INVESTIGATING THE MESOSCALE OF
β-LACTOglobulin Fibril Hydrogels

BY

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Abstract

The objective of this doctoral thesis was to investigate the relationship between the architecture of protein fibril networks and their macroscopic properties. This requires investigation of the mesoscale; the scale between the macroscopic and microscopic scales where fibril self-assembly processes occur resulting in structures organized in different hierarchical levels. The mesoscale of such networks is not extensively studied and this is where I want to add knowledge, in order for physical sciences to contribute to New Zealand agricultural food sectors by changing the way in which soft materials and biopolymer engineering is done and by taking biomaterials from commodities to specialties by adding knowledge.

The protein selected for the current study was β-lactoglobulin which forms amyloid-like fibrils when heated at 80°C under acidic conditions. Specifically, hydrogels were formed under three pH conditions; 1) pH 2.3, 2) pH 2.0, and 3) pH 0.9. These three conditions result in three different types of hydrogels being formed. The β-lactoglobulin hydrogels formed at pH 0.9, which have not previously been reported in the literature, exhibit completely different structure and macroscopic properties compared with the standard and widely reported in the literature β-lactoglobulin hydrogels formed at pH 2.3 and pH 2.0. Cryo-scanning electron microscopy (cryo-SEM) was used to investigate the intact 3D structure of the hydrogels. On the contrary, there are a lot of studies reported in the literature using other microscopy techniques, like atomic force microscopy (AFM) or transmission electron microscopy (TEM), which allow the fibril characteristics and not the intact interior of the hydrogel structure to be investigated. Cryo-SEM showed that β-lactoglobulin fibrils formed at pH 2.3 are the most flexible fibrils with the longest end-to-end fibril lengths, while the fragmented-particles fibrils formed at pH 0.9 are the thickest and least flexible with the shortest end-to-end lengths. Determination of fibril characteristics helps in predicting the macroscopic behaviour of fibril networks. Small-angle X-ray scattering (SAXS) was used as a complementary method to cryo-SEM. SAXS allows structural investigation of fibril networks, that cryo-SEM is not able to achieve. Specifically, SAXS showed that fibril hydrogels formed at pH 2.3 exhibit the least compact
structure with the least fibril surface roughness, while hydrogels formed at pH 0.9 exhibit the most compact structure and the roughest fibril surfaces. Another crucial point is that SAXS allows the thermodynamics of these systems to be probed. SAXS data confirmed that the more rod-like the network, the more favourable it is for the system to organize into a nematic phase.

Rheology was used to investigate the macroscopic properties of the hydrogels. Rheology demonstrated that there are two types of behaviour exhibited by these three types of hydrogels. Although it was assumed at the start of this project that these three types of hydrogels could exhibit different macroscopic behaviour, since their end-to-end fibril length scales are different, finally, it was demonstrated experimentally that hydrogels formed at pH 2.3 and pH 2.0 exhibit roughly the same macroscopic behaviour, while hydrogels formed at pH 0.9 exhibit a completely different macroscopic behaviour. Specifically, hydrogel networks formed at pH 2.0 are slightly stiffer than those formed at pH 2.3 and exhibit fast gelation time, while hydrogels formed at pH 0.9 are the softest and exhibit slow gelation time. Considering the cryo-SEM data, the fibrils formed at pH 0.9 are the least flexible. These two sets of data appear to contradict each other, but it must be noted that the length scales being probed are different. These results indicate that the characteristics of individual fibrils do not necessarily manifest themselves directly in the macroscopic behaviour of the whole fibril network. This means that the interplay of the fibrils with each other is important in defining longer length scale behaviour.

All in all, while most studies to date refer to the structural investigation of β-lactoglobulin fibril systems on a macroscopic or microscopic level, in this work it is addressed that thermodynamics, chain stiffness and thickness, electrostatic interactions, inter-fibril distances, orientation of β-sheet stacks, and the number of cross-links constitute the basic factors on the mesoscale that affect the architecture of the fibril networks and connect their architecture with their macroscopic properties. Hence, by controlling the mesoscale (self-assembly process), the manipulation of
biomaterials that already exist in the market is feasible, in order to exhibit novel macroscopic properties.

**Keywords:** self-assembly systems, β-lactoglobulin fibrils, cryo-SEM, SAXS, rheology
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Abbreviations

AFM=Atomic Force Microscopy
β-lg=β-lactoglobulin
Cryo-SEM=Cryo-Scanning Electron Microscopy
Cys=Cysteine
DLS=Dynamic Light Scattering
DM=Degree of Methyl-Esterification
ESEM=Environmental Scanning Electron Microscopy
FWHM=Full-Width Half-Maximum
GDL=Glucono Delta-Lactone
HPLC=High-Performance Liquid Chromatography
MALS=Multiangle Light Scattering
I-N=Isotropic-Nematic (transition)
LVE=Linear Viscoelastic Region
MM=Molecular Mass
MW=Molecular Weight
OT=Optical Tweezers
PEEK=Polyether Ether Ketone
PLAs=Pressure Limiting Apertures
PME=Pectin Methylesterase
SAXS=Small-Angle X-ray Scattering
SANS=Small-Angle Neutron Scattering
SALS=Small-Angle Light Scattering

SDS-PAGE=Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis

SEM=Scanning Electron Microscopy

TEM=Transmission Electron Microscopy

USALS=Ultra Small-Angle Light Scattering

WAXS=Wide-Angle X-ray Scattering

WPI=Whey Protein Isolate
CHAPTER 1

General Introduction

1.1 Self-Assembled Biopolymer Networks

Biomaterials present in nature, like cellulose fibers in fruits, keratin in wool, collagen in bones, elastin in lungs, and casein micelles in dairy products exhibit hierarchical structures that define their biological functions and uses. These structures emerge from molecular and supra-molecular self-assemblies organized on multiple length- and time-scales. Biophysical tools, like microscopy and scattering techniques, provide the opportunity to investigate such systems on the mesoscopic level; the level between the macroscopic (top) scale and the microscopic (bottom) scale, which is where protein and carbohydrate assemblies like fibrils and tubes exist, the so-called “mesocules”. Only a subset of these fibrils and tubes undergo self-assembly process. In order to characterise this subset of molecules which undergoes self-assembly process and to separate them from the molecules that are not able to self-assemble, a new definition is introduced into the literature, the so-called “mesocules”.

Under the right conditions mesocules undergo self-assembly to form gel networks. Probing the structure of such systems and their subsequent self-assembly enables understanding of for example the length- and time-scales on which the mechanical properties of the networks are established and how they emerge from interactions between constituent molecules. Given the wide occurrence of such networks, being able to access this type of information is invaluable.

Fibril formation, which is the first step of the amyloid fibril self assembly in several protein hydrogels, is well studied [1-3]. The next step is to understand the way in which fibrils in these systems interact together and how fibril self-assembly can be
controlled so that new biomaterials can be built that could have applications in a range of different fields. Understanding the properties of protein self-assembled systems is also a main area of research in the food industry. The structure of such systems is closely related to food properties, like taste, aroma, texture, and appearance [4]. For example, β-lactoglobulin has drawn special attention from the food industry, since β-lactoglobulin constitutes one of the two major globular proteins in milk [5]. “Smart foods”, like aerated gels, which increase the feeling of satiety are a common example that is already available in the market [6]. Advances in gel formulation and the production of new gels with defined macroscopic properties will see the advent of many new products as well as enhanced understanding of the structure of natural gelled systems.

Such advances in understanding also have spillover effects in other areas as diverse as the investigation of neurodegenerative diseases, where amyloidal fibril networks are believed to be important in Alzheimer’s, Parkinson’s, Huntington’s, and Prion diseases. Here the insoluble aggregations of amyloid fibrils in human brain tissues lead to the death of cells in healthy organs [1, 7]. In biomedical engineering silicone gels allow the cornea to breathe better than traditional hydrogel lenses and hence, eye infections decrease significantly [8-9]; and in art restoration and conservation special gel systems have been developed for surface cleaning without inflicting damage [10-11].

In this dissertation β-lactoglobulin is used as a model fibril and gel forming biopolymer.

1.2 β-lactoglobulin Protein

β-lactoglobulin protein is a well studied small protein which constitutes the major protein (~50%) in whey and occurs in abundance in bovine milk. It has a molar mass of 18.4 kDa and an isoelectric point ~5.1. It consists mainly of dimers at physiological
conditions with 38% β-sheets, 14% helical elements, and the rest random coils, while at pH values lower than 3.0 it largely exhibits a native monomer-like behaviour [12]. Each monomer consists of 162 amino acid residues with eight strands of anti-parallel β-sheet and one α-helix chain which wraps around [13], in order to form an anti-parallel β-barrel (secondary structure) [14]. Two disulfide bonds (covalent bonds) are formed between the amino acid residues Cys66-Cys160 and Cys106-Cys119 which stabilize the β-barrel structure and one free thiol group at Cys121 [15-16].

pH and ionic strength changes affect the fibril formation and the kinetics of the self-assembly process. Loveday et al. [17] demonstrated that there is no major effect on the long and thin fibril morphology or on the kinetics of the self-assembly process between pH 1.2 to 2.4. On the contrary, there are studies reported in the literature [18-20] showing that for pH>3.0 short β-lactoglobulin fibrils are observed. Generally, as the pH increases, H⁺ ions are removed from the carboxyl groups of Asp and Glu giving them a negative charge [21-22], while H⁺ are removed from the NH₃⁺ groups of Lys and Arg removing their positive charge. Aymard et al. [23] and Arnaudov et al. [24] suggested that ionic strength changes affect the kinetics of the fibril self-assembly process. By adding NaCl the anions of the salt interact with the positively charged amino acids (Lys and Asp) reducing the electrostatic repulsion interactions, while other studies [17-18, 22, 25-27] suggested that the fibrils start to curl as the ionic strength increases. Loveday et al. [17, 26, 28] demonstrated that the addition of cations (CaCl₂) in β-lactoglobulin fibril networks has a stronger impact on the fibril formation and on the kinetics of the self-assembly process than NaCl. Cations by screening side-chain charges of the same protein molecule (intra-molecular interactions) and behaving like inter-molecular "bridges" (π-interactions) between the aromatic groups (Phe, Tyr, Trp) [29], facilitate the self-assembly process and cause the curliness of the fibrils. Based on Loveday et al. [29], an addition of NaCl>60 mM is sufficient, in order to start the first fibril formation and kinetic changes to appear within the fibril network, while a smaller amount of CaCl₂ is required, in order similar effects within the network to be observed.
There are three β-lactoglobulin variants, A, B, and C with A and B to be the two main variants of β-lactoglobulin. The difference between them is that the Asp64 and Val118 amino acids in variant A are replaced by Gly64 and Ala118 in variant B and C, respectively, and Gln 59 in variant A and B is replaced by His59 in variant C [30]. The β-lactoglobulin A, B, and C variants exhibit negligible differences in their structural characteristics.

β-lactoglobulin is denatured at temperatures above ~70°C, and according to Jung et al. [13], based on transmission electron microscopy (TEM) images, upon heating it exhibits amyloid fibril formation at pH 2.0, spherical particles at pH 5.8, and worm-like fibrils at pH 7.0 (Fig. 1.1). In the Jung et al. [13] investigation, the β-lactoglobulin was heated at 90°C for 5 h, instead of 80°C for 10 h which are now the standard conditions reported in the literature [27, 31-34]. This was due to the better conversion rate of β-lactoglobulin monomers into fibrils under these conditions.

**Fig. 1.1** β-lactoglobulin denaturation during heating at 80°C-90°C for 5-10 h forming a) fibrils at pH 2.0, b) globular particles at pH 5.8, and c) worm-like particles at pH 7.0.

Upon heating to >70°C at pH 2.0 [13], the covalent bonds (disulfide bonds, Cys66-Cys160 and Cys106-Cys119 [15]) in β-lactoglobulin break and hence, the protein
starts to unfold. Based on Schleeger et al. [1], released peptides assemble into anti-parallel β-sheets (microscopic state, Fig. 1.2a). Each β-sheet consists of anti-parallel β-strands. These β-sheets are stacked together [1, 35] perpendicular to the direction of the fibril axis and are held together with hydrogen bonding thereby forming the protofibrils. These protofibrils can subsequently twist together, as presented in Fig. 1.2b, resulting in thin and long fibril formation (mesoscopic state). These thin and long fibrils consist of two or more twisted protofibrils which are termed mature fibrils. Finally, the fibrils can self-assemble (Fig. 1.2c) into fibril networks (macroscopic state, Fig. 1.2d) [1].

Historically, in 1959 Cohen and Calkins [36] showed, using electron microscopy, that amyloid fibrils are structured rather than being amorphous as had been reported previously based on light microscopy data. Cohen and Calkins [36] showed that the amyloid fibrils are elongated and unbranched. In 1968 Eanes and Glemer [37] elucidated the cross β-sheet core structure of amyloid fibrils from X-ray diffraction data. The cross β-sheet core structure, namely the parallel and aligned β-sheets when stacked perpendicular to the fibril axis as described above, constitute the fundamental building block of the amyloid structure.
The self-assembly process that is induced upon heating the protein is mediated by non-covalent bonds, like electrostatic and hydrophobic interactions, hydrogen bonding, ionic bonds and van der Waals attractive forces. Electrostatic interactions are mainly affected by pH changes, which alter the net charge and the interactions of the R-side chains (the chemical group attached to the central carbon atom of amino acid groups) with other molecules or ions. These net charge changes cause structural changes. Specifically, when the pH values are far away from the isoelectric point (pH ~5.1 for β-lactoglobulin protein), the net charge becomes high. When the pH becomes lower than the isoelectric point, the H⁺ neutralize [21-22] the negative charges of the carboxyl groups of Asp and Glu, while they give a positive charge to the N atom of the amino group of Lys and Arg. This results in the net charge becoming positive and the electrostatic repulsions dominating over the attractive interactions [38]. This allows fibril formation to occur as reported for β-lactoglobulin by Jung et al. [13] based on TEM data. On the contrary, when the pH increases and gets close to the isoelectric point (pH ~5.1, Fig. 1.1), H⁺ ions start to leave the carboxyl groups [21-22] and negatively charge amino acids (Asp, Glu) and H⁺ ions are removed from the N atoms of Lys and Arg removing their positive charge. As the pH gets closer to the isoelectric point the protein loses its net charge. This results in the reduction of electrostatic repulsions and the aggregates exhibit the form of globular particles densely packed in a random way [38], as was also shown by Jung et al. [13] via TEM investigation.

1.3 Dissertation Outline

Figure 1.3 presents a general outline for the present dissertation. Here β-lactoglobulin hydrogels were investigated to ascertain the main factors that contribute to the control of the fibril self-assembly process, and how these factors connect the top (macroscopic scale) to the bottom (microscopic scale) (top-bottom approach). It is hypothesized that there are unique relations between the structure and functions of biopolymer networks and that these relations can be altered by controlling the properties of the hierarchically assembled mesocules (eg. fibril length through the
initial formation conditions and therefore their function can be controlled, defined, and modified. The following questions were investigated:

- How do the factors that affect the fibril self-assembly process contribute to the macroscopic, mesoscopic, and microscopic properties of β-lactoglobulin fibril hydrogels?
- How does the mesoscopic state connect the macroscopic properties to the microscopic state (top-bottom approach)?
- What is the relationship between the protein networks’ architecture and their macroscopic properties?

