnets used in the majority of experimental studies is given in Ch. 3.3.1. The subsequent sections discuss in detail the technical aspects of the experiments performed in Chs. 5 and 6, focussing on purpose-built apparatus and specific procedures adopted. The low-temperature equipment used in the investigation of immobilised silk is described in Ch. 3.3.2 and the conversion of an electrical motor to be used during MRI in the "real-time silking" experiment is presented in Ch. 3.3.3.

3.3.1 Spectrometers

The high magnetic fields (1 — 20 T) required for NMR experiments are obtained from superconducting magnets. This type of electromagnet is constructed from a coil of superconducting wire, which has zero resistance when cooled to a temperature close to absolute zero by immersion in liquid helium. Current flowing in the coil will continue to flow for as long as the coil is maintained at superconducting temperatures, with a loss on the order of ppm per year. The solenoid and liquid helium are kept in a large dewar, surrounded in turn by a liquid–nitrogen dewar, which acts as a thermal buffer between the room–temperature air and the liquid helium [Fig. 3.2].

3.3.2 Low–temperature NMR

The NMR techniques described in Ch. 3.2.1 and 3.2.2 are applied to native liquid spider–silk dope in Ch. 4. Because local ordering among the silk protein is not detectable at
3.3 Instrumentation

Figure 3.2: External appearance (a) and cut-away view (b) of a superconducting magnet system as used for NMR spectroscopy. From outside to inside of the magnet the equipment consists of a vacuum chamber (a) enclosing a liquid-nitrogen reservoir (b). The vacuum chamber is filled with several layers of mylar film for the purposes of thermal reflection. Within the inside wall of the reservoir is a second vacuum chamber (c) filled with reflective mylar. Inside this is the liquid-helium reservoir (d), which houses the superconducting solenoid (e), inside which is the bore tube into which the probe head containing the detection coil and the sample is inserted.

At room temperature due to dynamic averaging effects, the sample was immobilised and studied with a solid-state NMR technique (3.2.3) at low temperature (Ch. 5). This section describes the experimental apparatus employed for the investigation of immobilised liquid silk samples prepared by the shock-freezing technique discussed in Ch. 2.3.2.

A schematic representation of the cryogenic apparatus used to perform experiments at 8 K is shown in Fig. 3.3. A probe head [Fig. 3.4] [116] capable of operating at liquid-He temperatures was constructed in-house and placed in an Oxford Instruments CF1200
cryostat. Prior to cooling, the cryostat, transfer line (P1–11626, A.S. Scientific Products) and probe–head chamber were evacuated over night to a pressure of $2.5 \cdot 10^{-6}$ mbar, and gaseous helium was blown through the cryogenic system to dry it. The sample was transferred from its liquid–nitrogen bath, at a temperature of 85 K, to the pre–cooled probe head by removing it from the chamber. During this process a stream of gaseous nitrogen was directed onto the probe head to reduce freezing of water vapour from the air on the sample and coil. After inserting the probe head the chamber was rinsed several times with gaseous helium before further cooling. Helium pressure in the chamber during the experiments was set to 300 mbar. The temperature for sample insertion was never lower than 85 K, to ensure that the melting points of oxygen and nitrogen were exceeded sufficiently that these gases were prevented from condensing on the probe head. Further cooling led to the desired temperature of 8 K for all experiments.

![Figure 3.3: Experimental apparatus for NMR measurements at 8 K. The cryostat (b) contains a heat exchanger (a) cooled by liquid helium, which is pumped (d) through a transfer line (c) from a 1001 dewar (l). The helium flow is controlled by a flow meter (e) and used gas exits by the He–recovery system (f). Temperature is controlled by a heater (h) and monitored by computer (g). The chamber where the probe head (j) is inserted is filled with gaseous helium from a gas bottle (k).](image-url)

The probe temperature was adjusted by regulating the helium flow by hand with a needle valve on the flow meter (VC30, Oxford Instruments) and a heater (ITC4, Oxford...
3.3 Instrumentation

Instruments) controlled by an external computer using LabVIEW 7 [117]. Both heater output and temperature were monitored continuously by the computer. A temperature stability of ±0.1 K, and at 8 K a liquid-helium consumption between 1.1 and 1.21/h was achieved.

Figure 3.4: Probe head (a) used for the experiments performed on immobilised silk dope at 8 K. Enlargement (b) shows the r.f.-coil, constructed of 0.75 mm wire and containing 4 windings with an inner diameter of 3 mm and a length of 4 mm. A piece of Teflon tape was wound around the soldered joint (arrow) to prevent arcing in the helium atmosphere.

The helium vessel was exchanged every 2.5 days over the three weeks required for data acquisition in the DOQSY experiments. In this procedure the transfer line was removed from the cryostat, the helium dewar was exchanged, and before reinserting the transfer line into the cryostat its tip was treated with a heat gun to prevent ice build-up. A temperature rise from 8 K to approximately 35 K was unavoidable. Although a stable temperature of 8 ± 0.1 K was achieved within minutes, helium flow and heater output had to be adjusted several times over the subsequent 4 h to maintain their optimal values.
3.3.3 Modification of an electrical motor to operate in an NMR spectrometer

The application of DC motors for functions requiring high magnetic fields is fraught with technical difficulties. These include damage to the motor itself because it may become magnetised, distortions of the homogeneous magnetic field due to electromagnetic interferences, and the occurrence of strong magnetic forces which complicate significantly the handling of the motor. This section describes how to modify a DC electrical motor in such a way that it remains usable in the high magnetic fields required for NMR experiments. The discussion leads from a description of the mechanical conversion in the motor to the application of the modified DC motor in the stray field (0.64 T) of a 500 MHz NMR spectrometer of the type employed in Ch. 6 to perform MRI experiments on spiders during silking [Fig. 3.5]. Measurements and graphical results obtained while characterising the operation of the modified motor are presented in App. C.

![Figure 3.5: Schematic representation of experimental apparatus used to perform real-time measurements during the silking of a spider. The silk thread (b) from the spider (a), fixed on the probe head (f), was drawn by the motor (d), placed in the bottom of the spectrometer (c), onto a reel (e).](image-url)
Motor

Figure 3.6 depicts in schematic form a diametrally-magnetised DC motor. A permanent magnet placed along the axis of the motor provides a magnetic field almost radial to the motor axis within the coil [Fig. 3.7(a)]. The field lines of this radial magnetic field are focussed by the magnetic return housing. The combination of the radial field with the currents in the coils surrounding the permanent magnet create the force driving rotation of the rotor [118–121].

Such a motor can only be used in high magnetic fields such as those required for NMR spectroscopy if all magnetic elements are removed. Otherwise strong induced forces may turn innocuous motor components into dangerous projectiles, or magnetic particles could be broken off the motor, distorting the homogeneity of the magnetic field. Removing magnetic elements means removing the permanent magnet and the magnetic
return housing, which removes the source of the static magnetic field. This “internal” magnetic field, essential for the function of the motor, is replaced by an “external” magnetic field [Fig. 3.7(b)] provided by the insertion of the motor into the NMR apparatus. Specifically, in this case the source for the external field was the stray field of the magnet in a 500 MHz NMR spectrometer.

![Figure 3.7: Cross-sections of (a) original and (b) modified diametrally-magnetised DC motors showing the field lines of their magnetic fields. The black circle in the centre represents the rotation axis. The letters I, B and F indicate respectively the current in the coil (light grey circle), the direction of the magnetic field and the resulting force. The force experienced at the coil leads to a clockwise rotation. (a) The permanent magnet (two coloured circular discs around the black circle) is placed along the centre axis, and the field lines of its magnetic field are focussed by the magnetic return housing (outer circle). (b) The permanent magnet and the magnetic return housing are both replaced by aluminium parts of the same size (circular disc around the black circle and outer circle respectively). The external field lines are provided by the magnet of the NMR spectrometer.](image)

Transmission of the rotation of the motor axle to a device is best effected by a spur gear. The spur gear lets the motor run at its optimal rotation frequency, which is close to its maximal rotation frequency, while the device to be driven may turn much more slowly. Because at constant voltage the rotation frequency reduces with increasing load, a gear in combination with a DC motor is considerably more favorable than a slowly turning motor. The MRI experiments [96] performed in Ch. 6 on spiders during silking [94] were therefore conducted using a modified DC motor together with a spur gear. The spider was fixed together with a reel (Ch. 2.4) on a probe head which was placed in the bore of the
3.3 Instrumentation

NMR magnet, and its silk thread was drawn from the spinnerets (Ch. 2.3) onto a reel. The motor, placed below the magnet, drove the reel through the spur gear and thus spooled the silk thread [Fig. 3.8].

![Figure 3.8: Silking a spider during MRI experiments. The silk thread (a) from the spider (b), placed in a half-shell (c) at the upper end of the probe head (d), is spooled by the motor (e) onto the reel (f).](image)

**Materials**

The diametrally-magnetised DC motor A-max16 (No. 110061) [122] was modified to be usable in magnetic fields by complete removal of the permanent magnet and magnetic return housing, and their replacement by cylinders machined in-house to be of the same shape but made of aluminium [Fig. 3.7]. This type of motor has an ironless rotor and commutates the current by graphite brushes. Electromagnetic interference arising from the graphite brushes was not observed in the MRI experiments; it is nevertheless advisable for delicate measurements to use metal brushes, which result in reduced high-frequency interference. Graphite brushes were chosen here because they are more robust to frequent switching on and off of the motor.

The MRI experiments on silked spiders presented in Ch. 6 were performed on a Bruker 500 MHz NMR spectrometer, with the modified motor placed at the bottom of the dewar where the stray field was measured to be 0.64 T. The location for optimal motor function was found by rotating the motor around its axle and moving it radially around the centre axis of the magnet. The rotation of the motor axle was transmitted by a spur gear unit GS16K (No. 201467) [122] to drive the reel spooling the silk thread. The body of the
spur gear unit is made of plastic, while the driveshaft is of corrosion-resistant steel and is therefore slightly magnetic. The silk was spooled onto the reel with a rotation frequency of 0.7 Hz. The operation of the spur gear is such that a rotation frequency of 0.7 Hz at the reel corresponds to a frequency of 54 Hz in the modified motor. In the course of a single imaging experiment the motor would operate for approximately 1 h, drawing some 35 m of silk.
4 Characterisation of native liquid spider dragline silk

The work described in this chapter was published in Ref. [123].

4.1 Introduction

The process of spinning silk from an aqueous, liquid dope to solid, almost insoluble fibres, occurs naturally in many insects and spiders, but remains poorly understood. A characterisation of this process requires detailed structural knowledge of the intermediate phases and phase transitions occurring as the dope is extruded and its hydration altered. Recent progress in this direction has included evidence for certain partially folded intermediates, some of these forming liquid–crystalline phases [9–11], but a full molecular–level model remains to be elucidated.