**Methodology**

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<td>High q SAXS (microscale-bottom)</td>
<td>• What is the relationship between the protein networks’ architecture and their macroscopic properties?</td>
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<td>• How do the factors that affect the fibril self-assembly process contribute to the macroscopic, mesoscopic, and microscopic properties of β-lactoglobulin fibril hydrogels?</td>
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**Fig. 1.3** Top-bottom approach. Methodology and main questions answered in the present dissertation.

The answers to these three questions will add knowledge to the literature about the mesoscale control for biopolymer networks, which is not as yet well understood. Hence, in short, it will connect the top to the bottom. In this way, physical sciences can contribute to New Zealand agricultural food sectors by changing the way in which
soft materials and biopolymer engineering is performed and by taking biomaterials from commodities to specialties by adding knowledge.

In order to answer these questions, my investigation is based on the controlled assembly of β-lactoglobulin fibril hydrogels from fibrils of controlled length. The reason why this study is based on this specific protein is because Dr Luigi Sasso, a member of Prof Gerrard’s Group (collaborator) at Canterbury University in Christchurch, New Zealand, had already found, using TEM (unpublished data, personal communication), that β-lactoglobulin fibril length varies upon changing pH at least when initiated in dilute solution. Specifically, the present work is focused on using long fibrils formed at pH 2.3, shorter fibrils formed at pH 2.0, and fragmented-fibrils formed at pH 0.9 as the building blocks of gelled networks. It should be highlighted here that it was clear from Dr Sasso’s work that β-lactoglobulin formed fragmented-fibrils at pH 0.9.

The form of the dissertation is divided into 5 main Chapters. Chapter 1 is a general introduction into hierarchically assembled biopolymer networks, the variety of their applications exhibited in different research fields, and the fundamental characteristics of β-lactoglobulin protein. Chapters 2-4 refer to my experimental work including the cryo-scanning electron microscopy (cryo-SEM), small-angle X-ray scattering (SAXS), and bulk rheology methods, respectively. Chapter 5 draws all the data together and presents the major outcomes from the work.

Chapter 2 presents a cryo-SEM investigation carried out on β-lactoglobulin gels assembled of different fibril length scales (formed at pH 2.3, 2.0, and 0.9). The reason why cryo-SEM was selected as an experimental tool in this study is because the intact 3D structure of the hydrogels was investigated rather than the fibril characteristics. This is the reason why there is no statistical analysis for the fibrils presented in this chapter. On the contrary, there are many studies in the literature investigating the fibril characteristics using microscopy techniques, like atomic force microscopy
(AFM), transmission electron microscopy (TEM), and dynamic light scattering (DLS), which do not allow the 3D interior of the hydrogel structure to be probed. Data on the microscopic and mesoscopic scales (100 nm-1 μm) in these protein systems is reported. Due to the introduction of freezing artefacts within the protein network during the sample preparation, firstly it was necessary to elucidate the most reliable freezing method for maintaining the native state of the protein fibril hydrogels during sample preparation. Considerable progress with regard to the development of the technique was made. Once established, the preferred sample preparation method was applied to the structural characterization of the gels allowing the fibrils and gels to be characterised including for example estimation of the mesh size and fibril dimensions. Based on this investigation important characteristics of the network architecture were determined for each of the β-lactoglobulin systems.

Chapter 3 presents a SAXS investigation for the same β-lactoglobulin hydrogels, here both during and after the gelation process, and on the microscopic and mesoscopic length scales (<220 nm). Specifically, the kinetic and structural changes occurring in these systems are described, and the role of entropy, molecular forces, and multiple inter-fibril interactions are considered, in determining the fibril self-assembly. These collectively allow a fuller description of the fibrils and hydrogels to be achieved. A comparison between the SAXS and cryo-SEM data is also presented in this chapter.

Chapter 4 describes the mechanical properties on the macroscopic scale (~mm) for the same gel systems using bulk rheology. It highlights structural and kinetic parameters, like the elastic modulus and the gelation time, respectively, during and after their gelation process. Based on this rheological structural and kinetic investigation for fibril self-assembly systems the relationship between thermal bending fluctuations and network architecture was determined.

Lastly, Chapter 5 represents an interpretation of the meanings of the combined cryo-SEM, SAXS, and bulk rheology data highlighting with respect to the application of
the top-bottom approach for analyzing protein hydrogels. Specifically, it is stated what knowledge has been added by this work and how it can be applied to achieve new materials that have defined structure and function.

Supplementary information where needed is presented in the Appendixes at the end of this dissertation.
References


CHAPTER 2

Cryo-Scanning Electron Microscopy: Structural Changes of Protein Amyloid Fibril Networks. Artefacts or The Real Structure?

Abstract

Visualization of the structure of "wet" samples with electron microscopy remains a significant challenge. Numerous methods have been used to prepare samples in order to reduce the introduction of artefacts which render structural elucidation difficult. Freezing of samples for example has been used for several decades using numerous techniques. Here, a major consideration is the formation of ice crystals during cryo-fixation. This is particularly an issue for high-water content hydrogel samples. Images of such cryo-fixed networks damaged by ice formation are unfortunately widely reported in the literature as being representative of the native structure of the fibrillar gels. In addition, this is one of the reasons why structures for proteins and polysaccharides seem to be the same in numerous cryo-EM imaging studies. Such artefacts can however be ameliorated or eliminated through the use of better cryo-fixation methods. Here, it was found that by rapidly freezing protein amyloid fibril hydrogels using a propane jet freezer, SEM imaging of biomaterials in their native state is feasible. Subsequently, fibril size and shape, pore size, and end-to-end and persistence lengths of fibrils within the protein networks are able to be directly extracted from SEM images. Persistence length expresses how easily a fibril could bend upon thermal fluctuations. In other words, persistence length is a measure of flexibility/rigidity of a polymer. The shorter the persistence length, the more flexible the fibril.

On the contrary, it is shown that slow freezing methods, like the use of slushy nitrogen, yield the same erroneous structures for both protein and polysaccharide fibril hydrogels. The use of cryo-protectants, also investigated here, affects the
bonding within protein hydrogels and hence, changes the initial structure of the gels making use of this method also problematic. The results demonstrate that by freezing protein fibril hydrogels rapidly it is possible to determine how fibrils within the gel assemble. This method should prove to be an appropriate and more consistent sample preparation method for biopolymer fibril networks and other high-water content materials.

2.1 Introduction

Sample preparation for solid-state systems in SEM imaging does not affect the native state of their structure. This makes the SEM imaging for solid-state systems to be straightforward. On the contrary, SEM is not an immediately appropriate tool for high-water content systems. It scans the sample using an electron beam along the x- and y-axes and the interaction between the beam electrons and the sample causes the emission of electrons from the sample. The emitted electrons are divided into two categories; low-energy secondary electrons (≤50 eV, produced by inelastic collisions) and backscattered electrons (>50 eV, produced by elastic collisions). SEM generally operates under high vacuum in order to prevent air scattering by either the incident electron beam or the produced electrons [1]. This operating condition requires the dehydration of samples, which can result in shrinkage [2-3]. It is for this reason that variants of classical SEM are used to investigate high-water content biological samples [4].

One variant is where wet and non-conductive samples, without any sample preparation, can be imaged in their native state. This is achieved by the presence of a gas, usually water vapour, in the sample chamber at a pressure of up to ~10 Torr. The presence of gas molecules scatters electrons and therefore degrades the beam and so the whole column of the microscope is still kept under vacuum (~10⁻⁵ Torr) [1] and is separated from the sample chamber by pressure limiting apertures (PLAs) [5]. The disadvantage in this case is that the resolution is quite low (~5 nm) [1]. This method is called Environmental SEM (ESEM).
Another variant is where high vapour-pressure specimens, like hydrogels, emulsions, and biological specimens can be imaged with higher resolution under cryogenic conditions and high vacuum. Cryogenic conditions avoid the evaporation of any volatiles from the samples under high vacuum [6]. Specifically, the samples undergo rapid freezing, fracture and sublimation under high vacuum, in order to reveal interior structural features, and finally, coating with carbon or platinum, in order to create a conductive layer for sample imaging without charging or thermal damage by the electron beam [4]. This particular method is called cryo-SEM and provides visual access to the internal 3D structure of hydrated samples under cryogenic conditions.

Cryo-SEM was first suggested in the mid 1970s. Taylor and Glaeser [7] showed that with appropriate sample preparation the structure of frozen hydrated catalase crystals seen in electron diffraction could be preserved with accuracy down to atomic resolution (~3 Å). Cryo-SEM did not become commercially available until the 1980s when Mayer and Brüggeller [8] and Dubochet and McDowall [9] succeeded in freezing small amounts of pure water in liquid ethane. The water molecules did not have time to rearrange into ice crystals and the result was the formation of glass-like water, which is called vitrified water [10-11]. Years later larger specimens, like cells and biological tissue, were successfully vitrified and examined using cryo-SEM [12-14].

Despite the positive aspects of cryo-SEM it is particularly susceptible to the introduction of sample distortion, especially in high-water content systems where water volume increases during freezing. This freezing artefact is very common when the standard cryo-SEM freezing method of boiling liquid nitrogen (slushy nitrogen) is used in conjunction with high water content samples. Here, the rate of cooling of the samples is not sufficiently rapid and ice crystals are formed damaging the sample structures (frozen samples). This results for example in the structure of protein and polysaccharide networks often appearing the same in cryo-SEM imaging. In contrast, fast freezing allows the formation of vitreous ice (glassy) and hence, for the “true structure” to be revealed [11]. In other words, fast freezing rate does not allow the ice
crystal formation within the sample structure to occur and hence, the native state of the structure can be imaged (vitrified samples).

The three main freezing processes used in cryo-SEM are 1) the boiling liquid nitrogen (standard) method which has a slow freezing rate (due to the Leidenfrost phenomenon) [15-16] and is optimal only for samples with thickness up to ~2-5 μm; 2) the propane or ethane jet freezing method which has a faster freezing rate and is able to freeze low concentration samples with thickness up to ~20-50 μm; [17-18] and 3) the high-pressure (~2100 bar) freezing method which has the fastest freezing rate of the three methods and can be used to freeze high concentration samples with thicknesses up to ~200-500 μm [18-20]. Although the freezing rate is increased with the high pressure freezing method, the sample thickness that can be vitrified is still limited due to the low thermal conductance of water. The faster the freezing rate, the less crystallized ice formation occurs. However, there is little information published about how different freezing methods affect the structure of biopolymer hydrogels used for cryo-SEM imaging. Leis et al. [21] have investigated rigid ionotropic carrageenan gels using high-pressure freezing for cryo-TEM imaging in an attempt to achieve accurate preservation of the gel structures, ensure a sufficiently high signal-to-noise ratio, and acquire comprehensive datasets of the gel structures.

The protein studied here is the globular protein β-lactoglobulin. As is reported in Chapter 1 (§1.2), β-lactoglobulin forms amyloid-like fibrils following heating (80°C) under acidic conditions (pH ~2.0). At ambient temperatures, most studies to date have investigated the shape, contour, and persistence lengths of individual β-lactoglobulin fibrils and the fibril precursors to gel formation, at pH 2 using TEM [22] and AFM [23]. As it is reported in the abstract, persistence length expresses how much flexible or rigid a fibril is. In other words, it expresses how easy or hard is for thermal fluctuations to bend a fibril. The larger the persistence length, the harder for the fibril to bend. Hence, it could be defined as fibril persistence length (Fig. 2.1) the fibril length over which thermal fluctuations start to bend the fibril into different directions and constitutes a measure of flexibility (or conversely rigidity) of the fibrils [24-26].
Persistence length varies with temperature, [26] with concentration, and ionic strength changes [24-25, 27]. Fibrils in networks can be flexible when the persistence length $\ll$ contour or entanglement length, semiflexible when the persistence length = contour or entanglement length, and rigid when the persistence length $\gg$ contour or entanglement length [28-29].

![Fibril Persistence Length](image)

**Fig. 2.1** Presentation of fibril persistence length $l_p$.

Different established methods for persistence length evaluation of semi-flexible and rigid macromolecules can lead to different results. Cifra [30] has highlighted four ways of estimating the persistence length and suggested the most accurate method is based on chain rigidity. The first method is based on the average projection of the end-to-end distance on the first bond of the chain and it is valid for any state of the chain; either self-avoiding or ideal random chain. This method is therefore appropriate for general use in persistence length estimation. The second method is based on the average cosine of the bond angle in the chain, but it is only valid for ideal coil regions of the chain. The third method is based on the orientation correlation of bonds in the chain and although it also is valid within the coil regions, it has been shown to be less accurate than the previous method. The last method is based on worm-like chain models and it is appropriate for more rigid macromolecules (non-coil regions).

A number of previous studies have focused on measuring the effect of salts on the formation of $\beta$-lactoglobulin fibrils using SEM. Loveday et al. [31] for example has
studied how monovalent (LiCl, NaCl, KCl) and divalent (MgCl$_2$, CaCl$_2$, BaCl$_2$) salts affect the diameter and formation of whey protein isolate (WPI) and β-lactoglobulin nanofibrils [24] around pH 2.0 using TEM and rheology. Loveday et al. found that β-lactoglobulin hydrogels with increased salts form shorter and worm-like fibrils in contrast to the purified β-lactoglobulin hydrogels which form longer and thinner fibrils. From the perspective of kinetics, divalent salts seem to accelerate the gelation process compared with the monovalent salts. Other studies have examined fibril characteristics using both SEM and scattering methods. Aymard et al. [27] and Pouzot et al. [32] found that the persistence and contour lengths for β-lactoglobulin fibrils at pH 2 and β-lactoglobulin worm-like fibrils at pH 7 decrease with the increase of ionic strength using TEM and X-ray and neutron scattering methods. Mudgal et al. [25] studied the concentration effects on β-lactoglobulin fibril hydrogels at pH 2.0 and 3.35 using capillary and rotational viscometry, high performance liquid chromatography coupled with multi-angle laser light scattering (HPLC-MALS), and TEM. In this study it is suggested that the increase of protein concentration causes the increase of conversion from monomers and dimers to fibrils during heating of the protein. This results in the reduction of inter-particle distances and hence, in the increase of inter-particle interactions which cause the formation of large aggregates. Akkermans et al. [33] investigated the contour and persistence lengths of soy glycinin fibrils and showed that their properties were similar to those of β-lactoglobulin or WPI.

In this study it was investigated the most appropriate freezing method for high-water content samples: the one that introduces the minimum extent of freezing artefacts during sample preparation, allowing, as close as possible, imaging of the native state of protein fibril networks. The different structural parameters will be characterized for protein fibril hydrogels at different pH. It is reiterated here, as stated in Chapter 1 (§1.3), that I attempt to control fibril length scales using pH changes from 0.9-2.3. Two different freezing methods are used; propane jet freezing and boiling liquid nitrogen. Based on the work of Dubochet et el. [11, 34] for the vitrification of liquid water, it is expected that the propane jet freezing method will enable data to be
obtained that most closely represents the true structures of the pre-frozen gels compared with the boiling liquid nitrogen method, due to the faster freezing rate.

Firstly, the presence of ice crystal formation (freezing artefacts) is examined within low concentration β-lactoglobulin fibril hydrogels using the two different freezing methods, in order to examine the possibility of imaging the native state of protein networks. It is also checked the slow freezing method (boiling liquid nitrogen) for both protein (β-lactoglobulin) and polysaccharide (pectin) hydrogels to examine if the method itself tends to determine the structure observed. These experiments confirmed that the slow freezing rate of boiling liquid nitrogen method does not allow the cryo-SEM imaging of the native state of high-water content sample, due to the ice crystal formation during cryo-fixation.