Spider dragline silk dope is a highly concentrated protein solution (exceeding 30% by mass), these proteins consisting of over 60% alanine and glycine (Ch. 1.1). A particularly mysterious property of the liquid phase is how such a concentrated, viscous and stress–sensitive dope can remain in this state without forming insoluble structures. The solid (fibre) form has long been known to contain both crystalline and amorphous domains, among which alanine–rich regions favour β–sheet formation and glycine residues are found predominantly in helical structures. Considerably less is known about the molecular structure of silk dope and of the intermediate phases formed during spinning.
process, although as detailed in Ch. 1 some forms of secondary structure have been suggested for the case of *Nephila edulis* and more detailed investigations have reported stable secondary structural elements in the silks of other species.

In this chapter the advantages of NMR for studying the structure and function of biological molecules are exploited to investigate the native liquid dragline silk stored within the lumen of the wide sac of the m.a. gland of *Nephila edulis*. Slow MAS is used to obtain $^1$H and $^{13}$C spectra with a resolution considerably enhanced by comparison with previously described spectra [73], permitting an almost complete assignment of the spectral features. From this assignment one may conclude that the silk dope is present in the m.a. gland in the form of an isotropic liquid of molecules with no evidence of secondary structure.

### 4.2 Materials and Methods

#### 4.2.1 Sample preparation

The supply of *Nephila edulis* spiders was assured by the captive breeding programme described in detail in Chs. 2.1 and 2.2. The general procedures for preparation of liquid silk samples is discussed in Ch. 2.3. The specific protocol for producing isotopically labelled samples was to keep mature female spiders for two weeks on a diet of 1–2 mealworms per week, supplemented with two daily doses of 6–8 droplets of an aqueous amino–acid solution (85 mg/ml) reflecting the amino–acid composition of m.a. silk (Tab. 2.1 on page 25), of which a particular amino acid was uniformly $^{13}$C–labelled. The spiders were silked twice during the first week to empty their m.a. glands of unlabelled silk [94]. After the 2–week feeding period the glands contain liquid silk uniformly $^{13}$C–enriched to 3–10% in the particular amino acid. To obtain silk enriched at every site, referred to henceforth as “fully labelled”, all amino acids in the feedstock were uniformly $^{13}$C–labelled and the spiders were fed with an additional 6–8 droplets per day of an aqueous solution of uniformly $^{13}$C–labelled glucose (50 mg/ml). The final liquid silk product has a uniform enrichment of approximately 10% in all of its constituent amino acids, essentially independently of the amino–acid type.
4.3 Results and Discussion

To preserve as closely as possible the native environment of liquid spider silk stored in the wide sac of the m.a. gland prior to the spinning process (dope), only freshly dissected glands were used for all experiments. For the experiments on native silk dope, the m.a. gland was dissected as described in Ch. 2.3.2 and the sample drawn into the MAS rotor by syringe. For the measurements on denatured silk dope, the wide sac of the m.a. gland was placed in concentrated urea and subjected to sonication and vortexing before being centrifuged to provide a supernatant which was loaded into the MAS rotor (Ch. 2.3.2).

4.2.2 NMR experiments

NMR spectra were obtained on a Bruker DMX 400 Avance Spectrometer at 400.13 MHz for $^1$H, using a Bruker 2.5 mm double-resonance magic-angle spinning (MAS) probe head. A carbon and proton field strength of 35 kHz was applied for the hard pulses. The field strength was reduced to 4.5 kHz for carbon decoupling and to 15 kHz for proton decoupling; the decoupling was performed using the GARP scheme [124]. For proton-detected spectra, water was presaturated with a field strength of 65 Hz. The recycle delays for proton and carbon spectra were set to 2 s and to 10 s, respectively. The experiments were carried out at a temperature of 296 K and a MAS frequency between 1.0 and 1.2 kHz (stable to 10 Hz). From 1D spectra it was judged that under these conditions the samples were stable for approximately four days. The chemical-shift scale was referenced to an external TMS sample, and an internal TMS sample was shown to yield an identical reference.

4.3 Results and Discussion

Typical one-pulse proton spectra of silk dope are shown in Fig. 4.1. Upon spinning the sample about the magic angle a reduction in linewidth by a factor of 4 is observed [Fig. 4.1(a)].

The aliphatic portion of the static $^{13}$C one-pulse NMR spectra of fully labelled silk dope of the spider Nephila edulis are shown in Fig. 4.2 in the presence (b) and absence
Figure 4.1: One-pulse $^1$H spectra of silk dope from *Nephila edulis* recorded with (a) and without (b) MAS. The resonance for (Ala)Hb in (a) has been scaled by a factor of 3. For both spectra 4 scans were taken.

(c) of proton decoupling. The splittings in the spectrum presented in Fig. 4.2(c) can be explained completely by the expected $^1$H ←$^{13}$C coupling constants for the alanine and glycine residues, and no trace of the presence of residual dipolar couplings is observed.
As shown in Fig. 4.2(a), the linewidth of the proton-decoupled $^{13}$C spectrum [Fig. 4.2(b)] is significantly reduced by the application of MAS, while no changes in the line positions were observed. The resolved splitting of the glycine $C_\alpha$ resonance at 43 ppm in Fig. 4.2(a) is due to the $^{13}$C–$^{13}$C $J$–coupling, and the remaining linewidth in the MAS spectrum is thought to be dominated by non–resolved homonuclear $J$–couplings. The experimental spectra are fully consistent with the assumption that the silk dope is an isotropic liquid. No indication was observed for liquid–crystallinity: with a liquid–crystalline sample one would expect the appearance of residual dipolar couplings and residual chemical–shielding anisotropy (CSA) effects which, without MAS, would lead to the appearance additional line splittings and to a variation in the line positions. Under MAS, these effects would vanish. In this case the spectra were found to be the same both with and without MAS, except for the significant reduction in linewidth observed under MAS. This can be attributed to the averaging of macroscopic susceptibility effects arising predominantly from the inhomogeneous sample distribution within the partially filled rotor, and to the unavoidable presence of particles of epithelium tissue. However, the possibility remains that liquid–crystalline phases may occur at certain other points in the silk–production process [10, 74, 125, 126].

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<th>$C_\alpha$</th>
<th>$C_\beta$</th>
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<td>Ala</td>
<td>51.0(50.0)</td>
<td>17.3(16.7)</td>
<td></td>
<td></td>
<td>176.3(a)</td>
<td></td>
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<tr>
<td>Gly</td>
<td>43.5(42.7)</td>
<td>(16.7)</td>
<td></td>
<td></td>
<td>172.3(a)</td>
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<tr>
<td>Leu</td>
<td>53.8(53.4)</td>
<td>40.5(40.0)</td>
<td>25.1(24.6)</td>
<td>21.6(20.9)</td>
<td>176.0(a)</td>
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<td></td>
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<td></td>
<td>17.3(16.7)</td>
<td>23.0(22.5)</td>
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<tr>
<td>Ser</td>
<td>a</td>
<td>62.1(61.4)</td>
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<td></td>
<td>172.4(a)</td>
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<tr>
<td>Tyr</td>
<td>56.4(56.0)</td>
<td>37.2(36.4)</td>
<td></td>
<td>a(a)</td>
<td></td>
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<td>116.3(115.9)[C3,5]</td>
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<td>155.5[C4]</td>
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Table 4.1: $^{13}$C chemical–shift (ppm) assignment for native liquid silk within the m.a. gland of Nephila edulis and (in brackets) for the same liquid silk denatured in 7.7 M urea. The chemical shifts refer to TMS. The accuracy of the chemical shift is 0.2 ppm. a: Could not be determined unambiguously.
Figure 4.2: Aliphatic region of one-pulse $^{13}$C spectra of fully labelled silk dope from *Nephipha edulis*. Spectrum (a) was recorded with slow MAS. Proton decoupling was applied in the two upper spectra (a, b). The three strongest resonances are assigned to (Ala)$_2$C$\alpha$ (51 ppm), (Gly)$_2$C$\alpha$ (43 ppm) and (Ala)$_2$C$\beta$ (17 ppm). The $^1$H$-^{13}$C J-coupling leads to the splitting of the resonances by approximately 135 Hz observed in panel (c). $^{13}$C$-^{13}$C J-coupling (50 Hz) is observed only for (Gly)$_2$C$\alpha$ (a, b). The spectra are scaled individually. The number of co-added transients was respectively 1230, 2970 and 4380 in (a), (b) and (c).
4.3 Results and Discussion

The $^{13}$C MAS spectrum of silk dope with natural isotopic abundance is shown in Fig. 4.3. The spectrum exhibits resonances arising from sidechain and backbone sites within the protein. Due to the absence of homonuclear $J$-couplings the lines are narrower (ca. 40 Hz FWHH) than for spectra from $^{13}$C -labelled liquid silk, and allow the assignment of the resonances to sites within the individual amino acids.

![Proton-decoupled one-pulse $^{13}$C spectra of silk dope from Nephila edulis with natural isotopic abundance (4096 scans). The peaks marked with letters are not assigned and may belong to molecules other than the silk protein.](image)

This assignment was obtained from $^{13}$C one-pulse experiments on liquid m.a. silk enriched specifically with a uniformly $^{13}$C -labelled amino acid (Fig. 4.4). Each spectrum shows a significantly increased resonance intensity with respect to the natural abundance spectrum (Fig. 4.3), almost exclusively at sites which can be assigned to the specific amino acid. The level of isotopic scrambling was taken to be low ($\leq 10\%$), in agreement with earlier findings [67, 93]. The assignment is listed in Tab. 4.1 and indicated in Fig. 4.3; it was obtained on the basis of the distinct chemical shifts, the observed $^{13}$C -$^{13}$C $J$-couplings and previously published chemical shifts for peptide systems [102].
Figure 4.4: Proton-decoupled one-pulse $^{13}$C spectra of silk dope from *Nephila edulis* enriched specifically with uniformly $^{13}$C-labelled amino acids. Asterisks denote the resonances enhanced by labelling, which are assigned to sites within the labelled amino acids. The two resonances around 30 ppm are not assigned and may belong to other molecules than silk. They occur in all spectra and are unaffected by the different isotopic labels applied here. The number of co-added transients was 1024 for alanine, glycine and serine, 2048 for tyrosine and 4096 for leucine.
In Tab. 4.2 the chemical shifts of the native liquid silk within the wide sac of the m.a. gland are compared with the chemical shifts both of its denatured form, representing the random–coil structure of liquid silk, and with $C_\alpha$ and $C_\beta$ chemical shifts from various databases [102, 127, 128]. The spectra of liquid silk denatured in 7.7 M urea (spectra not shown) are similar in appearance to the spectra of native liquid silk with only small (< 2 ppm) upfield shifts of the resonance lines. The values obtained in this study match to within experimental accuracy the range of random–coil shifts to be found in the databases (although one should note that these are strongly biased towards globular proteins).

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<tr>
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Table 4.2: Chemical–shift (ppm) distributions for $C_\alpha$ and $C_\beta$ in secondary–structure elements for particular amino acids. a: Could not be determined unambiguously. b: The chemical shifts refer to external TMS and the accuracy is 0.2 ppm. sheet: $\beta$–sheet, helix: $\alpha$–helix, rc: random coil [129]. The values for random–coil structures are taken from Refs. [127–130]. gland: silk dope, urea: silk dope denatured in urea.

The chemical shifts and the absence of any detectable distribution in the resonances, together with the relatively narrow lines, indicates that all amino acids of a given type experience, on the NMR time scale, the same environment. These results suggest strongly that native liquid silk within the wide sac of the m.a. gland is a dynamically disordered protein without well–defined secondary structure. However, it should be noted that the presence of other secondary structures having chemical shifts similar to random–coil values cannot be excluded.
A proton spectrum of the silk dope recorded under MAS is shown in Fig. 4.1(a). The lines are narrow (approx. 20 Hz FWHH), enabling the resolution of many resonances. The assignment of these resonances [Fig. 4.1(a) and Tab. 4.3] was obtained from the assigned $^{13}$C resonances in $^1$H–$^{13}$C–HSQC experiments performed on samples enriched specifically with a uniformly $^{13}$C–labelled amino acid [Fig. 4.5]. The proton shifts corroborate the conclusions drawn from the carbon shifts that silk dope is stored without a preferred secondary structure.

Figure 4.5: Example of a $^1$H–$^{13}$C–HSQC spectrum of silk dope from Nephila edulis enriched specifically with uniformly $^{13}$C–labelled leucine. The peaks marked with a single letter are not assigned and may belong to other molecules than silk. The number of co-added transients was 32 with 1024 data points in the direct and 512 data points in the indirect dimension. The acquisition time was 51.2 ms with an INEPT delay of 1.7 ms.
It is possible that the unassigned peaks in the aliphatic region (indicated A to E in Fig. 4.5) may belong to other amino acids within the liquid m.a. silk. However, no experimental data exist to date from the liquid m.a. gland silk of Nephila edulis enriched specifically with further uniformly $^{13}$C–labelled amino acids.

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<tr>
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<td>6.95(6.89)[H$_{3,5}$]</td>
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Table 4.3: $^1$H chemical–shift (ppm) assignment for native liquid silk within the m.a. gland of Nephila edulis and (in brackets) for the same liquid silk denatured in 7.7 M urea. The chemical shifts refer to TMS. The accuracy of the chemical shift is 0.2 ppm.

a: Could not be determined unambiguously.

### 4.4 Conclusions

Both the $^{13}$C and proton spectra of the liquid silk stored within the wide sac of the m.a. gland of Nephila edulis recorded under MAS are well resolved and permit an assignment of most resonances to particular amino–acid residues. The observation of a single narrow line instead of a distribution of resonance frequencies indicates that identical amino acids, irrespective of their position in the primary structure, are subject to a homogeneous local environment. This is an indication for the absence of partial pre-folding of the protein. The similar chemical–shift values found for native liquid silk and its denatured form, representing the random–coil structure, suggest that the molecule is not stored with well–defined secondary structure. Comparable results were found for silk dope of the silkworm Bombyx mori [78], while the silk dope from the silkworm Samia cynthia ricini forms an $\alpha$–helical structure [80,82]. CD spectroscopy on silk dope from the spider Nephila clavipes suggests the presence of $\beta$–sheet structures, but only in the small volumes and at the downstream end of the m.a. gland [75]. Other studies of spider
silk dope [73] suggested a “dynamic loose helical structure” similar to the silk I structure occurring in solid *Bombyx mori* silk. More recently, two somewhat similar models were postulated independently for silk I [76, 77]. Those models are thought to be stable only for (Ala–Gly)$_x$ sequences, and seem to be unlikely for the poly–Ala sequences found in spider silk.

As noted above, the results reported here are compatible with a random–coil arrangement but do not preclude the possibility of a different structure which is uniform about the entire macromolecule. While no signature of liquid–crystallinity was detected for the silk dope of *Nephila edulis*, the improved spectral resolution and the extensive assignment of the resonances performed here make possible the investigation of other intermediates occurring during spinning, and which may indeed be liquid–crystalline or partially folded.
5 Torsion–angle distribution in immobilised native liquid spider dragline silk

5.1 Introduction

In Ch. 4 the structure of the molecules in liquid silk were shown by high-resolution measurement of chemical shifts using MAS to be consistent with the hypothesis that the proteins are present as random coils. No evidence could be found of secondary protein structure, although this cannot be excluded unambiguously. More detailed structural investigations, in particular to detect possible local ordering of silk proteins, may be performed by NMR on samples which are immobile on the timescale of the experiment (Ch. 3.2.1).

The solid–state DOQSY spectrum (Ch.3.2.3) yields a pattern dependent on both the dihedral angles \( \phi, \psi \) in an amino–acid chain. The information contained in these data is therefore more valuable for structure determination than is that obtained from isotropic chemical shifts in liquid phases. This chapter describes the application of the DOQSY method to determine the distribution of dihedral angles between glycine pairs within the proteins of the immobilised silk dope. Immobilisation was achieved by the shock–freezing sample preparation technique presented in Ch. 2.3, and low–temperature measurements were performed at 8 K as described in Ch. 3.3.2.
5.2 Materials and Methods

5.2.1 Sample preparation

The process for obtaining isotopically labelled silk samples is summarised in brief. Mature female *Nephila edulis* spiders were kept for two weeks on a diet of 1–2 mealworms per week, supplemented with two daily doses of 6–8 droplets of an aqueous amino–acid solution (85 mg/ml). This solution was again composed specifically to mirror the amino–acid composition of m.a. silk (Tab. 2.1 given on page 25) but, in contrast to the samples employed in Ch. 4, only glycine residues were labelled isotopically, and only at the carbon atom of the carbonyl group. The spiders were silked twice during the first week to empty their m.a. glands of unlabelled silk [94]. As in Ch. 4, isotopic enrichment of the silk samples was expected to be on the order of 10 % [123], with low isotopic scrambling (also approximately 10 % [67, 93]). Shortly before the experiments, the m.a. gland was dissected and immediately shock–frozen in liquid nitrogen (Ch. 2.3.2).

5.2.2 NMR Experiments

Data acquisition

All of the experiments on immobilised silk dope were performed on a 220 MHz Varian InfinityPlus spectrometer using a double–resonance probe head constructed in–house (Ch. 3.3.2, Fig. 3.4). Details of the cryogenic equipment and operating procedure for conducting DOQSY measurements at low temperatures may be found in Ch. 3.3.2. The experimental temperature of 8 K was chosen to provide the optimal compromise between sufficiently low temperatures for high sensitivity (with complete sample immobilisation) and sufficiently short relaxation times for efficient measurement.

The one–dimensional $^{13}$C spectra were obtained using a 1D CP pulse sequence with proton continuous–wave decoupling (CW). $T_1$ was determined by a separate saturation–recovery experiment. A similar pulse sequence as in Ref. [112] was used for the DOQSY experiments (Fig. 5.1); this is comparable to that applied by Schmidt-Rohr [109], but the double–quantum coherence was excited by an improved multiple–pulse cycle due to
Antzukin et al. [131] and based on a method by Yen and Pines [132]. In order to avoid effects due to incomplete relaxation, the proton spin bath was saturated before each scan, thus ensuring identical initial conditions. While the 1D CP and DOQSY experiments were recorded at 8 K, the saturation–recovery experiment was performed at 10 K, where $T_1$ for immobilised liquid silk dope was determined to be 11 s.

Radio–frequency field strengths for the proton and carbon hard pulses were 69 kHz and 65 kHz, respectively, and continuous–wave proton decoupling was performed at 100 kHz. Cross–polarisation contact time was 4 ms, excitation time $\tau_m$ to generate a double–quantum state was set to 1.5 ms using a single cycle $\tau_c$, and acquisition time was 0.8 ms. The reconversion time was equal to the excitation time. The inter–scan delay was chosen to be $1.25 \cdot T_1$ for an optimal signal–to–noise ratio, and was set to 20 s. Optimising the cable length between probe head and pre–amplifier minimised phase transients. For each 1D CP experiment 32 transients with 64 data points were co–added. The time

![Diagram](image-url)
required to obtain a single spectrum was 2.75 min, and performing block acquisition led to a total measuring time of 25 min for the final 1D carbon spectrum.

For the DOQSY experiments the number of co-added transients was also 32 with 64 data points in the direct dimension, and 35 data points in the indirect dimension. Block acquisition was performed to average the effects of long-term instabilities. The time to obtain the individual spectra was 6.7 h, and hence the total measuring time for the final DOQSY spectrum was 21 days. Microarcs, typical for helium atmospheres and fortunately always occurring in the noise region and affecting at most individual slices, were replaced by random-noise blocks. Exchanging these artefacts for equivalent slices from other experiments would not generate new noise. These microarcs were observed in 19 out of 87 DOQSY experiments. Calibration of the spectra was performed with the one-pulse $^{13}$C spectrum of an external adamantane sample, where the two peaks are centred at 29.5 ppm and 38.6 ppm [133].

The final 15 out of the total of 87 DOQSY experiments were performed with only 17 data points in the indirect dimension, reducing the measurement time by a factor of 2. As observed in the 72 previous co-added time-domain signals, no data loss occurred because only data points in the noise region were neglected. Random noise was added afterwards in the time domain of the experiments performed with reduced coverage of the indirect dimension. This noise-filling was performed to obtain 87 DOQSY signals with equal numbers of data points in the indirect dimension.

Tests

To test the validity of the experiments and apparatus, this means a good r.f. performance to obtain undistorted CSA tensors upon double-quantum excitation, DOQSY experiments on $^{1-13}$C–alanine were performed at room temperature and at 7 K, and were compared with a DOQSY spectrum obtained at room temperature on a Bruker DMX 400 Avance spectrometer. Proton (carbon) hard pulses were performed respectively at radio–frequency field strengths of 55 kHz (60 kHz) and 65 kHz (69 kHz) for experiments on the Bruker and the InfinityPlus spectrometers. Radio–frequency field strengths for continuous–wave proton decoupling were set to 110 kHz on the Bruker spectrometer and 100 kHz for the InfinityPlus spectrometer. CP contact time for all experiments was 1.7 ms.
5.2 Materials and Methods

The excitation and reconversion time $\tau_m$ for the double-quantum state was 1.5 ms using a single cycle $\tau_c$. The recycle delay for the experiments at room temperature was 4 s. The inter-scan delay for the experiments at 7 K was set to 10 min, as $T_1 > 15$ min for alanine at 7 K.

Data analysis

Data processing. All spectra were processed using the matNMR package [134]. Block acquisition necessitated summation of the individual time-domain signals prior to further processing. The time-domain signal of 9 1D CP spectra was co-added and processed with 400 Hz Gaussian line-broadening [Fig. 5.2].

The DOQSY data was processed in two different ways: (I) the summed time-domain signal of all 87 DOQSY experiments was apodised in both dimensions with 400 Hz Gaussian line-broadening before Fourier transformation to yield the spectra in Fig. 5.2; (II) random-noise-filling of 1024 data points in both dimensions of the co-added DOQSY time-domain data with the noise level from the FID, prior to apodisation with 400 Hz Gaussian line-broadening in both dimensions and Fourier transformation, gave the spectrum used to calculate the probability distribution function (PDF) [Fig. 5.3(a)]. The object of noise-filling instead of zero-filling was to obtain a good Gaussian noise representation in the spectrum as required for the applied fitting algorithm [135]. Typically, the number of data points in the direct dimension is larger than in the indirect dimension. Assuming the signal is not truncated, zero-filling will induce different line-broadening in both dimensions for the noise, because a step function in the time domain convolutes the noise in the spectrum with a sinc function. Since the fitting algorithm assumes Gaussian (uncorrelated) noise in the experimental data, such correlated noise as obtained with zero-filling will be partially interpreted as signal, hence causing artefacts in the analysis. In an attempt to reduce this problem noise-filling instead of zero-filling was used. This involved adding Gaussian noise at the same level as occurred in the acquired FID before the dataset was apodized.