Secondly, the fibril structural characteristics, like fibril size and length, for each different fibril system are determined. In this way, structural differences for different β-lactoglobulin fibril architectures were investigated.

The propane jet freezing method is also applied to high concentration samples. Examination of these samples with the available SEM was not straightforward due to the low microscope resolution. In addition, the small vitrification depth of the propane jet freezer method does not always allow the vitrified freezing of the high concentration samples.
Materials and Methods

Before the cryo-SEM methodology description, it would be useful to highlight why except from β-lactoglobulin pectin was also used in this study. The first cryo-SEM results exhibit no fibrils within the β-lactoglobulin networks formed either at pH 2.3, 2.0 or 0.9 and under heat-treatment at 80°C, although based on the literature β-lactoglobulin under these conditions forms amyloid-like fibrils. This is the reason why it should be investigated, if the non-fibril β-lactoglobulin structures presented in the cryo-SEM images, when boiling liquid nitrogen method is used, are caused due to inappropriate sample preparation or due to inappropriate freezing method of the sample. In order to investigate this, β-lactoglobulin gels are compared with acid pectin gels which also exhibit fibril networks. β-lactoglobulin systems are amino-acid based systems, while pectin systems are carbohydrate based systems and hence, it is expected that these systems will exhibit a different network structure. Nevertheless, by using boiling liquid nitrogen as a freezing method for both systems (β-lactoglobulin and pectin), a similar non fibril structure is presented in the cryo-SEM images for both systems. This confirms that the similar non-fibril networks presented in cryo-SEM images for β-lactoglobulin and pectin when boiling liquid nitrogen is used derive from the use of inappropriate freezing method rather than the use of inappropriate sample preparation.

Although the inappropriate method of boiling liquid nitrogen was used for the fibril acid pectin gels, it was possible to image an intact (i.e. non-collapsed) pectin fibril network possibly, due to the rigid pectin fibrils. This was useful, since the pectin fibrils are non-amyloid; hence, they could be used as a contrast to the β-lactoglobulin amyloid fibrils.
2.2 Sample Preparation

2.2.1 β-lactoglobulin Purification

β-lactoglobulin was isolated from whey protein isolate (WPI) 8855 (Fonterra Cooperative Ltd., Auckland, New Zealand). The β-lactoglobulin purification process and the preparation of β-lactoglobulin gel systems reported below are based on Jung et al. [22]. WPI 8855 consists of 93.5% protein (on an as is basis), 4.7% moisture, and 0.3% fat, the remainder is carbohydrate and ash. WPI 8855 powder was dissolved in MilliQ water yielding solutions of 10% w/v. The undissolved protein was removed by centrifuging the solutions at a pH close to the isoelectric point of the protein (pH 4.6) at room temperature at 15,000 rpm for 15 min using a Sorvall Evolution RC (Thermo Scientific, USA). The pH was adjusted with 1 M HCl or NaOH by monitoring the pH with a Mettler Toledo SevenEasy S20 pH Meter until the desired pH was reached. The centrifuged supernatant of the protein solution was then adjusted to pH 2.0 with 1 M HCl or NaOH and filtered through Millex syringes (PES 33 mm, 0.22 μm, sterile PP housing), in order to remove any remaining undissolved protein. The protein solution was dialysed in two stages, in order to minimize the salt content. In the first stage, the protein solution was dialysed twice against pH 2.0 MilliQ water for 20 h with one water change after 4 h using ZelluTrans/Roth dialysis membrane T2 (MW: 6000-8000 Da, Flat width 40 mm). After this, the protein solution was dialysed against MilliQ water for 48 h with the first water change after 4 h and the remaining changes every 10-14 h. The volume ratio of the solvent to the protein solution was kept constant at around 35. In order to remove any heavy metal traces from the dialysis membrane, the membrane was soaked in MilliQ-water for 15 min, heated while stirring for 30 min at 80°C in a 10 mM sodium bicarbonate solution and, afterwards, soaked in a 10 mM Na2EDTA solution for 30 min. The membrane was reheated for 30 min at 80°C in a MilliQ bath and, finally, cooled down prior to use. After the dialysis process, the protein solution was adjusted to pH 6.7 using 1 M HCl and, finally freeze dried.

Analysis of the freeze-dried protein (Table 2.1) was undertaken at the Nutrition Laboratory, in Massey University, Palmerston North, NZ. The freeze-dried protein
analysis presented that 93.1% of the freeze-dried powder was β-lactoglobulin protein (Methodology: Leco, total combustion method. AOAC 968.06, Nitrogen - protein conversion factor = 6.41), 96.6% was dry matter (Methodology: Convection oven 105°C, AOAC 930.15, 925.10), and the salt content was 1.57 g/100 g (Methodology: by calculation from chloride).

During the second stage, the freeze-dried protein obtained in stage one was dissolved again in MilliQ-water, the pH was adjusted to pH 2.0, and the solution was dialysed again against MilliQ-water for 48 h with the first water change after 4 h and the remaining every 10-14 h. The dialysed protein solution was adjusted to pH 6.7, freeze-dried and, according to the protein analysis (Table 2.1), 90% of the freeze-dried powder is β-lactoglobulin protein, 95% is dry matter, the salts are <0.032 g/100 g, and the rest is moisture, fat, total carbohydrate, and ash. The freeze-dried β-lactoglobulin was preserved in sealed plastic containers at 4°C.

Table 2.1 Analysis of lyophilised β-lactoglobulin (β-lg) powder.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Dry Matter %</th>
<th>Protein %</th>
<th>Salt g/100 g</th>
<th>Chloride g/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpurified WPI 8855 powder (pH=3.8 when dissolved in water)</td>
<td>93.2</td>
<td>14</td>
<td>1.52</td>
<td>0.92</td>
</tr>
<tr>
<td>Lyophilised singly dialysed β-lg extracted from WPI 8855 (pH=6.7 when dissolved in water)</td>
<td>96.6</td>
<td>93.1</td>
<td>1.57</td>
<td></td>
</tr>
<tr>
<td>Lyophilised twice dialysed β-lg extracted from WPI 8855 (pH=6.7 when dissolved in water)</td>
<td>90</td>
<td>95</td>
<td>0.032</td>
<td>0.02</td>
</tr>
</tbody>
</table>
2.2.2 Preparation of β-lactoglobulin and Whey Protein Isolate Hydrogels

β-lactoglobulin and WPI hydrogels were prepared according to Jung et al. [22]. The purified β-lactoglobulin or WPI was dissolved in MilliQ-water over a period of 8 h at 4°C, in order to allow the complete hydration of the protein. The protein solution was adjusted to three different pH values, 0.9, 2, and 2.3, using 1 M HCl and 1 M NaOH, and centrifuged at 13,400 rpm for 20 min using a MiniSpin-Eppendorf, and then filtered through Millex syringes (PES 33 mm, 0.22 μm, sterile PP housing). The addition of HCl and NaOH to the protein solutions changed the volume of the high concentration protein solutions, and the final concentration solutions prepared for this study were 2% w/v and 8.4% w/v. All the filtered protein solutions with corrected concentrations were then placed in Kimax glass tubes with caps sealed with DuraSeal laboratory sealing film and heated in a water bath (Circulator HAAKE DC1 Bath) for 10 h. The duration of 10 h for heat treatment was selected, since fibril networks are already self-assembled in a steady state after 10 h of heating and also this heat time is widely reported in the literature [22, 24, 31]. The protein solutions started to gel upon heat treatment. The heated solutions were immediately cooled after the heat treatment in an ice bath for 10 min, in order to stop the aggregation process. The non-heated protein solutions and hydrogels were preserved at 4°C for 4 days.

2.2.3 Pectin Purification

Pectin hydrogels were formed based on the method of Schuster et al. [40]. Pectin lyophilisation was the first step. Pectin, extracted from apples, having an initial degree of methyl-esterification (DM) of 75 % (90 % galacturonic acid) was purchased from Fluka Biochemika. Pectin was dissolved in Milli-Q water to yield a 1% w/w solution which was then, pH adjusted to 7.5 using 0.1 N NaOH. pH was monitored with a Mettler Toledo SevenEasy S20 pH Meter until the desired pH was reached. The homogalacturonan component of pectin was demethylesterified using the enzyme pectin methylesterase (PME), in order to produce a pectin with increased calcium sensitivity owing to the increase in the amount and blockiness of unmethylesterified, charged, galacturonic acid groups. The calcium sensitivity refers to the hydrolysis of the ester bond between methanol and galacturonic acids by PME which causes the
release of carboxyl groups. Hence, theoretically the carboxyl groups could interact with the calcium groups. More carboxyl groups, higher calcium sensitivity. These blocky pectins (number of non-esterified galacturonic acid groups) have been shown to gel upon acidification [40-41]. 1.66% w/v PME was added into the pectin solution until the final DM reached from 75% to 40% (confirmed using capillary electrophoresis), while 0.1 N NaOH was slowly added, in order to keep the pH constant at 7. Subsequently, the solution was heated at 80°C for 5 min, in order to denature the enzyme PME. The water was then removed from the solution by freeze-drying.

2.2.4 Preparation of Acid Pectin Hydrogels

The formation of 2% w/v acid pectin hydrogels was the second step. 2% w/w lyophilised pectin with DM 40 % and a blockwise distribution of carboxyl groups, generated as described, was dissolved in sealable glass vessels under stirring conditions at room temperature overnight and the pH was adjusted to 4.0. After stirring glucono delta-lactone (GDL), concentration 8 % w/w, was then added to the pectin solution and it was slightly mixed with a spatula. Lastly, the sample was left for at least 12 h at room temperature prior to examination, in order for the GDL to reduce the pH of the sample and the gel properties to stabilize.

2.3 Cryo-Scanning Electron Microscopy

Following sample freezing as described below, samples were loaded into a Gatan Alto 2500 cryo-unit (Appen. A, Fig. A1a), fitted onto the electron microscope (Appen. A, Fig. A1b), and kept at -120°C. The frozen samples were sublimated at -100°C for 17 min and coated with Pt using a current of 10 mA for 240 s and, then, imaged at 3 kV using a JEOL JSM-6500F Field Emission Scanning Electron Microscope. The sublimation time for pH 0.9 samples was increased to 24 min. This increase was required since their gelation time was much faster and they were more viscous compared with the lower pH samples. Based on the literature review [42], the contour and persistence lengths were expected to be dependent on the ionic strength. This
could be rationalized as follows the increase of ionic strength causes a decrease of the contour and persistence lengths which result in the formation of smaller pore sizes within the gel structure. Hence, higher resolution was needed for the pore sizes to be resolved and this, in combination with the thicker layers of samples loaded into the sample holder, led me to increase of sublimation time from 17 min to 24 min.

2.3.1 Boiling-Liquid Nitrogen Freezing Method

Polymer solutions were pipetted onto a stub with a 1 mm wide slot (Appendix A, Fig. A2a) and the stub was then inserted into a holder. The holder, attached to a heat-transfer rod, was plunged into nitrogen slush (Appendix A, Fig. A3a) at -196°C.

2.3.2 Propane Jet Freezing Method

β-lactoglobulin hydrogels were loaded between two copper plates (specimen sandwich) (Appendix A, Fig. A2b) and any excess solution was removed by absorption into filter paper. The specimen sandwich was attached to a heat-transfer rod and rapidly frozen at -186°C using a propane jet freezer BALTEC JFD 030 (Appendix A, Fig. A3b).

2.3.3 Use of Cryo-protectants in Propane Jet freezing Method

10% w/v sucrose and 10% w/v glycerol were used separately as cryo-protectants for preventing ice crystal growth during the cryogenic sample preparation. Each cryo-protectant was added into the 2% w/v β-lactoglobulin solutions after the gel formation (heat treatment) and before their freezing, in order not to affect the gelation process during the heating process.
2.4 Image Analysis

Cryo-SEM images were analysed with Image J1.49v available from https://imagej.nih.gov/ij/index.html. Firstly, scale bars were converted from pixels to nm. End-to-end fibril lengths ($l_e$ in Fig. 2.2a, b), mesh sizes ($l_m$ in Fig. 2.2a, b), fibril diameters and pore sizes were measured directly from the cryo-SEM images using the straight-line tool of Image J and related histograms were obtained. 100 fibrils were interrogated from each of 3 images at different magnifications to obtain the representative data for each sample. The persistence length, $l_p$, is shown in Fig. 2.2a, b, where as reported in the introduction above, the persistence length is the typical length at which thermal fluctuations bend the fibril into different directions. Persistence length, $l_p$ (see §2.1 for details), was calculated based on the average cosine θ (Fig. 2.2a, b) of the bond angle in the chain, as reported in Cifra [30]. This method has been applied to β-lactoglobulin fibrils at pH ~2.0 in a number of studies, including Mudgal et al. [25] and Loveday et al. [24, 43]. Fibrils were subdivided into segments, $s$, using the multiple-point picker plugin [23, 44] and the following equation was applied to the segments:

\[
l_p = \frac{<s>}{1-<\cos\theta>}
\]

where $<s>$ is the average segment length and $\theta$ is the angle between the tangents of two points of a fibril separated by distance $s$ (Fig. 2.2). $<\cos\theta>$ is the average correlation between the segments. The angles $\theta$ were measured using the angle tool plugin.
Fig. 2.2 End-to-end and mesh lengths within a fibril network in a) cartoon and b) cryo-SEM image. Distance $s$ and angle $\theta$ were used for the persistence length, $l_p$, estimation based on eq. 1. $l_e$ and $l_m$ are the end-to-end and mesh size, respectively.

One of the difficulties with this type of analysis is that it assumes that the data relate to a 2D image. Rather here I have a 2D projected image of a 3D structure. The construction of 3D maps from 2D images is very complicated and requires an
innovative 3D reconstruction algorithm. Usov and Mezzenga [45] have recently suggested the software "FiberApp" which provides a statistical analysis for the structural and topological description of fiber-like objects (length $\gg$ width). Specifically, this software has been applied to AFM images obtained from fibrous systems, such as cellulose nanofibrils or $\beta$-lactoglobulin fibrils at an air-water interface. This software seems to be more useful for fibrils on surfaces and their interactions with a surface upon their adsorption, whereas here it is revealed through the sample preparation procedure the 3D structure, which is then captured as a 2D projection of that 3D structure. In the future it is likely that more sophisticated software tools might be developed to examine this further.
Results and Discussion

In this present work, two different freezing methods were applied; slushy nitrogen (sometimes called boiling nitrogen) and propane jet freezing, to the SEM sample preparation of thermally induced β-lactoglobulin hydrogels with a view to observing how the different fibril lengths varied with pH changes. Under high vacuum the liquid nitrogen is boiling and starts to freeze. Due to the coexistence of two phases; the liquid nitrogen and the solid nitrogen (frozen nitrogen), the word "slushy" is used for this method. Low concentration hydrogels (2% w/v at pH 2.3, 2.0, and 0.9) were examined first, and in the most detail, as they exhibit low viscosity behaviour and hence, a thin layer can be easily loaded into the sample holder used for both freezing methods. In contrast, high pressure freezing is an appropriate method for thick samples, like high concentration hydrogels which exhibit high viscous behaviour. A high pressure freezing experimental set up was not available. Hence, only the propane jet freezing method was used in the preparation of high concentration (8.4% w/v at pH 2 and pH 0.9) hydrogels. Note that loading the sample into the holder, in order to get a very thin layer of sample, was difficult. A thin layer is required to ensure rapid freezing with the minimum freezing artefacts introduced within the hydrogel structure.