The advantage of the noise-filling procedure was small but noticeable in the resulting probability distribution functions. The distributions were identical within the accuracy of the analysis, but the signals obtained with zero-filling were sharper. Noise-filling
Figure 5.2: Experimental 1D CP (upper) (blue line) and 2D-DOQSY (lower) spectra of the 1-\(^{13}\)C -glycine-labelled m.a. gland of Nephila edulis at 8 K. The principal values for the 1-\(^{13}\)C chemical shift, as determined from a nonlinear fit (red line), are \(\delta_{11} = 247\) ppm, \(\delta_{22} = 180\) ppm and \(\delta_{33} = 87\) ppm, with an accuracy of \(\pm 2\) ppm.

was chosen to be the best approach, a posteriori, after evaluation of the effects of linebroadening and grid resolution (as discussed in the next paragraph Ch. 5.2.2).

**Probability Distribution Function (PDF).** The PDF [Fig. 5.3(d)] was determined by a combination of three regulatory techniques, optimised discretisation, non-negativity and Tikonov regularisation [136]. The kernel matrix for the analysis was a set of basis spectra covering the parameter domains of \(\phi\) and \(\psi\). The basis spectra were calculated in the frequency domain, as functions of the backbone torsion angles, for two neighbouring spins in the primary structure, and were corrected for field strength and DQ excitation time. A single set of CSA principal values was taken independently of the torsion angles. The
Figure 5.3: Experimental 2D–DOQSY spectrum (a) and its corresponding best fit (b) for the m.a. gland of Nephila edulis labelled with l-13C–glycine at 8 K. (c) shows the difference of the two quantities and (d) the probability distribution function. Grid resolution is 20° and line–broadening was adjusted to optimise the fit.

GAMMA C++ simulation environment was used to calculate the kernel spectra [137]. The line–broadening of the spectra in the kernel was a free parameter to be determined from the fit of the experimental spectrum [Fig. 5.3(b)]. It was optimised using the least square of the difference plot of the experimental spectra and the fit [Fig. 5.3(c)]. In addition to the least–square value, direct inspection of the correspondence between calculated fits and experimental spectra was used to refine the fits.

The grid resolution for the resulting PDF, shown in Fig. 5.3(d), was also a free parameter, and was set to 20°. PDFs with higher resolutions, showing better–resolved signals, purport to have an accuracy which exceeds the limit of the analysis. The maximum attainable resolution is given by the $S/N$ ratio and the shape of the spectrum. A broad spectrum representing a wide variation in torsion angles leads necessarily to a broad PDF.
This is explained by the fact that the resolution of the PDF is expected to decrease if the complexity of the distribution of $\phi$ and $\psi$ underlying the spectrum increases.

To illustrate the last point, Fig. 5.4 shows the PDFs for immobilised liquid silk dope from the spider *Nephila edulis* at four different grid resolutions, 10°, 15°, 18° and 20°. All four PDFs contain the same information concerning the torsion angles represented in the sample, as the intensities in this Ramachandran map are found at same positions. Because the values of the torsion angles vary within a relatively broad range, the distribution of $\phi$ and $\psi$ is better represented by choosing a lower grid resolution. A second indicator that a lower resolution is required is the shape of the fitted spectrum itself [Fig. 5.5]: this broad spectrum indicates a wide variation in torsion angles, whence broad intensities are appropriate in the Ramachandran map.
5.3 Results and Discussion

Figure 5.5: Fits of the experimental DOQSY spectrum of immobilised liquid silk dope from the spider *Nephila edulis* at 8 K. (a), (b), (c) and (d) show fits using grid resolutions of 10°, 15°, 18° and 20°, respectively.

However, varying the grid resolution from 10° to 20° shows no major differences among the fits, as is to be expected for an appropriately chosen regularisation procedure. The difference plots of experimental spectra and corresponding fits are similar for the four different grid resolutions [Fig. 5.6], proving that this is the case.

5.3 Results and Discussion

Performing experiments at 8 K improves the sensitivity because a lower temperature $T$ enhances the Zeeman population ratio $(p_a/p_d)$. At these temperatures the liquid silk is expected to remain fully immobilised for the entire duration of the lengthy DOQSY measurement. However, experiments at this low temperature have two negative attributes, an
increased longitudinal relaxation time $T_1 (T_1(5 \text{ K}) = 16.5 \text{ s, } T_1(10 \text{ K}) = 11 \text{ s})$ and frequent changes of the helium dewar. The longer measurement time at very low temperatures was the primary reason for the choice of $8 \text{ K}$ as the experimental temperature, which also represented a suitable compromise between sensitivity and helium consumption. The frequent dewar changes led to unwanted temperature cycling and ice accumulation, which was controlled as described in Ch. 3.3.2.

The reliability and accuracy of experimental equipment and procedures were verified by measuring the DOQSY spectra of $1-^{13}\text{C} –$alanine at room temperature and $7 \text{ K}$ [Fig. 5.7(a)]. The superposition of the two spectra shows no significant differences, indicating that the temperature has no influence on the excitation of the double–quantum state. Comparing these two spectra with a DOQSY spectrum obtained on a Bruker DMX 400
Results and Discussion

Avance spectrometer at room temperature [Fig. 5.7(b)] shows all of the same characteristic features. Thus the experiments performed using the purpose-built He-temperature probe head are shown to reproduce the expected DOQSY spectrum for 1-\textsuperscript{13}C-alanine.

![Figure 5.7: Experimental DOQSY spectra of 1-\textsuperscript{13}C-alanine taken at the DQ frequency in the indirect dimension and the SQ frequency in the direct dimension. (a) Spectrum measured at room temperature on Bruker DMX 400 Avance Spectrometer. (b) Spectra obtained using purpose-built He-temperature probe head on a 220 MHz Varian InfinityPlus spectrometer: surface plot of spectrum obtained at room temperature superposed on contour plot of the spectrum obtained at 7 K.]

The experimental 1D CP and 2D DOQSY spectra of immobilised liquid silk dope at 8 K, labelled at 1-\textsuperscript{13}C-glycine residues, are presented in Fig. 5.2. The principal values for the 1-\textsuperscript{13}C chemical shift for this sample were \(\delta_{11} = 247\ \text{ppm}, \delta_{22} = 180\ \text{ppm}\) and \(\delta_{33} = 87\ \text{ppm}\) (with an accuracy of \(\pm 2\ \text{ppm}\)), as determined from a nonlinear fit of the experimental spectra by an analytical function fitting the line shape of the CSA tensor [138]. Because of the structural information encoded in NMR chemical shifts, these constitute a valuable adjunct to the procedure of structure determination. However, chemical shift values, which reflect the local structure of protein backbones, are not unique for a given secondary structure. Particularly for glycine residues, these are difficult to use because they are rather insensitive to changes in the backbone torsion angles \(\phi\) and \(\psi\). For this reason DOQSY measurements are invaluable in providing an accurate determination of the angle distribution \((\phi, \psi)\) among the glycine pairs in immobilised silk dope.

The experimental DOQSY spectrum used for the analysis is presented in Fig. 5.3(a). A spectrum with the same lineshape for both signal and noise is obtained. The additional
noise does not affect the information in the resulting PDF (Ch. 5.2.2). Figure 5.3(b) shows the corresponding best fit. The quality of the fit may be judged by the difference plot of the two spectra [Fig. 5.3(c)], which shows that the fit has a $S/N$ form very similar to the experimental spectrum, and no evidence of significant artefacts. The resulting PDF is shown in Fig. 5.3(d), and has the inversion symmetry \( (\phi, \psi) = (-\phi, -\psi) \) which is a characteristic feature of DOQSY experiments [108]. As noted in Ref. [136], the transformation \( (\phi, \psi) \leftrightarrow (\psi, \phi) \) also results for many cases in very similar DOQSY spectra. In this study, the interpretation of all PDFs is focussed only on the left half of the Ramachandran map. Abundant signal is found in the region around \( (\phi, \psi) = (-60^\circ, 140^\circ) \), and a less broad but equal intense maximum is found for \( (\phi, \psi) = (-140^\circ, 80^\circ) \). In addition, two less intense signals are found for \( (\phi, \psi) = (-80^\circ, -20^\circ) \) and \( (-60^\circ, -60^\circ) \). This relatively broad distribution of the torsion angles supports a structure close to that of a random coil for the glycine residues [139].

Figure 5.8 compares the best fits of experimental DOQSY spectra and the corresponding PDFs of four different silk samples: immobilised liquid silk dope (1) and solid dragline silk (2) of the spider *Nephila edulis*, and solid fibres from cocoon filaments (3) and cast film from an aqueous silk fibroin solution (4) of the silkworm *Samia cynthia richini*. Preparation of the silkworm samples is described in detail in Ref. [79]. All of the PDFs from these $^{13}$C–glycine–labelled samples exhibit a number of maxima distributed across the Ramachandran plot [Fig. 5.8(b)]. Those of the immobilised liquid silk dope are approximately twice as broad as those from the other two fibrous samples, which have a well-defined local structure. Partial $\beta$–sheet formation and a largely $3_1$–helical arrangement has been proposed for the glycine–rich domains in the fibrous silks [67, 70, 112]. By comparison, the broad maxima in the PDF of the immobilised silk dope support a disordered structure for the glycine residues. The PDF of the cast film from the silkworm corresponds to a significantly disordered system, and is also consistent with a random–coil structure for the glycine–rich domains [112]. By the definition of Flory [139] a random–coil structure is one in which the spatial configurations of a system of chain molecules show no recurring pattern of replication of bond conformations along the chain, and in addition comprise the overwhelming majority of all possible configurations of the long–chain molecule. The resulting chain statistics in polypeptides are described, approximately, by the statistics of pairs of amino-acid residues, since interactions between adjoining pairs are small. The steric interactions within a residue are mainly
5.3 Results and Discussion

Figure 5.8: Experimental 2D-DOQSY spectra (a) and the corresponding PDFs (b) of $\text{I}^{13}\text{C} - \text{glycine}-\text{labelled}$ silk samples: (1) immobilised m.a. gland silk from the spider Nephila edulis at 8 K, (2) solid dragline-silk fibre of the spider Nephila edulis at room temperature, (3) fibre of the silkworm Samia cynthia richini at room temperature, (4) cast silk film from the silkworm Samia cynthia richini at room temperature. Chosen grid resolutions were respectively 20°, 15°, 18°, 20° for (1), (2), (3) and (4). Data sets (2-4) are reproduced with permission from Ref. [136].
responsible for the energies of residues, which vary with the torsion angles, and these energies define the probability of occurrence in the random-coil state. Because the form of the PDF found for the immobilised liquid silk dope of *Nephila edulis* is rather similar to that of the cast film [Fig. 5.8(b)], the same random-coil interpretation is supported.