The first part of the experimental section (§2.6) refers to the freezing artefacts introduced via use of the slushy nitrogen method, whereas the second part (§2.7) refers to the determination of structural parameters within low and high concentration hydrogel networks vitrified by the propane jet freezing method. The third part (§2.8) refers to the use of cryo-protectants in cryo-SEM imaging and how they alter the native state of the fibril protein networks.

2.6 Boiling Liquid Nitrogen Method and Freezing Artefacts

Cryo-SEM experiments were performed on polysaccharide (acid pectin) and protein (β-lactoglobulin) gels loaded onto stubs with a 1 mm wide slot and frozen using slushy nitrogen. The data presented below indicate that this freezing method
introduces freezing artefacts due to the slow freezing rate. Specifically, the slow freezing rate allows the formation of ice crystals that increase the polymer concentration in the still as yet unfrozen water. Hence, as the crystal size is increased, the frequency of collisions between the fibrils [46] are increased which results in the formation of sequestered polymer walls (honeycomb-like wall structure), as shown schematically in Fig. 2.3. In this particular case, the samples were preserved and imaged at -130°C (devitrification temperature) after their initial freezing with slushy nitrogen and sublimation at -90°C.

According to Al-Amoudi et al. [47] sprayed water droplets imaged at -160°C were transformed into cubic ice around -135°C, whereas Dubochet [48] suggests that the transition of cubic ice to hexagonal ice probably takes place around -80°C. This phenomenon may aid in the interpretation of the honeycomb-like structure presented in Fig. 2.3 (right hand side). It should be highlighted here that even though a considerable number of investigations have been done examining the amorphous state of the water present in samples, there is still conflict regarding the glassy water polymorphism and the kinetic behaviour of water from form to form [49]. In Fig. 2.3 it is indicated that structural changes are likely occurring during the slow freezing process for biopolymers. These changes may explain why numerous studies in the literature present the same structures for both fibril protein and polysaccharide networks and also non-fibril gels when using slow freezing methods for cryo-EM imaging sample preparation [50-54]. Fig. 2.2b shows how ice crystal formation sequesters (squeezes) the native state of a fibril polymer, as presented in Fig. 2.2a, in the area of the unfrozen water. This results in the polymer being expelled from the aqueous phase and sequestered between the eutectic boundaries of the neighbouring ice crystals [55-56] resulting in the formation of a honeycomb-like structure (Fig. 2.2c). This means that the ice crystals are surrounded by eutectic phases [57]. The eutectic phases express the protein solution transformation into ice and sequestered protein, namely a liquid phase (protein solution) transformation into two solid phases (ice crystal and sequestered protein) [58].
Fig. 2.3 Proposed mechanism for the freezing process followed when using slushy nitrogen during sample preparation for high water content biopolymers. a) The first diagram presents the native state of the fibril biopolymers, b) the second presents the ice crystal formation which sequesters the biopolymer into unfrozen water, and c) the third presents the honeycomb-like structure formed due to ice crystal formation where the biopolymer is now mostly contained in the polymer walls.

The phenomenon described above for the honeycomb-like structure formation was exhibited here in two systems: an acid pectin gel (Fig. 2.4a-b) as a polysaccharide system and a β-lactoglobulin fibril gel (Fig. 2.4c-d) as a protein system. Several trials with different sublimation times and temperatures were undertaken, but all variations essentially result in the same honeycomb structures. Fig. 2.4a and c and Fig. 2.4b and d show that both systems exhibit strikingly similar structural features (honeycomb-like structure) when using the slushy nitrogen during sample preparation. Such similarity is unexpected considering that these polysaccharide and fibrillar protein gel systems are expected to have orders of magnitude differences in structural features, such as mesh size. Furthermore, the observed lengthscales for the structural features are comparable therefore more probably corresponding to the amount of water present and the freezing rate than truly representing features of the native gels. Hence, I strongly suspect that these similarities between the pectin and β-lactoglobulin hydrogels result from the freezing process method alone.
Fig. 2.4 Cryo-SEM images of 2% w/v acid pectin gels of a) 100 μm and b) 10 μm scale bars and 2% w/v β-lactoglobulin gels at pH 2.0 of c) 100 μm and d) 10 μm scale bars using the boiling liquid nitrogen method during sample preparation.

Fig. 2.5 shows an acid pectin fibril network that by contrast has not undergone collapse to the same degree as shown in Fig. 2.4. In this case, the same freezing method was used, but the sample was preserved and imaged at -120°C and sublimated at -100°C. Obtaining such an image was not the norm. This suggests that even though the freezing rate was slow, undertaking sublimation and sample preservation at temperatures sufficiently different from the devitrification and ice crystal phase transition temperatures can prevent the fibril network from entirely collapsing. Nevertheless, it should be highlighted that there are still regions of sequestered acid pectin (arrow in Fig. 2.5). The ability to capture some free regions in the pectin gels
may be due to the stronger inter-molecular interactions or stiffer fibrils within polysaccharide networks compared with protein networks.

The observed near native fibril networks presented in Fig. 2.5 will be used as a comparison for the images presented in the next section (§2.7) where β-lactoglobulin fibril hydrogels are studied using the propane jet freezing method during sample preparation. Although pectin features seem to be large for polysaccharides [40-41], it is expected that the protein fibrils (β-lactoglobulin) presented in §2.7 should exhibit the characteristic amyloid fibril form [60] and hence will be different from the polysaccharide non-amyloid fibrils (acid pectin) presented in Fig. 2.5.

![Fig. 2.5 Cryo-SEM image of a 2% w/v network within the acid pectin gel. Boiling liquid nitrogen was used for cryo-fixation during sample preparation. The non-fibril fragments at the left and bottom side of the image constitute the sequestered pectin formed due to ice crystal formation, as shown in Fig. 2.2.](image)

Overall, from the data obtained it is evident that the use of the slushy nitrogen method during sample preparation of the high water content hydrogel samples used here significantly disrupts the native structure of the gels and hence is not able to be used to reveal their true structure.
2.7 Propane Jet Freezing Method

2.7.1 Propane Jet Freezing Method for Low Concentration Hydrogels

Faster freezing methods, like the propane jet freezing method, increase the possibility for vitrification and hence for the true pre-frozen gel structure to be maintained during sample preparation. The propane jet freezing method, which provides more rapid heat conductance, was first used to study the fibril architecture of 2% w/v β-lactoglobulin and WPI gels formed upon heating protein solutions at 80°C for 10 h. This sample preparation technique is currently not widely used, since the vitrification depth is only a few micrometres at the freezing point, which results in the coexistence of amorphous and crystalline regions within the hydrogels [61]. In contrast, it is shown here that the SEM imaging of the native state of low concentration hydrogels using jet propane freezing is possible and hence that this method might be applied to a wide range of samples.

To act as a contrast control freshly prepared 8.4% w/v protein solutions (which were not heat treated) at pH 2.0 were also subjected to the freezing treatment and then examined using cryo-SEM. This allows me to determine that the fibrillar networks formed during heating are not present in the non heated sample. High concentration protein solutions of 8.4% w/v were selected in this case, because as the protein concentration increases the water volume decreases and hence, the freezing artefacts are reduced and the protein of high concentration behaves like a "cryo-protectant" within the solution. Hence, it was more likely that fewer artefacts would be introduced. Fig. 2.6 shows the resulting structure. In this case, a homogeneous layer of β-lactoglobulin protein is imaged with a few cracks possibly caused by the freezing or sublimation processes. Fibril networks are not observed as was expected. In contrast, Fig. 2.7a-c show the β-lactoglobulin fibril networks and Fig. 2.7d the β-lactoglobulin fragmented-fibril particle networks both formed during heat treatment for 2% w/v protein solutions at different pH values.
Fig. 2.6 Cryo-SEM image of a non heat-treated 8.4% w/v β-lactoglobulin solution at pH 2.0. The sample was cryo-fixed using the propane jet freezer method.

Images shown in Fig. 2.7a-d show the fibril structures of the gels formed once the protein solutions have been subjected to heat treatment. 2% w/v WPI gels at pH 2.0 and 2% w/v β-lactoglobulin gels at pH 2.3, 2.0, and 0.9 were examined. The presented networks clearly exhibit elongated fibrils except for the hydrogels formed at pH 0.9 (Fig. 2.6d) which exhibit fragmented-fibril particles that seem shorter, thicker, and more compact, in contrast to the images shown in Fig. 2.6 where there is no evidence of fibril formation.

Fibril networks formed at pH 2.0 and 2.3 (Fig. 2.7b-c) exhibit similar fibril formation. It should be highlighted here that Fig. 2.7c seems to exhibit a higher depth of field and hence lower image depth in the intermediate image plane. It is for this reason that the fibril hydrogel in Fig. 2.7c seems to be denser compared with the other gels shown.

Image analysis was applied, as presented in Fig. 2.8 and 2.10-2.13, in order to quantify the gel characteristics. Longer entangled fibrils are apparent at pH 2.3 (Fig. 2.7b) forming longer mesh lengths, whereas WPI and β-lactoglobulin fibril networks formed at pH 2.0 (Fig. 2.7a, c, respectively) exhibit no significant differences, despite
the fact that WPI contains a larger amount of ions due to the presence of considerable salt in contrast to the β-lactoglobulin protein. This could be explained due to the low WPI concentration used which therefore does not generate a significant increase of the ionic strength of the total solution used to form the hydrogels.

Fig. 2.7 Cryo-SEM image of 2% w/v a) WPI at pH 2.0, b) β-lactoglobulin at pH 2.3, c) β-lactoglobulin at pH 2.0, and d) β-lactoglobulin at pH 0.9 fibril gel networks prepared by heating protein solutions at 80°C for 10 h. Samples were cryo-fixed using the propane jet freezer method.

The SEM images obtained were analysed to obtain estimates of the diameters, end-to-end and persistence lengths, and mesh lengths for the fibrils in the 2% w/v protein hydrogels. The resulting data are presented as histogram distributions in Fig. 2.8 and Fig. 2.10-2.13.
Fibril diameters, as shown in Fig. 2.8 and summarized in Table 2.2, exhibit no significant difference for β-lactoglobulin hydrogels at pH 2.3 (Fig. 2.8b, fibril diameter range 3-17 nm) and β-lactoglobulin hydrogels at pH 2.0 (Fig. 2.8c, fibril diameter range 3.5-18.5 nm), possibly because the ionic strength is almost the same. In contrast, WPI hydrogels at pH 2.0 (Fig. 2.8a, fibril diameter range 2-14.5 nm) exhibit slightly thinner fibrils. It was expected, based on the literature, that there would essentially be no difference in the diameter size for the β-lactoglobulin hydrogels at pH 2.0 and 2.3 [24]. According to Jung et al. [22], based on SAXS data, the β-lactoglobulin fibril diameter at pH 2 is ~4 nm which is within the range measured here (Fig. 2.8c). Loveday et al. [24] using TEM measured β-lactoglobulin fibril diameters at pH 2.0 to be ~5-10 nm by TEM which also agrees with the fibril size distribution in Fig. 2.8c.

Table 2.2 Cryo-SEM distribution ranges for fibril diameters (D), fibril end-to-end lengths (l_e), mesh lengths (l_m), and fibril persistence lengths (l_p) of 2% w/v WPI gels at pH 2.0 and 2% w/v β-lactoglobulin (β-lg) gels at pH 2.3, 2.0, and 0.9 with different concentrations heated at 80°C for 10 h.

<table>
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<tr>
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<th>Cryo-SEM distribution range</th>
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<tbody>
<tr>
<td></td>
<td>D (nm)</td>
</tr>
<tr>
<td>2% w/v β-lg-pH 2.3</td>
<td>3.0-17.0</td>
</tr>
<tr>
<td>2% w/v WPI-pH 2.0</td>
<td>2.0-14.5</td>
</tr>
<tr>
<td>2% w/v β-lg-pH 2.0</td>
<td>3.5-18.5</td>
</tr>
<tr>
<td>2% w/v β-lg-pH 0.9</td>
<td>4.6-36.0</td>
</tr>
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</table>
Fig. 2.8 Fibril diameter distribution histograms of a) WPI gels formed at pH 2.0 and β-lactoglobulin (β-lg) gels formed at b) pH 2.3, c) pH 2.0, and c) pH 0.9. Gel networks were formed upon heating of 2% w/v protein solutions for 10 h at 80°C and pH 2.0. The jet freezing method was used during sample preparation for all samples.
Based on the β-lactoglobulin fibril size at pH 2.0 (~4 nm) estimated by Jung et al. [22], another interesting point presented here is that the fibrils at pH 0.9 are thicker (fibril diameter range 4.6-36 nm) compared with the other fibrils. It is already well-known [62] that the majority of amyloid fibrils are composed of parallel β-sheets in a cross-β conformation which are held together with hydrogen bonding. β-sheets consist of anti-parallel β-strands which lie perpendicular to the fibril axis. This could be used to interpret the formation process of long and unbranched fibrils [63-64]. Dahlgren et al. [65] suggested a fibril formation mechanism describing not only the fibril elongation, but also the fibril diameter variations and any branch morphologies. According to this mechanism, β-sheets can be stacked (β-sheets consist of β-strands and are connected parallel with hydrogen bonds) perpendicular to the fibril axis (fibril elongation) or lateral to the fibril axis (Fig. 2.9) with a possibility of arrangement of β-sheets along the fibril axis (generating fibril diameter variations and branched morphologies). This could mean that within pH 0.9 hydrogels the lateral orientation of β-sheets to the fibril axis dominates against the perpendicular orientation. This phenomenon results in the formation of short and thick fragmented-fibril particles rather than long and thin fibrils.

Fig. 2.9 Cartoon of the structural differences for β-lactoglobulin fibrillar gels at pH 2.3, 2.0, and 0.9. D represents the protein fibril diameter which is larger at pH 0.9 compared with the fibrils formed at pH 2.3 and 2.0. The β-sheet planes stack perpendicularly and laterally to the fibril axis (with possible arrangement of the planes along the fibril axis for the latter case) representing the mechanism for fibril elongation and fibril thickness variations, respectively. The end-to-end (l_e) and mesh (l_m) length decrease as the pH decreases from pH 2.3 to 0.9.
In addition to the freezing artefacts (ice crystal-formation) introduced by the cryogenic sample preparation, 2D analysis of 3D fibrils in the cryo-SEM images introduces an additional limitation here to the quantitative analysis of the SEM images. Furthermore, the low resolution of the cryo-SEM experimental set up used for this study could also constitute an additional limitation. Thus, the smallest fibril sizes present in a hydrogel network may be absent in the present fibril diameter distributions (Fig. 2.8). For these reasons SEM imaging may not be the most robust method for fibril diameter determination. To explore this, the SEM data are therefore compared with SAXS data in §3.4.3.1.

The end-to-end fibril length distribution ranges (l_e) given in Fig. 2.10 and summarized in Table 2.2 highlight that WPI gels at pH 2.0 (Fig. 2.10a, l_e range 650-1900 nm) exhibit roughly the same end-to-end length scales as those formed in β-lactoglobulin gels at pH 2.0 (Fig. 2.10c, l_e range 500-1650 nm). β-lactoglobulin fibrils at pH 2.3 (Fig. 2.10b) exhibit the longest end-to-end length scales (l_e 1030-3300 nm), while β-lactoglobulin gels formed at pH 0.9 (Fig. 2.10d) are the shortest (l_e 150-800 nm). Here, it is proposed that the variation of end-to-end length scales at different pH values could be attributed to a combination of factors (Fig. 2.9). As the pH increases, the hydrophobic interactions decrease and this inhibits the self-assembly process. On the contrary, as the pH decreases, the hydrophobic interactions increase and this facilitates the self-assembly process. Hence, the inter-fibril distances become smaller and the fibril lengths shorter [66-67] for pH 0.9 hydrogels. The fibrils formed at pH 0.9 exhibit also larger diameter compared with the fibrils formed at pH 2.0 and 2.3 and this is also another factor that contributes to the reduction of inter-fibril distances within pH 0.9 hydrogels.
Fig. 2.10 End-to-end fibril length: a) WPI 8855 gels formed at pH 2 and β-lactoglobulin (β-lg) gels formed at b) pH 2.3, c) pH 2.0, and d) pH 0.9. Gel networks were formed upon heating the 2% w/v protein solutions for 10 h at 80°C. Cryo-fixation was achieved using the jet freezing method. TEM data values supplied by Dr Luigi Sasso. TEM experiments were conducted on WPI 895.
It has been already reported that ionic strength and pH variations induce changes to the morphology of fibrils and the kinetics of the fibril formation process \[24, 27, 31, 42, 66\]. Specifically, upon reduction of pH, the $H^+$ could bind to the carboxyl group (COO$^-$) of Asp or Glu neutralizing their negative charge, while they give a positive charge to the amino group HN$_2$ of Lys or Arg. This results in the net charge becoming positive and the electrostatic repulsions dominating over the attractive interactions \[24, 67\]. It could be suggested that this phenomenon in combination with the perpendicular orientation of $\beta$-sheets to the fibril axis \[65\] upon decreasing pH reduces the end-to-end length.

The cryo-SEM data above were also compared with the corresponding TEM data obtained through a personal communication with Dr Luigi Sasso (Prof. Juliet Gerrard’s Group) at Canterbury University, Christchurch, NZ. It should be emphasised that in the previous work TEM imaging applied to whey protein isolate (WPI) 895 (Fonterra Cooperative Ltd., Auckland, New Zealand) fibril networks and not to $\beta$-lactoglobulin or WPI 8855 (Fonterra Cooperative Ltd., Auckland, New Zealand) fibril networks as was the case in this study. WPI 895 is a mixture of $\beta$-lactoglobulin protein and other globular proteins (e.g. $\alpha$-lactalbumin, bovine serum albumin) and a variety of salts. Despite the complex protein mixture, only $\beta$-lactoglobulin forms fibrils upon heating under acidic conditions. The WPI 8855 is also further purified compared with the WPI 895 and hence, contains less salts. WPI 895 end-to-end fibril length ranges were at pH 2.3 1750-6250 nm, at pH 2.0 1 range 500-3250 nm and at pH 0.9 250-1250 nm. This shows that the measured TEM end-to-end length distributions cover a wider range of end-to-end fibril lengths compared with the cryo-SEM distributions presented in this study. It should be taken into account that the TEM images are of dehydrated fibrils removed from heated solutions with a concentration of 0.1% w/v WPI 895 rather than the hydrogel network examined here and that the process of gel formation itself could limit the length of the fibrils in this study. Lastly, it is quite difficult to follow a single fibril’s direction in the SEM images due to overlap with other fibrils \[24\] in comparison to the individual fibrils imaged for the TEM images in the case of Sasso’s work.
The mesh size ($l_m$) distribution ranges are given in Fig. 2.11 and summarized in Table 2.2. β-lactoglobulin mesh lengths at pH 2.3 (Fig. 2.11b, $l_m$ range 8.5-57.5 nm) exhibit larger mesh lengths compared with the WPI (Fig. 2.11a, $l_m$ range 5.5-30 nm) and β-lactoglobulin (Fig. 2.11c, $l_m$ range 3.5-51.5 nm) mesh lengths at pH 2.0. On the contrary, pH 0.9 hydrogels (Fig. 2.11d, $l_m$ range 4.5-61.5 nm) exhibit the smallest mesh lengths. It should be highlighted here that pH 2.3 and pH 2.0 β-lactoglobulin hydrogels most frequently have mesh lengths within the range of 8.5-42 nm and 3.5-22 nm, respectively, while pH 0.9 hydrogels most frequently exhibit mesh lengths within the range of 4.5-23 nm. The observed long tails in the high end mesh length distributions may result from the presence of freezing artefacts. It should also be mentioned here that β-lactoglobulin gels at pH 0.9 are much denser than the other gels and the lower resolution of the images does not allow high accuracy analysis for these systems.
Fig. 2.11 Mesh length yielding information on pore size: a) WPI gels formed at pH 2.0 and β-lactoglobulin (β-lg) gels formed at b) pH 2.3, c) pH 2.0, and d) pH 0.9. Gel networks were formed upon heating the 2% w/v protein solutions for 10 h at 80°C. Samples were cryo-fixed using the propane jet freezer method.
The persistence length \( l_p \) distribution ranges shown in Fig. 2.12 and summarized in Table 2.2 indicate that persistence lengths are smaller than the end-to-end fibril lengths in Fig. 2.10 and that based on this the fibrils are flexible to semi-flexible. The smaller the fibril persistence length is compared with the end-to-end fibril length, the more flexible the fibril. Comparing the persistence lengths \( l_p \) with the end-to-end fibril lengths \( l_e \) in Table 2.2 and the corresponding most frequently presented values in Fig. 2.10 and 2.12, it seems that fibrils at pH 0.9 are the least flexible, while fibrils at pH 2.3 are the most flexible. Furthermore, the WPI fibrils at pH 2 are slightly more flexible than the \( \beta \)-lactoglobulin fibrils at pH 2 and pH 2.3. \( \beta \)-lactoglobulin fibrils at pH 0.9 are also shorter and thicker compared with the other \( \beta \)-lactoglobulin fibrils. The results presented in this study are close to those of Mudgal et al. [25] who found \( l_p \sim 788 \) nm for 1.5% w/v \( \beta \)-lactoglobulin fibrils at pH 2.0 using TEM imaging. This difference between the result of this study and that of Mudgal et al. [25] could be attributed to the different protein concentration and ionic strength of the samples and the 2D instead of 3D image analysis.
Fig. 2.12 Persistence length: a) WPI gels formed at pH 2.0 and β-lactoglobulin (β-lg) gels formed at b) pH 2.3, c) pH 2.0, and c) pH 0.9. Gel networks were formed upon heating the 2% w/v protein solutions for 10 h at 80°C. The propane jet freezing method was used to cryo-fix all samples.
2.7.2 Propane Jet Freezing Method for High Concentration Hydrogels

Propane jet freezing method is also used to study the fibril architecture of high concentration β-lactoglobulin gels (8.4% w/v) formed upon heating protein solutions at 80°C and pH 2.0 (Fig. 2.13a) and 0.9 (Fig. 2.13b and c) for 10 h. Generally, the β-lactoglobulin gels with high concentration (8.4% w/v) formed structures with features of different length scales compared with the low concentration (2% w/v) β-lactoglobulin gels. This means that the high concentration β-lactoglobulin gels formed smaller pores compared with the low concentration β-lactoglobulin gels as expected (this made direct observation of the pore sizes much more difficult to characterise). Thus, the execution of SEM experiments on high concentration β-lactoglobulin hydrogels was very difficult and this is the reason why good results were only obtained for the 8.4% w/v β-lactoglobulin gels formed at pH 2.0 (Fig. 2.13a) and 0.9 (Fig. 2.13b and c) and not at pH 2.3.

![Image](image1.png)

Fig. 2.13 Cryo-SEM images of 8.4% w/v β-lactoglobulin gels a) at pH 2.0 (magnification 30,000) and b-c) at pH 0.9 (magnification 30,000 and 43,000, respectively) prepared by heating protein solutions at 80°C for 10 h. Samples were cryo-fixed using the propane jet freezer method.
High concentration hydrogels are more viscous than the low concentration hydrogels are more viscous. This means that the freezing artefacts introduced into the high concentration protein hydrogel structures due to the ice crystal formation upon heating process are reduced compared with the freezing artefacts introduced into the low concentration protein hydrogel structures described earlier.

Based on analysis of representative SEM images, such as these shown in Fig. 2.13a-c, estimated diameter (Fig. 2.14), end-to-end (Fig. 2.15) and persistence fibril length (Fig. 2.17), and mesh length (Fig. 2.16) distribution histograms for 8.4% w/v β-lactoglobulin gels at pH 2.0 and 0.9 were obtained. The corresponding distribution ranges are summarized in Tables 2.3.

Fig. 2.14 Fibril diameter distribution histograms of 8.4% w/v β-lactoglobulin (β-lg) gels formed at a) pH 2.0 and b) pH 0.9. Gel networks were formed upon heating at 80°C for 10 h at the adjusted pH. The jet freezing method was used during sample preparation for all samples.
Table 2.3 Cryo-SEM distribution ranges for fibril diameters (D), fibril end-to-end lengths (l_e), mesh lengths (l_m), and fibril persistence lengths (l_p) of 8.4% w/v WPI gels at pH 2.0 and 8.4% w/v β-lactoglobulin (β-lg) gels at pH 2.3, 2.0, and 0.9 with different concentrations heated at 80°C for 10 h.

<table>
<thead>
<tr>
<th>Cryo-SEM distribution range</th>
<th>D (nm)</th>
<th>l_e (nm)</th>
<th>l_m (nm)</th>
<th>l_p (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.4% w/v β-lg-pH 2.0</td>
<td>1.0-28.5</td>
<td>75-705</td>
<td>14.0-85.0</td>
<td>25-255</td>
</tr>
<tr>
<td>8.4% w/v β-lg-pH 0.9</td>
<td>2.5-28.0</td>
<td>25-105</td>
<td>9.5-82.0</td>
<td>15-50</td>
</tr>
</tbody>
</table>

Fig. 2.14a and b and Table 2.3 show that the range of distributions for fibril diameters (D) for 8.4% w/v β-lactoglobulin gels at pH 2.0 and 0.9 exhibit slight difference (D range ~1.0-28.5 nm & 2.5-28.0 nm), while based on the % frequency values it seems that the distribution for 8.4% w/v gels at pH 0.9 follows a Gaussian-like distribution, while the distribution for gels at pH 2.0 is flat. However, the most frequently presented fibril diameters for gels formed at pH 2.0 are larger (up to 19.5 nm, Fig. 2.14a) compared with the most frequently presented fragmented-fibril diameters (up to 11 nm, Fig. 2.14b) for gels formed at pH 0.9. On the contrary, low concentration β-lactoglobulin gels (2% w/v) at pH 2.0 exhibit thinner fibril diameters (Fig. 2.8c, D range 3.5-18.5 nm) compared with the β-lactoglobulin gels formed at pH 0.9 (Fig. 2.8d, D range 4.5-36 nm). This shows that as the protein concentration increases significantly the fibril diameters increase and hence, the fibril diameter deviations between the fibrils formed at pH 2.0 and 0.9 become smaller. Based on the fibril diameter changes and the data presented below upon protein concentration changes, it is apparent that gel properties are affected by protein concentration changes. These changes are presented in Fig. 2.15 and discussed further below.
The fibril end-to-end length ($l_e$) distribution histograms (Fig. 2.16a and b) and their corresponding end-to-end length ranges summarized in Table 2.3 for high concentration β-lactoglobulin gels (8.4% w/v) show that fibrils formed at pH 2.0 are longer ($l_e$ range 75-705 nm) compared with the fragmented-fibril particles formed at pH 0.9 ($l_e$ range 25-105 nm) as was also determined at lower protein concentration. On the other hand, the data indicates that β-lactoglobulin fibrils of low concentration hydrogels (2% w/v) formed at pH 2.0 and 0.9 exhibit longer fibril end-to-end lengths (Fig. 2.10c, $l_e$ range 500-1650 nm and Fig. 2.10d, $l_e$ range 150-800 nm, respectively) compared with the corresponding fibril end-to-end lengths of high concentration gels (8.4% w/v). This can be explained in terms of increased cross-links within the gel structure caused by protein concentration increase.
Fig. 2.16 End-to-end fibril length distribution histograms of 8.4% w/v β-lactoglobulin (β-lg) gels formed at a) pH 2.0 and b) pH 0.9. Gel networks were formed upon heating the protein solutions at 80°C for 10 h at the adjusted pH. Cryo-fixation was achieved using the jet freezing method.

Although the range of the mesh length ($l_m$) distributions for high protein concentration gels (8.4% w/v) formed at pH 2.0 (Fig. 2.17a, Table 2.3) and 0.9 (Fig. 2.17b, Table 2.3) exhibit slight differences, the most frequently observed mesh lengths for gels formed at pH 2.0 are slightly larger (Fig. 2.17a, 23.5-56 nm) compared with the most frequently observed mesh lengths for gels formed at pH 0.9 (Fig. 2.17b, 11.5-49.5 nm). The same is observed for low protein concentration gels (2% w/v) formed at pH 2 (Fig. 2.11c) and 0.9 (Fig. 2.11d). Here, it should also be highlighted that the mesh lengths for high concentration gels (8.4% w/v at pH 2.0 and pH 0.9, Table 2.3) seem
to be larger compared with the mesh lengths for low protein concentration gels (2% w/v, Table 2.2). Although it was expected that the mesh lengths for high concentration gels would be smaller, since the protein concentration increases and the number of cross-links increases and the fibril end-to-end lengths decrease. These conflicting results may be caused due to the 2D image analysis of the 3D objects in the EM images or due to the introduction of ice crystals within the protein network during the freezing process which is part of the sample preparation for EM imaging. Another possible explanation consistent with the data in this study is that the fibrils formed at pH 0.9 during heat treatment at 80°C are thicker hence, the protein is concentrated into a smaller volume than at pH 2.0 and this "opens" up the pores, and hence longer mesh lengths are formed.

**Fig. 2.17** Mesh length distribution histograms of 8.4% w/v β-lactoglobulin (β-lg) gels formed at a) pH 2.0 and b) pH 0.9 yielding information on pore size. Gel networks were formed upon heating protein solutions at 80°C for 10 h and at the adjusted pH. Samples were cryo-fixed using the propane jet freezer method.
Comparing the fibril persistence lengths ($l_p$) and end-to-end fibril lengths ($l_e$) in Table 2.3 and their corresponding most frequently observed values in Fig. 2.16 and 2.18, respectively, for high concentration gels (8.4% w/v), it seems that fibrils at pH 2 (Fig. 2.18a) are more flexible compared with the fragmented fibrils at pH 0.9 (Fig. 2.18b). The same is observed for low concentration gels (2% w/v) formed at pH 2.0 (Fig. 2.12c, Table 2.2) and 0.9 (Fig. 2.12d, Table 2.2), although in this case the fragmented-fibril particles formed at pH 0.9 are thicker (Fig. 2.8d, diameter range 4.5-36 nm, Table 2.2) compared with the fibrils formed at pH 2 (Fig. 2.8c, diameter range 3.5-18.5 nm, Table 2.2). This shows that the fibril diameter possibly does not significantly affect the fibril persistence length and hence, its sensitivity to thermal fluctuations/bendings. This is possible only if the major defining factor for the persistence length is electrostatic in origin and this means that the persistence length changes mainly due to electrostatic interactions rather than thermal energy changes [68].