By comparing the best fits [Fig. 5.8(a)], clear similarities may be observed between the fibrous samples and between the immobilised liquid silk dope and the cast film. The best fits of the fibrous samples show a clear X-shaped pattern, while the other two samples show enhanced signal around the centre of their spectra. Because all samples with similar PDFs show similar best fits, previous predictions based on PDFs alone may be assumed to be reliable. Thus the glycine-rich domains within immobilised liquid silk dope do not appear to contribute to a well-defined structure.

### 5.4 Conclusions

The structure of immobilised native liquid dragline silk from the spider *Nephila edulis* was investigated using solid-state NMR techniques and an isotopic labelling strategy giving access to local protein conformation. Specifically, the distribution of backbone torsion-angle pairs $\phi$ and $\psi$ between glycine–glycine pairs within the immobilised silk dope was determined successfully by applying a regulatory analysis procedure to measured 2D-DOQSY spectra.

The wide variation in torsion angles, together with a PDF pattern very similar to that found for the random-coil structure of the glycine-rich domains in cast films from the silkworm *Samia cynthia richini* [112], supports a disordered structure for the glycine residues within immobilised silk dope. This is in accordance with conclusions from previous studies of native liquid dragline silk (Ch. 4), where it was postulated on the basis of chemical-shift measurements that the viscous silk dope forms an isotropic liquid consisting of molecules with no well-defined secondary structure. Although the results from these earlier experiments were consistent with a random-coil arrangement, a different structure, uniform about the entire macromolecule could not be excluded.
5.4 Conclusions

Native liquid spider dragline silk was shock-frozen with the aim of studying the native molecular structure in the absence of dynamic averaging. However, both protein-folding processes and shock-freezing occur on the same timescale (i.e. ms), and thus it is debatable whether the molecules are in fact trapped instantaneously in exactly the conformation at the moment of immersion in liquid nitrogen. The “snapshot” obtained by shock-freezing shows that the glycine-rich domains within immobilised liquid silk dope do not contribute to a well-defined local structure, or to any other uniform secondary-structure elements. Under the assumption that no conformational changes, such as denaturation or protein-folding, occur during the timescale of the shock-freezing process, one may conclude that the glycine-rich domains of the silk proteins contained in the viscous liquid of the wide sac of the m.a. gland form random coils.

Other investigations of liquid spider silk dope have postulated \( \beta \)-sheet structures in the small volume at the downstream end of the m.a. gland of the spider *Nephila clavipes* [75], or suggested a “dynamic loose helical structure” similar to the silk I structure of solid *Bombyx mori* silkworm silk [73,76,77]. Because the vast majority of residues show backbone angles corresponding to \( \alpha \)-helices and \( \beta \)-sheets, even in the absence of defined secondary structure [140], these possibilities cannot be excluded unambiguously.
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6 Micro-imaging of spiders and localised spectroscopy

6.1 Introduction

MRI is an attractive, noninvasive, in-vivo tomographic technique which provides anatomical and physiological information in three dimensions at a high spatial and temporal resolution.

This chapter reports a set of procedures which make possible the imaging of live spiders, presents high-resolution pictures of anatomical details within the body of the spider and discusses the advantages of MRI in comparison to other classical techniques such as histological preparations. However, the focus of this study remains the native liquid silk within the wide sac of the m.a. gland of *Nephila edulis*. In this regard, 1D $^1$H spectra obtained by localised spectroscopy on the A-zone, the central region and the B-zone of the wide sac are compared to the MAS spectra discussed in Ch. 4, and feasibility studies are presented for monitoring in real time the changes within the wide sac which occur during silking. Feeding of the spider with chelates containing gadolinium as a paramagnetic contrast agent allows the study of the intestine and adjunctive organs involved in digestion and food storage and permit the dispersion of gadolinium-labelled water to be monitored. When conducted in combination with spider silking these techniques may provide a basis for further experiments investigating the role of water in this unique process.

The presentation of three-dimensional objects in only two dimensions requires the specification of the perspective (the side from which the object is viewed). Common
expressions used in MRI to determine the position of a three-dimensional object in a two-dimensional image are coronal, sagittal and axial views. These three terms are explained in Fig. 6.1.

![Diagram](image)

Figure 6.1: Coronal, sagittal and axial views of an object are defined by slices taken parallel to the planes (a), (b) and (c) respectively. Thus a coronal imaging plane bisects the body into top and bottom, a sagittal imaging plane bisects the body into left and right parts and an axial imaging plane bisects the body into front and back parts.

Another expression used frequently in the visualisation and analysis of medical and scientific data is voxel. This is a portmanteau of the words “volumetric” and “pixel”, and describes a volume element representing a value in three-dimensional space. Here voxel is used to define the size of the observed volume element.

### 6.2 Material and Methods

#### 6.2.1 Sample preparation

Because all MRI measurements were performed in-vivo, it was essential to keep the spider still to prevent blurred images caused by unwanted movements. This immobilisation
was achieved by fixing the spider with elastic medical gauze sewn onto a piece of Styrofoam (Ch. 2.4). The abdomen was positioned in the center of a 1.0x1.5x7.0 cm Styrofoam block with the 4 front and hind legs elongated and oriented along the axis of the block. This construction of the immobilised spider and its holder was placed into the resonator of the probe head and fixed by transparent tape [see Fig. 2.4 on p. 30].

For the "real-time silking" experiments, where the spider was silked while the m.a. glands were monitored, the spider was placed on its back to allow the required access to its spinnerets. The Styrofoam block holding the spider was taped into a half-shell containing the reel to spool the silk thread [Fig. 2.5, p. 31], and the shell inserted into the resonator of the probe head.

MultiHance [97] was used as a contrast agent for the experiments monitoring the intestine. Spiders were fed with 15 drops of an aqueous 1 % (v/v) solution immediately prior to fixation on the Styrofoam block.

### 6.2.2 Contrast agent: MultiHance

MultiHance [97] is a clear, colourless aqueous solution containing gadobenic acid as the dimeglumine salt [Fig. 6.2]. The paramagnetic gadolinium ion is complexed with a chelating agent to form gadobenate, which consists of a stable coordinating sphere around the gadolinium ion. It is salified with meglumine to yield a 0.5 M solution; the generic name of this complex compound is Gd–BOPTA/Dimeg.

Because it is a paramagnetic compound, Gd–BOPTA/Dimeg shortens the longitudinal ($T_1$), and, to a lesser extent, transverse ($T_2$) relaxation times of surrounding (tissue) water protons, and thus improves the contrast of the image. MultiHance is indicated for use in diagnostic MRI of the liver and the central nervous system (brain, spine and surrounding structures) to visualise lesions or metastatic diseases. After rapid intravascular distribution, the highly hydrophilic gadobenic ion diffuses rapidly into the extracellular fluid space. The substance is excreted partly by the kidneys (75 – 90 %) and partly by the biliary system (10 – 25 %) [141–143].
6.2.3 Experiments

The experimental programme reported in this chapter was made possible by the generous assistance of Bruker Biospin (Germany) in donating three two-day blocks of instrument time.

MRI experiments were performed on a Bruker AV-500 spectrometer with a gradient microimaging accessory, at a frequency of 500.134 MHz for $^1$H, and using a Bruker “micro-mouse probe head” with a 30 mm $^1$H resonator. All experiments were conducted at a temperature of 283±2 K to sedate the spider and to assist in minimising stress factors which could conceivably alter its metabolic processes. The spooling system and technical equipment to perform the real-time silking experiments are described in detail in Chs. 2.4 and 3.3.3.
1D proton-detected spectra were obtained using point-resolved spectroscopy (PRESS) \[144-146\]. Synchronous r.f. pulses were applied to effect localised excitation. Water suppression was achieved by chemical–shift–selective r.f. pulses with a bandwidth of 180 Hz (VAPOR) \[147-150\]. The echo time (TE) and repetition time (TR) were set to 20 ms and 1.5 s, respectively.

Two– and three–dimensional MRI datasets were obtained using either $T_1$–weighted 3D Spin–Echo (SE3D) experiments \[151, 152\] or one of two fast imaging techniques, $T_1$–weighted 3D FISP \[151, 153–156\] or $T_2$–weighted 2D RARE \[151, 157–159\]. Table 6.1 indicates the acquisition parameters, including the pulse sequences, employed to obtain the images displayed in the figures within this chapter.

The time required to obtain an image dataset was dependent on the desired quality (e.g. spatial resolution and $S/N$), the dimension (2D or 3D) and the temporal resolution. Thus the measurement times of the experiments presented here range from 4 min to 14 h \[Tab. 6.1\].

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Table 6.1: Acquisition parameters used for the experiments shown in Figs. 6.3, 6.4, 6.7, 6.8 and 6.9. Abbreviations: Meth. = Method, Thi. = Excited slice thickness, MTX = Matrix size, FOV = Field of view, Res. = Resolution, TE = Echo time, TR = Repetition time, TA = Total acquisition time.
6.3 Results and Discussion

Figure 6.3(b), (c) and (d) shows projections from different directions of a three-dimensional spider MR image. Extracted 2D slices [Fig. 6.3(a)] provide detailed structural information within the selected layer, similar to those obtained from histological transections. The schematic representation inserted in Fig. 6.3(a) gives an overview

Figure 6.3: Sagittal (b), coronal (c) and axial (d) projections of a 3D FISP image of the spider Nephila edulis with the two m.a. glands appearing as the bright, shining features. Panel (a) shows the difference in information provided by a sagittal slice of this image in comparison with a schematic drawing based on a histological transection of a spider. The time required to record the data was 1 h. Drawings courtesy of Rainer F. Foelix (see picture credits in App. D)
of the organs within the opistoma and prosoma, but does not represent their precise locations and proportions. A case in point is provided by the m.a. silk glands, which can be recognised in Figs. 6.3(b), (c) and (d) as two bright, crescent-shaped objects. This type of experiment (3D FISP) shows enhanced signal intensity for tissue with short $T_1$ because the signal is predominantly $T_1$-weighted. The relative high protein concentration within the gland (30%) reduces $T_1$ of its content and therefore the glands appear bright. In fact the m.a. glands may be identified not only by their contrast but also by their location and shape, which are known from previous dissection studies (Ch. 2.3.2, Fig. 2.3). The difference in brightness of the two glands in Figs. 6.3(b), (c) and (d) may be due to unequal protein concentrations within the glands, but may also be explained by technical aspects of the data-acquisition procedure.

Localised spectroscopy provides a means of accessing further specific information concerning the m.a. silk glands and their contents. In this experiment 1D $^1$H spectra were measured at specific locations within the gland: the A–zone, the central region and the B–zone. The positions and sizes of the voxels defined to obtain the spectra from these specific regions are shown in the three consecutive slices of the 3D FISP dataset in Fig. 6.4. Because the gland is crescent-shaped the three voxels differ in size: the selected volumes within the A– and B–zones were of necessity smaller than that used for the central region. These voxels were positioned to be fully inside the required part of the gland, specifically avoiding the selection of external volume, by using the water resonance for their localisation. Thus the voxels shown in Fig. 6.4 apply only for the water resonance, and for the other resonances are displaced in space in proportion to their chemical-shift difference from the water resonance. The maximal spatial displacement of the localised volume for a resonances appearing at the edges of the spectrum (i.e. with a chemical-shift difference of ±4 ppm) were 0.40 mm for the A–zone, 0.33 mm for the B–zone and 0.45 mm for the central region [115]. These voxel displacements are responsible for distortions of the resonance ratios within the measured spectra.