![Persistence length distribution histograms of 8.4% w/v β-lactoglobulin (β-lg) gels formed at pH 2.0 and pH 0.9. Gel networks were formed upon heating the protein solutions at 80°C for 10 h at the adjusted pH. The propane jet freezing method was used to cryo-fix all samples.](image-url)
2.8 Use of Cryo-protectants in Propane Jet Freezing Method

While liquid nitrogen is useful in maintaining low temperatures, it is not able to be used in the vitrification of high water content samples, like colloidal suspensions and cell interiors, due to its poor heat transfer behaviour [61]. Hence, the use of cryo-protectants in cryo-SEM have been widely used, since they delay ice crystal formation when the samples are immersed into liquid nitrogen; and hence, are purported to prevent the distortion of the structure. High concentrations of agents (e.g. 20% w/w), like glycerol, are widely used as cryo-protectants for cell banking. These agents however often introduce structural rearrangements into the samples even before freezing.

Although, nowadays, it is accepted that cryo-protectants introduce structural artefacts, due to ionic bonding changes [69], they continue to be widely used. Here, I show that cryo-EM imaging for amyloid fibril networks within hydrogels is not possible with the use of cryo-protectants, since the native state of the samples is altered by their presence. Specifically, Fig. 2.18a and b present the change of the 2 % w/v β-lactoglobulin fibril networks at pH 2.0 when cryo-protectants are added. Two cryo-protectants were used: sucrose (Fig. 2.19a) and glycerol (Fig. 2.19b). Comparing the β-lactoglobulin gels with added sucrose and glycerol with the purified β-lactoglobulin, it is highlighted that the β-lactoglobulin structure with sucrose (Fig. 2.19a) is somewhat collapsed. Fibril length appears reduced and the resulting shrinkage results in a very dense structure. This shows that cryo-protectants, like sucrose, affect the bonding within the protein fibril network and hence, their network architecture. Possibly, this is the reason why large void spaces are created. This shows that although cryo-protectants were used in conjunction with the propane jet freezing method, the solute was sequestered.

β-lactoglobulin gels with added glycerol (Fig. 2.19b) contain well-defined small void spaces and hence, it seems that the amount of 10 % w/v glycerol did not prevent the ice growth. It is also highlighted that uniform and continuous areas of glycerol can be
seen. Fig. 2.19c presents a uniform fibril structure with smaller void spaces. Hence, although ice crystal formation artefacts may be reduced, other modifications of the networks owning to the change in solvent conditions are introduced.

Fig. 2.19 Cryo-SEM images of 2% w/v β-lg fibril gel network at pH 2.0 heated at 80°C for 10 h with addition of a) 10% w/v sucrose and b) 10% w/v glycerol. Samples were cryo-fixed using the propane jet freezer methods.
Conclusions

The present work suggests that the freezing method giving the highest fidelity of accurate preservation of high-water content biopolymer samples for cryo-SEM imaging is the propane jet freezing method. It produces a fast freezing rate that introduces fewer freezing artefacts and allows for more truthful SEM imaging of high-water content samples compared with the slushy nitrogen method. In contrast, it has been shown that the slushy nitrogen method displays similar hexagonal-like structures for both protein and polysaccharide gels which reveal the distortion of the samples caused by ice-crystal growth. Hence, the proper cryogenic sample preparation constitutes a crucial step for reliable cryo-SEM imaging.

An alternative way of reducing the freezing artefacts is the addition of cryo-protectants within the sample. However, it has been demonstrated by cryo-SEM images that the use of cryo-protectants affects the solvent quality and polymer bonding and hence, the use of them does not allow cryo-SEM imaging of the native state of protein fibril hydrogels. In the case of sucrose, it changes the fibrils from long to short fibrils and causes shrinkage of the network structure. In the case of glycerol use, freezing artefacts are not avoided and the water is not easily sublimed which introduces new artefacts into the system.

The cryo-SEM imaging indicated that the fibril length scales can be controlled by adjustment of pH. As the pH increases, the mesh and end-to-end fibril lengths increase, while the fibril diameter decreases. This is attributed to the decrease of hydrophobic interactions with pH increase that inhibits the self-assembly process. In order to explain this in more detail, the self-assembly process is facilitated, the mesh lengths become smaller and the fibril lengths become shorter, since the number of nodes increases and this interrupts the perpendicular [63-64] stacking of β-sheet planes to the fibril axis. As the self-assembly process is facilitated and the hydrophobic interactions increase, the lateral [65] stacking of β-sheet planes to the fibril axis also causes the fibril diameter to increase.
It is suggested that a new type of β-lactoglobulin hydrogel was also presented here with fragmented-fibril particles formed at pH 0.9 being very closely aggregated. These particles are thicker, shorter, and less flexible compared with the β-lactoglobulin amyloid fibrils formed at pH 2.0 that have been widely studied. Fibrils at pH 2.3 exhibit the longest end-to-end length scales and highest flexibility compared with the fragmented-fibril particles at pH 0.9 which exhibit the shortest end-to-end length scales. Further, the presence of salts in the WPI hydrogels seems also to affect the fibril persistence length, since they form more flexible fibrils compared with the purified β-lactoglobulin fibrils at pH 2.0 as reported previously.
References


CHAPTER 3

Structural Characterisation of Amyloid-Fibril Networks Using Small-Angle X-ray Scattering

Abstract

Here, the structural changes are investigated occurring in β-lactoglobulin fibril gels at different length scales during and after the gelation process using SAXS at ambient temperatures (25°C). Based on the SEM data presented in Chapter 2, it is expected to find that fibril length is increased upon increasing pH from 0.9 to 2.3. Using these same systems here, changes in the SAXS data revealing information about the different hierarchical levels presented in these systems during and after the gelation process are studied. The different hierarchical levels relate to different length scales which reveal information on the fibril interactions, the mass and surface fractal dimensions, and the inter-atomic interactions. It is shown that the fibril roughness and compactness within these structures are dependent on pH, protein concentration, and heating time. It was found that the gel systems formed at pH 0.9 exhibit the shortest fragmented-particle fibrils, the most compact structure, the highest fibril surface roughness, and a less-ordered gel structure. The gels formed at pH 0.9 with protein concentration above 7% w/v exhibit similarities with the gels formed at pH 2.0 with protein concentrations of ~11.4% w/v. This study is a starting point for a more sophisticated understanding of the relationship between fibril interactions and architecture of the assembled biopolymers.
3.1 Introduction

The SAXS method is a useful experimental tool which allows the study of structures existing at different length scales from low (large length scales) to high (small length scales) \( q \) wave vectors. The intensity of the SAXS pattern is measured as a function of the scattering vector (Fig. 3.1), \( q \), where \( q = \frac{4\pi\sin\theta}{\lambda} \), with \( \theta \) the scattering angle and \( \lambda \) the X-ray wavelength. SAXS covers spatial resolution from tens to several hundred nanometres \((2\pi/q)\) [1].

![Diagram of SAXS experimental setup](image)

**Fig. 3.1** Basic SAXS experimental setup.

Biomaterials present in nature with particulate disordered structures (high dispersed molecular density), like gels, soft tissues, and glasses, can be described in terms of the concept of fractal geometry which was born out of the lack of geometric tools, like spheres, squares, etc. [2] that could be used to describe such systems. The meaning of the term fractal was first introduced by Mandelbrot [3], in order to describe natural shapes of topographical features and landforms (e.g. mountains and coastlines). The cornerstone of the concept of fractals is the meaning of dimension. For example, in order to describe an amorphous material in nature, a box of size 'R' is drawn and the mass 'M' inside the box is summed. The mass 'M' of the amorphous material within that box follows the power law behaviour \( M \sim R^D \) where \( D \) is defined as the mass fractal dimension. In the case of a Euclidean object the mass would follow the relation \( M \sim R^3 \) [4].
In the case of SAXS methods, the relation between the scattered X-ray beam intensity at small angles and the q wave vector typically follows a power law $I(q) \sim q^{-\alpha}$ within a defined region. If $1 < \alpha < 3$ then the power-law exponent $\alpha$ corresponds to the mass fractal dimension $D_m$ which expresses the way that the scattering atoms fill space [5]. Specifically, when $\alpha = 3$ aggregates are densely packed, whereas when $\alpha = 1$ aggregates are weakly packed [6]. On the other hand, if $3 < \alpha < 4$ (Porod regime-short wavelength limit) [7], the power-law exponent $\alpha$ is equal to $6 - D_s$, where $D_s$ is the surface fractal dimension and expresses the surface roughness [5]. Specifically, smooth and rough surfaces are exhibited when $\alpha$ equals to 4 and 3, respectively [8].

Another characteristic that can be derived from the Porod regime of SAXS patterns is the Porod volume. According to Armbrüster et al. [9], the Porod volume is defined as the excluded volume of the hydrated particles. By investigating changes in the Porod volume, important information about polymer systems in connection with thermodynamics and specifically entropy can be extracted. Entropy expresses the order of ataxia within a system. Onsager et al. [10] studied the isotropic (orientationally disordered) to nematic (orientationally ordered) transition (I-N transition) of monodisperse hard rigid rods and found that this transition originates from competition between orientational and translational entropy. Specifically, assuming an athermal solution with thin rods where only repulsive forces act between them [11], orientational entropy dominates over translational entropy (isotropic phase) [12]. As concentration is increased the two entropies compete and at a critical concentration translational entropy dominates over orientational entropy (nematic phase) and hence, excluded volume per particle is decreased [12-13]. Investigating the orientational vs translational entropy on molecular level, it can be defined that the orientational entropy expresses the number of different angles at which a molecule (fibrils in this case) can orientate [14-15]. At high entropy the molecule is free to rotate at any angle, while at low entropy the molecule can rotate at only one or two directions. On the other hand, translational entropy [14-15] on a molecular level expresses the number of different spatial positions that a molecule can occupy. At high entropy the molecules are distributed randomly within the structure, while at low energy the molecules are distributed in a specific way within the structure.
Several literature investigations have explored the structure of the fibrils forming networks using scattering techniques. Specifically, Jung et al. [16] have previously used scattering techniques, such as small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), small-angle light scattering (SALS), and ultra small-angle light scattering (USALS), in order to investigate the internal structures and fractal dimensions of β-lactoglobulin gels formed at pH 2.0, 5.8, and 7 and also the different particle sizes and morphologies. On the other hand, Aymard et al. [17] and Pouzot et al. [18] studied the length scales of β-lactoglobulin fibrils and ovalbumin, respectively, revealing the relation between ionic strength and fibril persistence length.

The study presented here addresses gaps in the literature and aims to relate the structural changes found in protein amyloid fibril hydrogels after the gelation process to the competition between orientational and translational entropy as the excluded volume of the fibrils changes. In other words, this study will investigate at how many different angles the fibrils can rotate and how much randomly the fibrils are distributed in each hydrogel system. It also considers how these structural and kinetic changes are connected to the related changes which are manifest on different scales upon the gelation of fibril hydrogels.
Materials and Methods

In this present work, SAXS experiments are performed on thermally induced β-lactoglobulin gel systems at different pH values and ionic strengths. The first part of the experimental section refers to changes in thermally induced gel networks after the gelation process, whereas the second section refers to kinetic differences between the β-lactoglobulin networks during the gelation process.

3.2 Preparation of β-lactoglobulin and Whey Protein Isolate Hydrogels

Lyophilised β-lactoglobulin protein and unpurified WPI powder (see §2.2.1) were used for the fabrication of β-lactoglobulin and WPI hydrogels, respectively, as described in §2.2.2. In this case the filtered protein solutions were placed in tubes with screw caps, sealed with DuraSeal laboratory sealing film, and heated in a Contherm oven (Design series oven, Scientific Ltd) at 80°C for the heating periods of 2, 6, 10, and 16 h. The protein solutions start to gel upon heat treatment. The heated solutions were immediately cooled after the heat treatment in an ice bath for 10 min, in order to stop the aggregation process. The samples were studied immediately after their preparation.

3.3 Small-Angle X-Ray Scattering

SAXS experiments were carried out at the Australian Synchrotron, Clayton, Australia. The beamline operated from an in-vacuum undulator of 3 m length and 22 mm period with energy resolution 10^{-4} obtained from a cryo-cooled Si (111) double crystal monochromator. The beamline energy was set at 8.2 keV for the structural study of the protein gels and at 16 keV for the kinetic study of the gelation process. The SAXS patterns were collected using a Dectris-Pilatus 1M detector (Appen B, Fig. B1). Two different camera lengths were selected, because different length scales were of different significance at different times. The detector-sample distance was determined to be 7198 mm and 500 mm for the two beamline energies, respectively.
Gelled β-lactoglobulin samples were loaded into holes into stainless steel sample holder plates (316 SST) of thickness 2 mm (Appen. B, Fig. B2a). These plates were appropriate for the low pH conditions used in these experiments, ensuring no corrosion of the plates occurs. Mica windows of thickness 0.035 mm were used to make windows on one side of the sample holders. The pre-gelled samples were loaded into the holes and a second mica window was secured in place using tape (Scotch duct tape). Mica windows were used instead of Kapton, in order to avoid high background scattering. Mica windows of small thickness produced low scattering compared with the weak scattering coming from the amorphous structure of the protein gel networks. All SAXS experiments on pre-gelled samples were performed at room temperature (25°C).

SAXS experiments were also performed during the gelation process at 80°C. Protein solutions were loaded into flame sealed glass thin-walled capillary tubes (Appen. B, Fig. B2b) (Charles Super Company, diameter 1.5 mm) with needles (Braun, 0.80 x 120 mm), in order to record the SAXS patterns. Capillaries were preferred in this case as loading liquids between mica windows was problematic.

The raw scattering data (I(q)-q) were analysed using the Scatterbrain software (http://www.synchrotron.org.au/aussyncbeamlines/saxswaxs/software-saxswaxs, Australian Synchrotron) [19]. Specifically, the scattering intensities I(q) were corrected for beam fluctuations and exposure times and scattering from the background (water with mica windows) was subtracted from the data.

3.3.1 Dimensionless Kratky Plots

Kratky considered the relation between X-ray scattering intensity and the scattering vector q in the form of q^2I vs q using Gaussian coil models (the probability distribution for the end-to-end distance of a chain follows a Gaussian distribution), in order to characterize protein conformation and flexibility [20-21]. The Kratky plots
focus on the high scattering angles (high $q$ values) and specifically, in the case of globular folded proteins, the Kratky plot (Fig. 3.2) exhibits a bell shape with a pronounced maximum with the intensity decreased at high scattering angles following the asymptotic behaviour $I \sim q^{-4}$ (Porod’s law) [20]. On the other hand, in the case of unfolded proteins the Kratky plot exhibits a plateau over a specific range of $q$ and then follows a monotonic increase at high scattering angles. The latter behaviour is usually observed in experiments, whereas on a theoretical level the intensity for ideal Gaussian chains follows the asymptotic behaviour $I \sim q^{-2}$ over a specific range of $q$ and exhibits a plateau at high scattering angles [22].

**Fig. 3.2** Dimensionless Kratky plot $(qR_g)^2 I/I(0)$ vs $qR_g$ presenting the expectations for folded, unfolded, and partially unfolded protein.

The advantage of using normalized versions of these Kratky plots for different macromolecules is that scattering profiles of different protein mass and conformational states are compared. Thus, following the method proposed by Durand et al. [23], the Kratky plots are made dimensionless $[(qR_g)^2 I/I(0)$ vs $qR_g]$ by normalizing the scattering data to $I(0)$ and the wavevector by $R_g$. By dividing the scattering data $(I-q)$ by $I(0)$, the scattering data become independent of molecular
weight, since \( I(0) \) is proportional to the protein’s molecular weight, whereas by multiplying the scattering vector by \( R_g \) the angle scale becomes independent of the particle size. In this way, scattering profiles of protein particles with different mass and size can be compared.