Figure 6.5 shows the in–vivo localised spectra together with an ex–vivo MAS spectrum from Ch. 4 [Fig. 4.1]. The chemical–shift scale of the in–vivo spectra was referenced to the water resonance of the ex–vivo spectrum, under the assumption that no changes in pH occurred either within the wide sac or during dissection. Comparison of the three in–vivo 1D proton spectra shows distinct $S/N$, but no significant changes in the distribution of the individual resonances. The differences in $S/N$ are explained by the unequal voxel
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Figure 6.4: Three different sagittal slices of a 3D FISP image of the spider Nephila edulis displaying one of the two m.a. glands as a bright feature. The head of the spider is oriented upwards while the front of the abdomen is oriented to the left. Panels (a), (b) and (c) indicate the position and size of the voxel used to perform localised spectroscopy on the A-zone, the central region and the B-zone of the gland.

sizes and the different numbers of scans taken. The weaker S/N for the spectra of the A- and B-zones is due primarily to the smaller voxel size. The variation in FWHH of the water peak indicates different local magnetic-field inhomogeneities around the individual voxels. This variation may be explained by the different environments of the three voxels within the sample, and contributes slightly to differences in the resolution among the three spectra. Changes in the proton chemical shift which would indicate structural alterations in the silk-protein molecules occurring along the wide sac of the m.a. gland, as postulated in previous studies [75], are of order 0.2 ppm [160–162]. Within the resolution of the spectra such changes are not observed. The distortion of the intensity ratios for resonances above 7 ppm and below 2 ppm is a consequence of the spatial displacement of the voxels discussed above. This effect is stronger for the A- and B-zone spectra, where even small displacements cause part of the voxel to be moved outside the gland [Fig. 6.4].

Comparison of the ex-vivo and in-vivo spectra reveals that the chemical shifts are very similar. The differences in the linewidths may be explained by inhomogeneities within the environments of the three voxels, and by the fact that the ex-vivo spectrum was
6.3 Results and Discussion

MAS
NS: 4
A-zone
V: 0.90 mm$^3$
NS: 1024
FWHH: 85 Hz

Tyr
Tyr
^Mk^Ji

Central
V: 1.00 mm$^3$
NS: 1024
FWHH: 36 Hz

B-zone
V: 0.75 mm$^3$
NS: 1500
FWHH: 26 Hz

Figure 6.5: $^1$H spectra of silk dope from Nephila edulis. (a): spectra of a dissected native m.a. gland at 400 MHz, recorded under 1 kHz MAS (Ch. 4, p. 52). The resonance at 1.5 ppm (Ala $H_B$) has been scaled by a factor of 3. (b), (c) and (d): in-vivo spectra at 500 MHz from the A–zone, the central region and the B–zone of the m.a. gland, respectively. The water resonance at 5.1 ppm is cut at 45%, 35% and 65% of its original intensity in the three panels. Its FWHH is 85 Hz, 36 Hz and 26 Hz. 4 (a), 1024 (b, c) and 1500 (d) scans (NS) were taken, with voxel sizes (V) of 0.90 mm$^3$ (b), 1.00 mm$^3$ (c) and 0.75 mm$^3$ (d). Spectra (b), (c) and (d) were apodised with 10 Hz Gaussian line-broadening.
Figure 6.6: $^1$H spectra of silk dope from Nephila edulis. (a, b) and (c): spectra of a dissected native m.a. gland at 400 MHz, recorded under 1 kHz MAS (Ch. 4, p. 52). 4 scans were taken and apodised with 2 Hz, 20 Hz and 40 Hz Gaussian line-broadening (lb) in the three panels. The resonance at 1.5 ppm (Ala $H_β$) in panel (a) has been scaled by a factor of 3. (d): in-vivo spectra at 500 MHz from the central region of the m.a. gland. 1024 scans were taken with voxel sizes of 1.00 mm$^3$. The spectra was apodised with 10 Hz Gaussian line-broadening. The water resonance at 5.1 ppm is cut at 35% of its original intensity.
6.3 Results and Discussion

recorded under MAS. The similarity of ex-vivo and in-vivo spectra is better seen when the MAS spectrum is apodised with 20 Hz or 40 Hz Gaussian line-broadening [Fig. 6.6]. The different intensities for the (Gly)Hα resonance at 4.12 ppm are not understood. Nevertheless, the overall similarity supports the assumption that no changes to the overall folding were caused during sample preparation for the measurements performed on dissected m.a. glands (see Ch. 4), and that the conclusions drawn from these latter experiments do indeed refer to native liquid spider dragline silk.

The effect of silking on the wide sacs of the m.a. silk glands is presented in Fig. 6.7. This figure shows slices of a T₁-weighted 3D FISP image selected to display the wide sac in sagittal, axial and coronal views. Images were taken during silking (Ch. 2.4 and 3.3.3) for a test period of 45 min, and show distinct reductions in gland size and brightness. This loss of contrast and size can be attributed primarily to the removal of silk material due to the spinning process. More specifically, a reduction in brightness indicates an increase in T₁. This may be attributed to an increase of unbound water ("long-T₁ pool") within the wide sac due to removing silk protein. The observed reduction in size and brightness then indicates a process where the silk molecules leaving the wide sac are accompanied by bound water (i.e. "short-T₁ pool"), which is then retained in the duct and is probably returned to the digestive system as the thread is spun. However, definitive judgements based on such relatively short silking periods are complicated by the fact that spiders with completely full glands can spin a fibre for more than 8 h. The techniques employed here are readily extendable for the longer-term experiments required to obtain a more detailed picture of the emptying and filling of the m.a. gland during and after silking. Such experiments could not be performed here due to the limited measurement time available. Further real-time silking experiments combined with the administration of a contrast agent, which should shed additional light on the role of water during the spinning process, are discussed later in this chapter.

The effect of feeding MultiHance to Nephila edulis is shown in Fig. 6.8(1), which displays sagittal, axial and coronal projections of a T₁-weighted 3D FISP image of the prosoma and opistoma of the spider. The gadolinium reduces the longitudinal relaxation time T₁ of the surrounding water protons and leads to an increased signal intensity for regions containing gadolinium-labelled water. The intestine and its associated structures appear extremely bright 10 min after administration of the contrast agent, and the cacea
Figure 6.7: Sagittal (a), axial (b) and coronal (c) slices of the opistoma of *Nephila edulis* during real-time silking. Slice thicknesses are $156\,\mu m$ (a), $117\,\mu m$ (b), and $156\,\mu m$ (c). In panels (a) and (b) the head of the spider is oriented upwards, while in (c) it points away from the observer with the front of the abdomen oriented downwards. This series of images shows the behaviour of the m.a. gland immediately before (1) and during the silking process, after 15 min (2) and 45 min (3).
are observed as a particularly well-resolved ramification. The time-dependent distribution of the contrast agent before and after silking, for a period of 4.5 h, was also monitored for a subsequent period of approximately 13 h [Fig. 6.8(2)]. Soon after silking ends, the previously clear, well-defined structures vanish, and are replaced by more blurred appearance of the opistoma. Over the next 12 h high contrast of the intestine and its associated structures reestablishes. This disappearance and reappearance of the high intestinal contrast indicates a redistribution process of both gadolinium-labelled and unlabelled water within the spider. While it is tempting to relate this redistribution of water to the spinning process, a direct connection is only possible after the analogous time-series measurements without silking have been performed.

A detailed investigation of the role of water in the spinning process (i.e. water retention in the duct and water management in the spider’s metabolism; see Ch. 1.1.3) would require more specific experiments of this type. It would be possible to obtain more detailed information with the aid of experiments performed using different concentrations of the contrast agent: in the event that there is water transfer from the digestive tract into the surrounding tissue, different signal intensity would be observed for the different concentrations of gadolinium administered; if such a transfer process occurs opposit (i.e. from surrounding tissue into the digestive tract), the contrast of the surrounding tissue would remain constant.

Completely different experiments which would deliver further understanding of the digestive process itself may also be designed. One example would be to investigate the criteria governing the filling of the coxae, by performing a series of experiments where in the place of water the spider is fed with different kinds of prey infused with contrast agent, and the process of digestion and food storage is monitored not only for a few hours but for several days.

Figure 6.9 shows a variety of in-vivo MR images displaying anatomical details within the body of the spider. These were obtained by using standard imaging techniques ($T_1$-weighted 3D SE3D, $T_2$-weighted 2D RARE) applied routinely in clinics or medical studies to give explicit information on specific structures and their native environments. Images of the prosoma [Fig. 6.9(3)] of the relatively small spider *Nephila edulis* were the basis for a series of experiments monitoring the dispensing of venom by the much larger
Figure 6.8: Projections of 3D MR images of Nephila edulis after feeding with the contrast agent MultiHance, before (1) and after (2) silking for 4.5 h. Panels (1a), (1b) and (1c) show sagittal, axial and coronal views of the intestine, appearing as a bright feature 10 min after feeding. The caeca are seen as a clearly resolved ramification. Panels (2a), (2b), (2c) and (2d) show the distribution of the contrast agent 0.5, 4.5, 8.5 and 12.5 h after silking. The storage of the contrast agent in the coxa is observed most clearly in panel (2d).
hunting spider *Cupiennius salei*, and were used to support the biochemical investigation of gland cells involved in the previously unexplained mechanism of venom ejection [163].

While the implementation of these imaging techniques is well-known for phenotyping studies of rats and mice, their application to spiders is, to the best of our knowledge,
new. The high quality of the images obtained here verifies that these MRI methods are suitable for studies of spiders. The combination of such MR images with other biological and (bio)chemical methods then represents a technique for a detailed investigation of the life-sustaining processes (e.g. lung and "kidney function", digestion, silk and venom production) in spiders, as well as analogous studies of other small animals. This in-vivo characterisation and measurement of biological processes at the cellular and molecular level, termed "molecular imaging" [164, 165], is a growing research discipline; it is, at least for rats and mice, a well developed and rather active field. One example taken from spider research would be the direct injection of contrast agent into the m.a. glands to monitor the changes in protein/water concentration along the spinning duct. Using MRI as a control step prior to dissection of m.a. glands also has the advantage of avoiding the possible killing of a spider while the ovaries are full of eggs [Fig. 6.9(1)].

6.4 Conclusions

MRI was used to perform noninvasive, in-vivo experiments on the spider Nephila edulis, and a number of applications of the technique were illustrated by feasibility studies.

It was shown to be possible to obtain proton spectra by localised spectroscopy on voxel sizes as low as 0.75 mm³ within the wide sac of m.a. gland. Although these spectra have relatively poor $S/N$, they were of sufficient quality for meaningful comparison with spectra obtained from ex-vivo glands under MAS. The similarities between these spectra support the assumption that the conclusions drawn from the experiments on dissected m.a. glands are indeed appropriate to native liquid spider dragline silk. The ex-vivo MAS spectra (presented in chapter 4) are obtained using the entire wide sac and represent a superposition of the spectra from different parts of the sac; by contrast the in-vivo spectra allow in principle the possibility of monitoring conformational changes in silk-protein structure along the wide sac. Furthermore, the in-vivo method could allow the observation of conformational changes that are induced by the shear forces during spinning. However, in this preliminary investigation the available spectral resolution and the rather weak $S/N$ preclude the observation of any changes in the spectrum indicative of potential structural differences. Extensive shimming and longer measurement times may be expected to improve the resolution and $S/N$, respectively. In fact the observation of tiny
6.4 Conclusions

Conformational changes along the wide sac, as postulated in Ref. [75], would probably require $^{13}$C spectroscopy because in this case structurally induced changes of chemical shifts are greater, and thus more readily detectable. The disadvantage of $^{13}$C spectroscopy is the low natural abundance of the NMR-active carbon isotope, for which specific labelling strategies similar to those described in chapter 2.3.1 would be necessary.

The experiments within this chapter demonstrated two specific applications for this combination of spatial and temporal imaging, namely monitoring of the m.a. gland during silking and the dispersion in the metabolic system of water containing a contrast agent, both before and after silking. These preliminary analyses indicate the feasibility of significantly more sophisticated studies of, among other processes, the digestion, silk production and dispensing of venom in spiders.

Feeding the spider with different types of prey or artificial food of diverse consistencies, both containing contrast agents, may be exploited to investigate the digestive cycle, and specifically the process of enzyme secretion, which remains to be elucidated in full. Real-time silking experiments in combination with the application of contrast agents can be expected to provide the basis for potential experiments studying the role of water uptake and retention during the spinning process. Experiments measuring the amount of water consumed, and the weight of the spider, the silk produced and the material excreted, could be taken together with volume calculations based on 3D images of the silk gland during silking, to obtain a better macroscopic understanding of fibre formation along the spinning duct.

With the advent of stronger magnetic fields allowing better $S/N$ in MRI images, as well as the development of stronger field gradients which at the time of writing extend the spatial resolution of MRI images to the $10 - 50\, \mu m$ range [166], it is clear that MRI technology is advancing to a level where it is capable of providing qualitatively new insight into spider phenotyping. While MRI will not replace classical (bio)chemical and biological techniques such as histological staining, light scattering and electron microscopy, when employed in parallel with these methods it is an outstanding technique for addressing fundamental biological questions.
7 Concluding Summary and Outlook

This thesis reports on the NMR–spectroscopic investigation of the spider *Nephila edulis* and its native liquid dragline silk.

Initial experiments analysed native silk dope at room temperature by applying slow magic–angle sample spinning. The NMR data obtained were consistent with an isotropic liquid phase because (I) the reduction in linewidth could be ascribed to averaging susceptibility effects arising from an inhomogeneous sample distribution within the rotor rather than to residual dipolar couplings and (II) no chemical–shielding anisotropy effects were observed. The narrow width of the resonance lines in the proton and carbon spectra allowed these to be assigned almost completely. From the observed chemical shifts one may conclude that the silk protein within the wide sac of the m.a. gland is dynamically disordered throughout the molecule, by which is meant that each amino acid of a given type is subject on average to an identical environment.

A sequence of experiments was then initiated to investigate possible local ordering among the silk proteins of the native liquid dope, which however would be dynamically averaged to appear disordered at room temperature. For this the silk molecules, labelled isotopically at specific amino–acid sites, were immersed in liquid nitrogen, and thus assumed to be trapped in their specific conformations at the moment of shock–freezing, although in principle folding processes triggered by the shock–freezing process cannot be excluded because both occur on the same timescale (ms). The immobilised molecules were studied by DOQSY experiments at low temperature (8 K) to obtain the backbone
torsion angles ($\phi$, $\psi$) of adjacent glycine–glycine pairs. The distribution found in the Ramachandran map for these torsion angles suggested that the glycine–rich domains of the silk protein adopt a random–coil conformation with no local ordering.

The last part of this thesis described in–vivo MR–spectroscopic studies, performed for the first time on Nephila edulis. These provided new insight into spider phenotyping, from both static images and real–time imaging of the silk glands and digestion processes during spider silking. The spatial resolution of MRI was exploited to apply localised spectroscopy to the A–zone, central region and B–zone of the wide sac of the m.a. gland, to extract additional information relevant to the initial set of experiments performed under MAS. The spectra from these locations indicate that no structural changes detectable by NMR, occurred during the dissection of the m.a. glands studied, and therefore that the ex–vivo experiments were conducted on native m.a. silk dope. The MR images and spectra took the form of feasibility studies of techniques and procedures introduced to the study of spiders, and therefore provide an important foundation on which to build detailed programmes of investigation.

Equipment was built in–house for the purpose of obtaining samples and performing experiments monitoring the spider during the silking process. Protocols were established in spider preparation and silking by an electrical motor drive adapted specifically to operate in the magnetic fields of an NMR spectrometer.

The experiments on liquid and immobilised silk dope show a random–coil structure for the silk molecules stored in the lumen of the wide sac of the m.a. gland prior to spinning. Conformational changes in the silk–protein molecules which have been postulated to occur along the wide sac could not be observed with the techniques applied. More detailed information about such changes may be obtainable by performing localised spectroscopy using $^{13}$C chemical shifts, rather than those of $^1$H; with appropriate labelling the former are better suited for monitoring differences in the chemical–shift distribution among the spectra obtained from distinct regions along the silk production pathway.

Insight into the spinning process, and the role played in it by water, may be obtained by real–time experiments monitoring water uptake, retention and excretion before, during and after a long silking period. The apparatus for such experiments would be that developed to monitor the silk glands during silking, augmented by a microcamera for remote
control of the spooling of the silk thread, which in experiments of long duration may be cut.

Finally, effort was put into the structural investigation of native liquid dragline silk, where the results suggested that it retains a random-coil structure and is stored as an isotropic liquid solution, and experimental equipment was designed for real-time imaging of spider physiology during silking.
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Appendix A

List of abbreviations

1D    One-Dimensional
2D    Two-Dimensional
AD    Anno Domini
A-zone Upstream end of m.a. gland of Nephila edulis
B-zone Downstream end of m.a. gland of Nephila edulis
BC    Before Christ
cemf  counter-electromotive force
CD    Circular Dichroism (Spectroscopy)
CP    Cross-Polarisation
CSA   Chemical-Shielding Anisotropy
CT    Computer Tomography
dope  see Silk dope
DOQSY Double-Quantum Single-Quantum Spectroscopy
DQ    Double-Quantum
FID   Free-Induction Decay
FISP  Fast Imaging with Steady-State Precession
FOV   Field Of View
FWHH  Full Width at Half Height
GARP  Globally optimized Alternating-phase Rectangular Pulses
       (used as a decoupling sequence)
HSQC  Heteronuclear Single-Quantum Coherence
Appendix A. List of abbreviations

MAS  Magic–Angle Spinning
m.a.  major ampulla (gland of Nephila edulis)
MR  Magnetic Resonance
MRI  Magnetic Resonance Imaging
MTX  Matrix (size)
NMR  Nuclear Magnetic Resonance
NS  Number of Scans
PDF  Probability Distribution Function
PET  Positron Emission Tomography
ppm  Parts per million
PRESS  Point–Resolved Spectroscopy
(\(\phi, \psi\))  Backbone torsion angles in peptides
RARE  Rapid Acquisition with Refocused Echoes
r.f.  Radio–Frequency
SE3D  Spin– Echo 3D– Experiment
Silk dope  Native liquid silk within the lumen of wide sac
of m.a. gland of Nephila edulis
Spiderling  Nymphal or immature spider, fully mobile
and no longer dependent on yolk
\(S/N\)  Signal to noise
SQ  Single–Quantum
\(T_1\)  Longitudinal or spin–lattice relaxation time
\(T_2\)  Transverse relaxation time
TA  Total Acquisition time
TE  Echo Time
TR  Repetition Time
TMS  Tetramethylsilane
VAPOR  Variable Pulse power and Optimized Relaxation delays
(Volume localized, solvent Attenuated, Proton NMR)
Appendix B

Food supply for the spider *Nephila edulis*

**B.1 Fruit fly**

The fruit fly, *Drosophila melanogaster*, is a small insect about 3 mm in length, of the kind which accumulates around spoiled fruit. Fruit flies are one of the most valuable organisms in biological research, particularly in genetics and developmental biology, because of their reproductive speed and the simplicity of their genetic structure. They are also an ideal food source for many young, carnivorous insects and small spiders because they are high in sugars and minerals.

The flies were kept on yeast media (protocol below) in glass jars with a wad of cotton wool as a lid (Fig. B.1). The fruit flies not only eat the yeast medium but also lay eggs of approximate size 0.5 mm on it, and these develop quickly into worm-like maggots. Within 2 – 3 days several moults follow in quick succession to form an immobile pupa which hatches to give the adult winged form and is fertile within about 12 hours. At 20 °C they breed very rapidly, and their life cycle lasts only around 10 days, while at 18 °C this development takes twice as long. To sustain this culture, either larvae or adult flies were transferred at intervals into new glass jars containing fresh yeast medium.
Yeast-medium protocol: 12 g agar are dissolved in 1000 ml of water. 100 g ground corn, 70 g sugar and 5 g dry yeast are added and boiled for 20 min. Adding 5 ml of propionic acid prevents mildew. Sterile glass jars are filled to a depth of 3 cm with the liquid mixture and air-dried. A teaspoon of fresh yeast is dropped onto the dry, cold and solid surface of the medium and the jars closed with a sterile wad of cotton wool ([167]).

B.2 House Fly and Blow Fly

The common house fly, Musca domestica, is one of the most common of all insects. Adults are 6 – 8 mm long with a wingspan of 13 – 15 mm, and have a grey thorax with four dark, longitudinal stripes. Being a strong flier, it is a nuisance as well as a carrier of disease to man and domestic animals. The life cycle from egg to adult usually takes approximately 8 days.

Blow flies, Lucilia Cuprina, are slightly larger than common house flies with a somewhat longer life cycle of 9 – 21 days. These strong fliers usually show a metallic blue-green colour on their thorax and make a loud droning buzz.

Because arthropods, such as insects, are the native source of nutrition for the spider Nephila edulis, a diet of house and blow flies is suitable feeding material for captive spiders. There are four distinct stages in the life of the fly: egg, larva or maggot, pupa and adult. The larvae of most species are slender, white, legless maggots which develop rapidly, passing through three instars. They form a capsule-like case, the puparium, within which the transformation from larva to adult takes place, and at the end of which the fly pushes open the top of the case and works its way out. Soon after emergence the fly spreads its wings and the body dries. The time required for this development depends on the species as well as the temperature, and usually takes 4 – 12 days for house flies and blow flies. The procedure most economical in time, effort and space was to buy house- or blow-fly larvae from a fishing shop, and to store these at 4°C in a small plastic box to slow their metabolism. When required, a quantity of larvae was transferred to another plastic box and stored at room temperature to obtain the desired flies for feeding.
Figure B.1: Breeding drosophilae on yeast medium in glass jars with a wad of cotton wool as a lid. (a) Fresh medium, no flies. (b) Fruit flies before laying eggs onto the medium. (c) Worm–like maggots in medium with immobile pupae sticking to glass wall. (d) Glass jar 20 days after inserting fruit flies.