The scattering pattern of a globular folded protein follows the Guinier law: \( I(q) = a_0 \exp \left( -R_c^2 q^2 / 3 \right) \) [21] (Fig. 3.2, folded protein). Hence, the dimensionless Kratky plot for a globular folded protein will exhibit a bell shape with the peak maximum at \( qR_g \approx \sqrt{3} \) and \( (qR_g)^2 I(0) \approx 3e^{-1} \) [23]. In the case of unfolded proteins (Fig. 3.2) the relation between scattering data \( I(q) \) and scattering vector \( q \) does not follow the Guinier law. Specifically, Gaussian chain scattering profiles follow Debye’s law: \( I = I(0) \frac{2}{x^2} (x - 1 + e^{-x}) \) where \( x = (qR_g)^2 \). In this case, the Kratky plot exhibits a horizontal asymptote at high scattering angles at \( (qR_g)^2 I(0) \approx 2 \) [23-24]. On the other hand, in the case of a non-Gaussian chain (Fig. 3.2, partially unfolded) the rigidity affects the horizontal asymptote which starts to show a linear increase at high angles [25]. In this way, Kratky plots can be used to distinguish disordered systems from folded proteins and to qualitatively characterize the flexibility of protein structures.

3.3.2 Guinier Plot (Reciprocal Space)

The radius of gyration \( R_g \) is the root-mean square of the distance of all electrons from the centre of mass of the chain [26]. Based on the radius of gyration, an overall "particle size" can be estimated from the SAXS patterns within the range of \( qR_g \leq \sim 1 \) where the wavevector \( q \) and the scattering intensity \( I(q) \) from the sample do not depend on the particle shape [27]. In the case of \( \beta \)-lactoglobulin fibrils, it is assumed that the fibrils are elongated thin particles with a circular cross-section. Use of the Guinier plot (see Fig. 3.3), \( \ln q I(q) - q^2 \), [28-29] is a popular method for the radius of gyration \( R_g \) estimation for globular particles and the radius of gyration of the cross-section \( R_c \) estimation for elongated particles. According to the Guinier plot, the
relation between I and q for elongated thin rods with length $L \approx \sqrt{2}R_g$ is expressed as [27-28]:

$$qI(q) \cong I \exp \left( -R_c^2 q^2 / 2 \right) \quad (1)$$

$$\ln qI = \ln I(0) - \frac{R_c^2 q^2}{2} \quad (2)$$

where $q$ is the wave vector, $I$ the intensity, and $I(0)$ the intensity at $q=0$ which depends on the number of particles and the number of electrons in each particle. The plot displays linear behaviour only for the region $\frac{2\pi}{L} < q < \frac{1}{R_c}$.

The geometric radius $R$ of the cross-section $R_c$ of long fibrils can be extracted from the slope $-R_c^2 / 2$ (see eq. 2) of the Guinier plot (Fig. 3.3) and is given by $R = \sqrt{2}R_c$. Hence, the diameter $D$ of the fibril is given by $D=2R$. The Guinier plot was obtained using the software Primus (Primus Qt) [30] from the ATSAS (package https://www.embl-hamburg.de/biosaxs/software.html).

![Guinier plot](image)

**Fig. 3.3** Here the Guinier plot for a $\beta$-lactoglobulin gel heated at 80°C and pH 2.3 is shown allowing the particle’s cross-section $R_c$ to be determined. The zero-angle intensity $I(0)$, is used to exemplify the various elements of the plot as described in the text.
3.3.3 Persistence length

The persistence length, according to Cros et al. [31] expresses the stiffness of fibrils, since it is indicative of the distance through which the longitudinal axis of a chain can be considered as linear. The persistence length was calculated according to Kratky et al. [32] and Porod [33]. The persistence length \( l_p \) is extracted from the q-I log-log plot from the intersection, \( q^*_{\text{Kratky/Porod}} \) (see Fig. 3.4), of two lines fitted to the two mass fractal power law regions with slopes -2 (Gaussian region), -1 (persistence region). \( q^* \) is related to the persistence length via the equation \( l_p = \frac{6}{\pi q^*} \) [34]. It should be highlighted here that in this study (in concentrated materials), the mesh lengths within the fibril gel networks are smaller than the persistence lengths (see Chapter 2) hence, in this case it is being measured the mesh length rather than the persistence length.

![Fig. 3.4 I-q log-log plot for β-lactoglobulin gel heated at 80°C and pH 2.3, in order to determine the persistence length \( l_p \) from the equation \( l_p = \frac{6}{\pi q^*} \).](image)

3.3.4 Porod Volume and Molecular Weight Estimation (Real Space)

The Porod volume is dependent on the intensity at zero scattering angle \( I(0) \) [35]. Hence, a prerequisite step for the Porod volume evaluation is the creation of a Guinier plot, as described above. By using the same value of the radius of gyration of the
cross-section, as defined in § 3.3.2, and the same software, Primus (Primus Qt) [30] from the ATSAS package (selecting Distance Distribution which recalls the GNOM [36] programme), the pair-distance distribution analysis p(r)-r, which expresses a continuous r²-weighted histogram of all electron pair distances, r, in the protein, can be extracted [37]. The scattering intensity I(q) is transformed to p(r) by taking an inverse Fourier transform [20, 38]:

\[ p = \frac{r}{2\pi^2} \int_0^\infty q^2 I(q) \frac{\sin(qr)}{qr} dq \quad (3) \]

The useful parameter obtained by the p(r)-r analysis is the maximum diameter r=D_max of the particle in the hydrogel. D_max was found from the distance scale beyond which the electron density is zero (p(r)=0). The recommended limit for the p(r)-r distribution is q_max < \frac{8}{R_g}. Petoukhov et al. [39] set this limit, because of algorithm approximations within the software which require limited wide angle regions for the reliable computation of p(r)-r analysis and Porod volume (V_p, see below).

Subsequently, the radius of gyration R_g [20] and the intensity I(0) [40] at the origin were derived from eq. 3 and estimated by the equations 4, 5, respectively:

\[ R_g^2 = \frac{\int_0^{D_{\text{max}}} r^2 p(r) dr}{2 \int_0^{D_{\text{max}}} p(r) dr} \quad (4) \]

\[ I(0) = 4\pi \int_0^{D_{\text{max}}} p(r) dr \quad (5) \]

The Porod volume V_p (eq. 6) [39, 41] was computed based on the following:

\[ V_p = \frac{\int_0^\infty 2\pi^2 I(0)}{\int_0^\infty q^2 [I(q) - A] dq} \quad (6) \]

where A is an appropriate constant subtracted from each data point to force the q^-4 decay of the intensity at higher angles following Porod's law for homogeneous particles. Q = \int_0^\infty q^2 [I(q) - A] dq is determined as Porod invariant and corresponds to the integrated area under the curve for folded proteins. Thus, the Porod invariant and the Porod volume estimation is not possible for fully unfolded proteins [35].
After the Porod volume estimation, the molecular mass (MM) of the protein was estimated from the data. There are several ways to estimate the MM. One of the most widely used ways to estimate the MM of a protein using SAXS experiments is that described in Mylonas et al. [42]. According to this method, the experimental protein MM can be estimated from SAXS experiments (using a standard protein with the requirement of precise knowledge of MM (error ~10%) and concentration (accuracy ~5-10%)) following eq. 7:

\[ \text{MM}_p = \frac{I(0)_p}{c_p} \frac{\text{MM}_\text{st}}{I(0)_\text{st}/c_{\text{st}}} \]  

(9)

where \( \text{MM}_p \) and \( \text{MM}_\text{st} \) are the molecular mass, \( I(0)_p \) and \( I(0)_\text{st} \) are the scattering intensities at zero angle, whereas \( c_p \) and \( c_{\text{st}} \) are the concentrations of the studied (p-index) and standard protein (st-index), respectively. The standard protein should be monodisperse, monomodal, and globular in an appropriate buffer. However, in this study this was not feasible due to a lack of precise knowledge of the sample concentrations.

In this study the Porod volume is used to estimate the MM since it does not require the knowledge of the exact sample concentration. According to Petkouhov et al. [39], the Porod volume (in \( \text{nm}^3 \)) for globular proteins is equal to 1.6-1.8 times the MM (kDa) \( (\text{MM} \approx \frac{V_p}{1.6}) \).
Results and Discussion

In order to evaluate the data presented below, the SAXS data are divided into five regions based on the information they reveal (Fig. 3.5); (i) the inter-particle interactions, (ii) the form of particle, (iii) the mass fractal ($D_m$), (iv) the surface fractal ($D_s$), and (v) the inter-atomic interactions regions [43]. These different regions correspond to critical length scales of the gel systems. The inter-particle interactions and the size of particle regions were investigated using the long camera length (length scales 3.5-210 nm), whereas the mass fractal and surface fractal dimensions, and inter-atomic interactions regions were investigated using the short camera length (length scales 0.3-16 nm).

![Decreasing length scale](image)

**Fig. 3.5** Various structural information obtained from analysis of the SAXS data, here for a 8.4% w/v β-lactoglobulin gel heated at 80°C.
3.4 pH and Concentration Dependence of β-lactoglobulin Networks after the Gelation Process

3.4.1 Normalised Kratky Plots

Here, Kratky plots are presented, in order to investigate the β-lactoglobulin conformational state (pH 2.3 (Fig. 3.6), 2.0 (Fig. 3.7), and 0.9 (Fig. 3.8) heated at 80°C) and the rigidity of the polypeptide chains [44]. Data in Fig. 3.6-3.8 show that the β-lactoglobulin is partially unfolded (bell shape with pronounced maximum followed by monotonic intensity increase) at pH 2.3 (Fig. 3.6a-c), 2 (Fig. 3.7a-e), and 0.9 (Fig. 3.8a-c). The disulfide bridges (Cys66–Cys160 and Cys106–Cys119) [45] and the thiol group at Cys121 in the protein tertiary structure are responsible for the protein shape. Upon heating of protein solutions at 80°C the disulfide bridges break and hence, the protein unfolds. The bell shape curve corresponds to a folded protein domain. Upon the increase of protein concentration the number of monomers (162 amino acids each) [45] and disulfide bridges are increased and hence, the protein unfolding is more evident.

Fig. 3.6 Kratky plots of a) 7% w/v, b) 5% w/v, and c) 2% w/v β-lactoglobulin gels heated at 80°C and pH 2.3 for different heating times.
Fig. 3.7 Kratky plots of a) 11.4% w/v, b) 8.4% w/v, c) 7% w/v, d) 5% w/v, and e) 2% w/v β-lactoglobulin gels heated at pH 2.0 for different heating times.
Fig. 3.8 Kratky plots of a) 8.4% w/v, b) 7% w/v, and c) 5% w/v β-lactoglobulin gels heated at pH 0.9 for different heating times.

The loss of the bell shaped peak in the Kratky plots expresses the loss of the protein folded domain within the system [44]. The data here show that all gel networks with concentrations of 2% w/v at pH 2.3 (Fig. 3.6c) and pH 2 (Fig. 3.7e) exhibit a larger unfolded domain (lack of bell shape) compared with gel networks with higher concentrations than 5% w/v (Fig. 3.6a-b and Fig. 3.7a-d). Generally, at concentration hydrogels (2% w/v) at pH 2.3 and pH 2.0 unfold more easily compared with the high concentration hydrogels. A more reasonable explanation is that the protein at pH 2.3 and 2.0 acts as a stabilizer (resists to unfold) in high concentration systems unlike in low concentration systems. Generally, the role of non-covalent bonds in the protein unfolding process is important within the different protein hydrogel systems. Gel networks at pH 0.9 (Fig. 3.8) exhibit the largest folded domain. This means that these hydrogels exhibit a high resistance to protein unfold during the heating process,
possibly because the protein at pH 0.9 exhibits a higher secondary structure and a more compact conformation than native protein.

Here it is shown that as the concentration decreases to 2% w/v, the bell shape peak value is shifted to smaller q values and starts to vanish for gel networks at pH 2.3 (Fig. 3.6c) and 2.0 (Fig. 3.6e). This means that the oligomers (peptide) become larger [46] before the protein starts to denature. Another interesting point here is that the bell shaped peak for the pH 0.9 networks is followed by monotonic intensity increase at higher q values compared with the other hydrogels, as reported above. This happens because this type of hydrogel exhibits the largest oligomers (peptide) at high q values.

Kratky plots can also be used to enable a qualitative approach to the estimation of the rigidity and flexibility of the fibrils within the network [47]. Specifically, when the scattering curve in a Kratky plot decreases towards zero and flattens at high q values, then the fibrils are rigid [48]. On the contrary, when the scattering curve in a Kratky plot increases at intermediate and high q values, the fibrils are flexible. Here, the change of undulating slope to asymptotic [47] at q ~2.2 nm\(^{-1}\) at pH 2.3 (Fig. 3.6) and 2 (Fig. 3.7) and at q ~4 nm\(^{-1}\) at pH 0.9 (Fig. 3.8) reveals the change of rigidity for the polypeptide chains. Special cases constitute the 2% w/v fibril networks at pH 2.3 and pH 2.0 where the asymptote becomes steeper at high q values specially, for only 2 h heating times, and hence, these systems exhibit more flexible chains. On the contrary, the scattering curve for the pH 0.9 networks with concentrations 8.4% w/v and 7% w/v (Fig. 3.8a-b) and the high concentration networks (11.4% w/v) at pH 2.0 (Fig. 3.17a) seems to flatten at high q values and this suggests the presence of rigid fibrils. In this case it should be highlighted that hydrogels at pH 0.9 and hydrogels with high concentration at pH 2.0 seem to exhibit larger diameter particles compared with the low concentration samples at pH 2.3 and 2.0. This particle size difference could also contribute to the increase in rigidity for pH 0.9 hydrogels and pH 2.0 hydrogels with high concentration.
3.4.2 Structural Hierarchy

Scattering patterns were recorded for different concentrations, heating times, and pH. The changes along the SAXS curves reveal information about the different protein hierarchical levels [41], namely about the protein inter-particle interaction region, the inter-domain and intra-domain structure (mass ($D_m$) and surface ($D_s$) fractal dimensions), and the interatomic interaction region ($q_o$), as presented in Fig. 3.9-3.11.

**Fig. 3.9** SAXS data of a) 7% w/v, b) 5% w/v, and c) 2% w/v β-lactoglobulin gels heated at pH 2.3 for different heating times. The cryo-SEM image of 2% w/v β-lactoglobulin gel shown in c is reproduced from Fig. 2.6b.
Fig. 3.10 SAXS data for a) 11.4% w/v, b) 8.4% w/v, c) 7% w/v, d) 5% w/v, e) 2% w/v β-lactoglobulin and f) 8.4% w/v WPI gels heated at pH 2.0 for different heating times. The cryo-SEM image of 2% w/v β-lactoglobulin gel shown in e is reproduced from Fig. 2.6c.
Fig. 3.11 SAXS data of a) 8.4% w/v, b) 7% w/v, and c) 5% w/v β-lactoglobulin gels heated at pH 0.9 for different heating times. The cryo-SEM image in c is reproduced from Fig. 2.6d.