B.3 Mealworms

Mealworms are the larvae of the miniature darkling beetle, *Tenebrio molitor*, and are rich in protein and fats with an indigestible exoskeleton. Fully grown larvae closely resemble wireworms in appearance. Beetles undergo complete metamorphosis (egg, larva, pupa, adult). The female mealworm lays hundreds of minuscule, white, oval eggs, which hatch within 4 – 9 days into small, white larvae. The mealworms become a yellowish–brown colour, moulting many times (~20) as they grow in length from 2 to 25 mm or larger before entering the pupal stage. A white adult beetle (12 – 15 mm in length) emerges and quickly turns brown, then almost black. The adult lives for 2 – 3 months, while the entire life cycle takes approximately a year. With prior knowledge of this year–long life cycle, the larvae, in one of their last instars, were bought in a pet shop and stored at 4 °C, which was possible without feeding for several month with no damage to the beetles. The larvae were kept slightly humid with a water spray, but never wet.
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Appendix C

Characterisation of a modified electrical motor operating in high magnetic fields

Chapter 3.3.3 describes the modification of a diametrally–magnetised DC electrical motor mandated by the requirement that it be able to operate in the high magnetic fields of an NMR spectrometer. These adaptations essentially take the form of a complete replacement of all metallic parts, with the magnetic field of the spectrometer itself taking the place of the static permanent magnet of a conventional motor. The behaviour of this modified motor was then measured in detail at a representative selection of points in the bore of the NMR magnet, and thus in fields varying from 0.2 to 9.4 T. For the studies reported in this thesis the motor was operated only at 0.2 T for the real–time imagery performed during silking in chapter 6. However, with a view to later experiments in high field, and for the possibility of completely different applications of the equipment, the motor was characterised at all fields accessible within the spectrometer. The behaviour of the motor, its operating specifications and its influence on the homogeneous field of the magnet are reported here.
C.1 Materials and Methods

The specifications of the modified motor (Ch. 3.5) were measured while using as the source of its external magnetic field the magnet of a Bruker 400 MHz NMR spectrometer. The field along the bore of the magnet varies from 9.4 T at the magnetic centre to 0.3 T at a distance of 50 cm from this. The dependence of rotation frequency, terminal voltage and current on the field strength was monitored by placing the modified motor at points successively deeper in the bore of the magnet. The frequency of rotation was detected optically. Of primary interest were the behaviour of frequency, terminal voltage and current while either the terminal voltage or the current was maintained at a constant value. In a second experiment the motor was operated for 3–4 h at the centre of the magnet to observe its influence on the magnetic field. The drift of the spectrometer magnet was obtained by measuring the chemical shift of Tetramethylsilane (TMS) at intervals of 0.5–1 h, for which the motor was replaced by a probehead containing the TMS sample.

C.2 Results and Discussion

From the Lorentz force one may at first expect an increase of the “external” magnetic field to induce an increase in rotation frequency. Figure C.1 shows the behaviour of the rotor frequency as a function of field: the measured decrease is explained by two effects both arising from the induced counter-electromotive force (cemf), eddy currents along the axis of rotation and a generator voltage being developed in the coil [118–121]. A greater rotation frequency or “external” field causes a larger induced cemf. The counter-currents developed along the rotation axis are known as eddy currents and lead to strong forces which decelerate the motor. The induced cemf in the coil, known as the generator voltage, leads to a direct reduction of the coil current. A reduced current diminishes the Lorentz force and hence the frequency of rotation.

A constant coil voltage was maintained by keeping the current constant, and the rotation frequency and terminal voltage were monitored. Also in this case the rotation frequency decreases at higher fields (Fig. C.2), again due to eddy currents and generator voltage. The behaviour of the terminal voltage is explained by two antagonistic effects.
A higher magnetic field increases the emf and leads to a reduced terminal voltage, because the voltage at the coil is kept constant. However, a reduced rotation frequency at higher fields leads to a smaller induced emf, therefore permitting a higher terminal voltage. The frequency and terminal voltage collapse at 0.4 T because the required current of \( \sim 500 \text{ mA} \) (Fig. C.1) cannot be delivered; the maximum current available from the supply were 200 mA and 300 mA for the curves shown.

The rotation frequency is strongly influenced by the position and orientation of the modified motor with respect to the magnetic field, varying between its maximal value and stagnancy. This is explained by the fact that the motor position and orientation may cause the field lines of the net magnetic field to be no longer perpendicular to the coil [Fig. 3.7(b), p. 46]. The maximal rotation frequency, achieved in a stray field (0.4 T), is approximately 50\% of the frequency of the original motor at equal voltage (Figs. C.1, C.2). The frequency, current and nominal voltage of the original motor without load

---

Figure C.1: Rotation frequency (top) and current (bottom) of the modified DC motor as a function of magnetic field at constant terminal voltage. The voltage at the coil and therefore its current are suppressed at higher fields, causing a frequency reduction. The accuracy of the measurements is 10\% for the rotation frequency and 20\% for the current.
Appendix C. Characterisation of a modified electrical motor operating in high magnetic fields

Figure C.2: Rotation frequency (top) and terminal voltage (bottom) of the modified DC motor as a function of magnetic field at constant current. The frequency decreases at higher fields; the terminal voltage is reduced both by higher fields and by higher rotation frequencies occurring at lower fields. The limit of the available current leads to a collapse of the voltage and frequency at 0.4 T (where the required current would be \( \sim 500 \) mA, Fig. C.1). The accuracy of the measurements is 10% for the rotation frequency and 5% for the terminal voltage.

are respectively 230 Hz, 187 mA and 2.4 V. The linear relationship between voltage and rotation frequency is maintained after the modification of the motor.

Operating the modified motor in the centre of the spectrometer magnet at 9.4 T for 3 - 4 h causes only a small field drift which is of the same magnitude as that occurring on inserting or changing probe heads. This observation means that the modified motor may be used to drive a variety of devices, such as gears changing the orientation of a sample [168], without disturbing the measurement.
C.3 Conclusions

DC electrical motors may be modified to operate under the high magnetic fields used in NMR spectroscopy. High values of the spectrometer field cause a systematic suppression of the rotation frequency, terminal voltage and operating current of the motor due to the strong induced counter-electromotive force. These motor characteristics can be measured for all locations (fields) in the bore of the magnet. The destabilising influence of a motor operating at the center of the magnet is small, being comparable to the effect of changing probe heads. Thus the combination of the motor with a variety of devices is conceivable, including the positioning, reorientation and spinning of NMR samples inside the bore of the magnet.
Appendix D

Picture credits

Fig. 1.1, p. 2

Web
Yellowstone National Park (USA),
National Park Service,
http://www.nps.gov/yell/slidefile/index.htm

Arachne
Image from “Greek and Roman Mythology A to Z”,
by Kathleen N. Daly, New York Public Library Picture Collection

Arachnophobia
http://www.moviegoods.com

Spiderman
http://www.sony.com/spider-man

Tarantula
Universal Studios

Eiger
Uli Aussermann (info@uliaussermann.de)
http://www.uliaussermann.de
Appendix D. Picture credits

Fig. 1.2, p. 4
Raw silk, larva  DESC O de Schulthess Ltd, Zürich (Switzerland)
Patrick Gendoud (patrick.genoud@desco-group.com),
Sericulture  National Geographic Magazine
http://www.nationalgeographic.com
through John R. Meyer (john_meyer@ncsu.edu)
http://www.cals.ncsu.edu:8050/course/ent425/text01/sericulture.html
Moth, larva  ManYee DeSandies (manyee_desandies@nhusd.k12.ca.us),
Alvarado Elementary School, Union City, California (USA)
http://www.pclaunch.com/kayton/Silkworms/lifecycle.htm
Fabric  Info-G Corporation, Ooya, Suzaka (Japan)
http://www.info-g.co.jp/silk

Fig. 1.3, p. 7
Qing-dynasty  Odon Wagner Gallery, Toronto (Canada),
http://www.odonwagnergallery.com/
Liao-dynasty  The Cleveland Museum of Art, Ohio (USA),
www.clevelandart.org
Map  Prepared using Google Earth
http://maps.google.com/

Fig. 1.4, p. 9
Schema  Fritz Vollrath (fritz.vollrath@zoology.oxford.ac.uk), Oxford (England),
Fig. 1.6, p. 15
Spiders  Jurgen Otto (katejotto@optusnet.com.au), St Ives (Australia),
Ed Nieuwenhuys (ednew@xs4all.nl), Badhoevedorp (The Netherland),
www.xs4all.nl/ednieuw

Fig. 1.7, p. 16
Anatomy  Rainer F. Foelix (Rainer.Foelix@ag.ch), Aarau (Switzerland)

Fig. 1.8, p. 17
Anatomy  Rainer Foelix (Rainer.Foelix@ag.ch), Aarau (Switzerland)

Fig. 3.2, p. 41
Open magnet  JEOL USA, Inc (nmr@jeol.com)
http://www.jeolusa.com/nmr/mag_view/magnet_destruction.html


[27] Brooklyn Eagle. Text obtained from the Brooklyn Public Library. Newspaper, Okt. 9, 1902.


118 Bibliography


[97] Bracco Suisse SA, 6850 Mendrisio.


[117] LabVIEW is a graphical programming language from National Instruments mainly used to monitor and control measured data, [http://www.ni.com/labview/](http://www.ni.com/labview/).


MatNMR is a toolbox for processing NMR/EPR data under MATLAB and can be downloaded freely at http://www.nmr.ethz.ch/matnmr.


http://www.documed.ch/ch.


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Curriculum Vitae

31.08.1974  Born in Zürich, Switzerland.
1981–1994  Education in Zürich, Switzerland.
January 1994  Maturität, Type C (Natural Sciences).
1994–1999  Study of Biological Chemistry at ETH Zürich, emphasis on Molecular Biology, Crystallography Organic, Inorganic and Biological Chemistry.
Spring 1999  Diploma thesis (Masters) with Prof. K. Wüthrich, Liquid–State NMR on a Mouse Prion–Protein–Mutant.
          Supply teacher for biology, Dübendorf grammar school (Kantonsschule Zürich Oberland, Filliale Glattal).
2000–2001  Educational travel and Spanish study in South America.
          Courses in didactics, paedagogics and psychology at University of Zürich.
          Teacher–training for Chemistry at ETH Zürich.
June 2006  Ph.D. thesis at ETH Zürich (this work).
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List of Presentations


4. *NMR on Spiders and Spidersilk*, M. Hronská and Isabelle Marcotte, Talk, Zoologisches Institut - Colloquium, University Bern Switzerland (2004).

5. *NMR on Spiders and Spidersilk*, M. Hronská, Talk, Institute of Biomedical Engineering - Colloquium, ETH/University Zürich Switzerland (2005).
List of Publications