The slope of each region in Fig. 3.9-3.11, each representing a different hierarchical level of the β-lactoglobulin hydrogels at pH 2.3, 2.0, and 0.9, is presented in detail in Table 3.1a, b, and c, respectively. The data show that β-lactoglobulin fibril networks exhibit steeper slopes (region of inter-particle interactions) in the low q-region (q < ~0.05 nm\(^{-1}\) in Fig. 3.9-3.11) as the pH is reduced from pH 2.3 to 0.9 irrespective of the protein concentration. The steeper the slope (region of inter-particle interactions in Fig. 3.9-3.11), the stronger the inter-particle interactions. This suggests that the β-lactoglobulin fibril networks [44] exhibit larger agglomerates as the pH is decreased from pH 2.3 to 0.9. This is in agreement with Matson et al. [49] where the steeper the slope at low q values corresponds to peptide amphiphile nanofibers of higher-order aggregates (bundles). This is attributed to the increase of inter-fiber hydrogen...
bonding. On the other hand, unpurified WPI hydrogels formed at pH 2 (Fig. 3.10f) also exhibit a steeper slope (region of inter-particle interactions) when compared with the desalted β-lactoglobulin gels formed at the same pH (Fig. 3.10b). This suggests that the inter-particle interactions in WPI gels at pH 2.0 are stronger compared with the β-lactoglobulin gels because the presence of salts in the WPI gels [50] decreases the repulsions between charged amino acids and hence, accelerates the self-assembly process [51] and makes the aggregation stronger.

**Table 3.1** Hierarchical structural levels of β-lactoglobulin fibril gel networks of different concentrations heated at 80°C for different heating times at pH a) 2.3, b) 2.0, and c) 0.9 where $q_o$ and $q_{o2}$ are SAXS peak positions of oligomers with different size.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Slope (Interparticle interactions)</th>
<th>Form of particle</th>
<th>Slope $D_m$</th>
<th>Slope 6-Ds</th>
<th>Peak Position $q_{o1}$ (nm$^{-1}$)</th>
<th>FWHM (nm)</th>
<th>Peak position $q_{o2}$ (nm$^{-1}$)</th>
<th>FWHM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7% w/v</td>
<td>2 h</td>
<td>-3.39±0.03</td>
<td>-0.81±0.02</td>
<td>-0.09±0.01</td>
<td>-3.57±0.03</td>
<td>5.33±0.01</td>
<td>1.78</td>
<td>2.95±0.01</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>-2.99±0.02</td>
<td>-1.01±0.01</td>
<td>-0.52±0.01</td>
<td>-3.34±0.02</td>
<td>5.37±0.01</td>
<td>1.85</td>
<td>2.84±0.01</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>-2.57±0.11</td>
<td>-1.05±0.01</td>
<td>-0.95±0.01</td>
<td>-3.11±0.03</td>
<td>5.52±0.01</td>
<td>1.91</td>
<td>2.73±0.01</td>
</tr>
<tr>
<td>5% w/v</td>
<td>2 h</td>
<td>-3.39±0.01</td>
<td>-0.85±0.03</td>
<td>-0.09±0.01</td>
<td>-3.12±0.02</td>
<td>-</td>
<td>-</td>
<td>2.92±0.01</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>-3.29±0.01</td>
<td>-0.97±0.02</td>
<td>-0.07±0.01</td>
<td>-2.91±0.01</td>
<td>-</td>
<td>-</td>
<td>2.76±0.01</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
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<td>-0.56±0.01</td>
<td>-2.92±0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2% w/v</td>
<td>2 h</td>
<td>-3.37±0.01</td>
<td>-1.05±0.04</td>
<td>-0.12±0.01</td>
<td>-2.23±0.01</td>
<td>-</td>
<td>-</td>
<td>2.86±0.01</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
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<td>-0.71±0.02</td>
<td>-0.18±0.01</td>
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### pH 2.0

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<th>Slope 6-( D_s )</th>
<th>Peak position ( q_{\text{el}} ) (nm(^{-1}))</th>
<th>FWHM (nm)</th>
<th>Peak position ( q_{\text{el}} ) (nm(^{-1}))</th>
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<td>5% w/v</td>
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<tr>
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<td>-1.04±0.01</td>
<td>-0.64±0.01</td>
<td>-2.88±0.01</td>
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<tr>
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<td>-1.08±0.02</td>
<td>-0.27±0.01</td>
<td>-2.31±0.02</td>
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<tr>
<td>16 h</td>
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<td>-1.08±0.02</td>
<td>-0.27±0.01</td>
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### pH 0.9

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<th>Slope 6-( D_s )</th>
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<th>FWHM (nm)</th>
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<tr>
<td>2 h</td>
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<tr>
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<tr>
<td>2 h</td>
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<td>-3.06±0.04</td>
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<tr>
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<td>2 h</td>
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<td>-2.97±0.02</td>
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<td>-0.81±0.01</td>
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<td>-3.09±0.04</td>
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<td>-1.02±0.02</td>
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<tr>
<td>16 h</td>
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<td>-2.49±0.04</td>
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</table>
Another significant hydrogel characteristic presented in Table 3.1a-c is the determination of the particle shape (form of particle). The particle is rod-like when the exponent in the power law of the form factor at small angles equals to -1 [29]. Rollovers towards lower exponents indicate smaller entities in the q range that extend beyond the scale at which they change. Specifically, Table 3.1c shows that the slopes determining the form of the particles formed at pH 0.9 (Fig. 3.11a-c) vary between -0.68 and -1. This is attributed to the shorter and larger particles formed at pH 0.9, the so-called fragmented fibrils compared with the particles formed at pH 2 (Fig. 3.10a-e) and 2.3 (Fig. 3.9a-c), as was also highlighted in Chapter 2. Data in Table 3.1b show that at a high concentration (≥ 8.4% w/v) β-lactoglobulin hydrogels formed at pH 2.0 (Fig. 3.10a-b) exhibit exponents of the power law of the form factor between -0.72 and -1.12. The low exponent -0.72 is attributed to the highly compact fibril aggregations which result in the SAXS scattering derived from small compact agglomerates instead of extended individual fibrils. On the other hand, low exponents of the power law of the form factor is also presented for different heating times in lower concentration (≤ 2-7% w/v) hydrogels formed at pH 2.3 (Table 3.1a). This happens because oligomers (oligomers) start to be converted to protofibrils and are trapped as smaller entities [52].

The data presented here show that SAXS is a reliable method for particle size estimation for low concentration samples. However, it is not clear if the scattering comes from individual rod-like particles or from a bundle of particles when the fibrillar samples are highly concentrated (≥ 8.4% w/v). Nevertheless, SAXS can be an appropriate method for the investigation of length scales larger than the particle size for samples with high concentration. This means that in the case of highly concentrated samples the lower q values can be studied with higher accuracy, as "zooming out" of the structure (investigation of larger length scales), rather than the high q ranges.

In addition, the mass (D_m) and surface (D_s) fractal dimensions provide important information about the compactness of the fibril aggregations and the roughness of the
fibril surface (Fig. 3.12), respectively. Table 3.1 shows that the mass fractal ($D_m$) for β-lactoglobulin fibril networks formed at pH 2.0 (Table 3.1b, Fig. 3.10a-e) is larger than the mass fractal dimension for networks formed at pH 2.3 (Table 3.1a, Fig. 3.9a-c) and roughly equal with the mass fractal dimension for networks formed at pH 0.9 with concentrations above 5% w/v (Table 3.1c, Fig. 3.11). This means that the networks formed at pH 2, due to hydrophobic interactions, and the high concentration networks formed at pH 0.9 exhibit a more compact structure compared with the networks formed at pH 2.3. On the other hand, WPI gels formed at pH 2.0 (Fig. 3.10d) exhibit slightly less densely self-assembled fibrils compared with the β-lactoglobulin gels formed at pH 2.0 (Fig. 3.10b). Generally, as the protein concentration is increased, the number of cross-links and the hydrophobic interactions are increased hence the mass fractal dimension is also increased. It should be highlighted here that there is big difference between the mass fractal dimension for 11.4% w/v at pH 2.0 samples (Fig. 3.10a) heated for 2 h or 6 h and those heated for 10 h or 16 h. It is proposed that this happens, because the fibril networks become very compact after 10 h of heating and single fibrils cannot be detected. This also applies for 5% w/v samples formed at pH 0.9 (Fig. 3.11c).

![Fibril Roughness and Compactness](image)

**Fig. 3.12** Fibril roughness and fibril network compactness.
The surface fractal dimension (degree of surface roughness $D_s$) for gels formed at pH 2.3 (Table 3.1a, Fig. 3.9a-c) and 2.0 (Table 3.1b, Fig. 3.10a-e) is larger than the surface fractal dimension for gels formed at pH 0.9 (Table 3.1c, Fig. 3.11a-c). This means that the fibril surface in gel networks formed at pH 2.3 and 2.0 are smoother compared with the fragmented fibrils in gel networks formed at pH 0.9 (Fig. 3.13). This is attributed to the smallest persistence length or mesh size and hence, the less flexibility of the polymer chain than the fragmented fibrils exhibit at pH 0.9. Another characteristic of these networks is that the surface of semi-flexible pH 2.3 and pH 2.0 fibril networks become rougher as the heating time is increased and the protein concentration is decreased. This implies that as the heating time increases, the rate of oligomers to protofibril conversion increases and the fibrils become longer and as the protein concentration decreases, the fibril length decreases and the mesh length and hydrophobic interactions increase. This possibly allows the fibrils to exhibit larger persistence length as the heating time is increased and the protein concentration is decreased. Large persistence length means that a fibril cannot bend easily during thermal fluctuations and concentration increase. This translates to a rougher surface.

The last structural hierarchical level studied for these systems refers to the size of $\beta$-lactoglobulin oligomers. The q-position ($q_{o1}$ and $q_{o2}$ in Fig. 3.9-3.11) of the SAXS oligomer peaks between $q\sim1$-10 nm$^{-1}$ and the full width at half maximum (FWHM) of these oligomer peaks reveal information about the oligomer size and network homogeneity [53], respectively. The larger the q, the smaller the oligomer size and the broader the SAXS peak between $q\sim1$-10 nm$^{-1}$ or the larger the FWHM, the less ordered the hydrogel structure. Table 3.1a-c show that there are two sizes of oligomers ($q_{o1}$ and $q_{o2}$) presented in the $\beta$-lactoglobulin networks formed at pH 2.3 (Table 3.1a), 2.0 (Table 3.1b), and 0.9 (Table 3.1c). The presence of the two sizes of oligomers could be attributed to the direct or indirect conversion of monomers or paranuclei to protofibrils or fibrils or that the internal structure of dimeric proteins is not considerably changed when they bind together to form the amyloid fibrils. The presence of the two sizes of oligomers ($q_{o1}$ and $q_{o2}$ in Table 3.1) refers to the networks with protein concentration higher than 5% w/v, whereas the presence of any oligomers is rare for networks with concentration lower than 5% at pH 2.3 (Table
3.1a) or 2.0 (Table 3.1b) and for networks with concentration lower than 7% w/v at pH 0.9 (Table 3.1c). The $q_0$-position of the oligomer peaks is not affected by the pH variation, but the peaks become broader (FWHM increase) with the reduction of pH from 2.3 to 0.9. This suggests that the oligomer size is stable during the pH changes, but the oligomers exhibit the less ordered structure [54] at pH 0.9 and the highest at pH 2.3 (Fig. 3.13).

![Fig. 3.13 Cartoon of network compactness and surface roughness changes for purified β-lactoglobulin gels heated at 80°C and pH 2.3, 2 and 0.9.](image)

**3.4.3 Determination of Structural Parameters**

Based on the SAXS curves presented in Fig. 3.9-3.11, the particle size, the mesh length, the intensity at the zero scattering angle, the Porod volume, and the molecular weight were estimated and are summarized in Table 3.2a-c. The particle size was firstly estimated in the reciprocal space and then in real space, as described in §3.4.2.
Table 3.2 Comparison of structural parameters of β-lactoglobulin fibril gel networks of different concentrations heated at 80°C for different heating times at a) pH 2.3, b) 2.0, and c) 0.9 where \( R_c^a \) and \( R_c^b \) are the radius of gyration of the cross-section in reciprocal and real space, respectively, \( L_m \) the mesh length, \( I_o \) the zero-angle scattering intensity, and MM the molecular mass. The oligomerisation state was characterized as M: Monomeric, D: Dimeric, Hex: Hexameric, Hep: Heptameric, or O: Octameric state.

<table>
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<th>( R_c^b ) (nm)</th>
<th>( D_{\text{max}} ) (nm)</th>
<th>( L_m ) (nm)</th>
<th>( I_o ) Porod Vol. ((\text{nm}^3))</th>
<th>MM (KDa)</th>
<th>Oligomerisation State</th>
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<td>-</td>
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<tr>
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<td>1.19±0.01</td>
<td>115±0.01</td>
<td>3.5±0.1</td>
<td>20.6±0.6</td>
<td>0.059±0.001</td>
<td>47±1</td>
<td>29±1 M, D</td>
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### pH 2.0

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<th>$R_e^b$ (nm)</th>
<th>$D_{max}$ (nm)</th>
<th>$L_m$ (nm)</th>
<th>$I_o$</th>
<th>Porod Vol. (nm$^3$)</th>
<th>MM (KDa)</th>
<th>Oligomerisation State</th>
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<td>11.4% w/v</td>
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<td>0.22±0.002</td>
<td>860±10</td>
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<td>0.22±0.002</td>
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<td>0.54±0.013</td>
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</tr>
<tr>
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<tr>
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<td>42±1</td>
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<td>1.36±0.01</td>
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<td>0.17±0.003</td>
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<td>32±1</td>
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</tr>
<tr>
<td>10 h</td>
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</tr>
<tr>
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</tr>
<tr>
<td>10 h</td>
<td>1.01±0.01</td>
<td>1.01±0.01</td>
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<td>0.02±0.001</td>
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### pH 0.9

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<th>$R_e^b$ (nm)</th>
<th>$D_{max}$ (nm)</th>
<th>$L_m$ (nm)</th>
<th>$I_o$</th>
<th>Porod Vol. (nm$^3$)</th>
<th>MM (KDa)</th>
<th>Oligomerisation State</th>
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<tbody>
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<td></td>
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<tr>
<td>2 h</td>
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<td>225±9</td>
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</tr>
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<td>2.39±0.01</td>
<td>2.32±0.01</td>
<td>7.1±0.1</td>
<td>6.2±0.6</td>
<td>0.36±0.001</td>
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</tr>
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<td>2.19±0.01</td>
<td>2.12±0.01</td>
<td>7.1±0.1</td>
<td>6.9±0.7</td>
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<td>144±6</td>
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<td>2.35±0.01</td>
<td>2.28±0.01</td>
<td>7.3±0.1</td>
<td>6.9±0.1</td>
<td>0.33±0.001</td>
<td>312±10</td>
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<td>7% w/v</td>
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</tr>
<tr>
<td>2 h</td>
<td>2.36±0.01</td>
<td>2.31±0.01</td>
<td>7.2±0.1</td>
<td>8.2±1.3</td>
<td>0.45±0.004</td>
<td>355±10</td>
<td>222±9</td>
<td>&gt; O</td>
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<tr>
<td>6 h</td>
<td>2.32±0.01</td>
<td>2.28±0.01</td>
<td>7.1±0.1</td>
<td>10.6±1.9</td>
<td>0.28±0.001</td>
<td>308±10</td>
<td>192±8</td>
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<tr>
<td>10 h</td>
<td>2.45±0.01</td>
<td>2.44±0.01</td>
<td>7.2±0.1</td>
<td>11.2±0.9</td>
<td>0.28±0.004</td>
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<td>7.3±0.1</td>
<td>11.1±0.8</td>
<td>0.28±0.003</td>
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<td>234±9</td>
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<td>5% w/v</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>1.78±0.01</td>
<td>1.73±0.01</td>
<td>5.5±0.1</td>
<td>7.1±0.1</td>
<td>0.30±0.006</td>
<td>282±10</td>
<td>176±7</td>
<td>&gt; O</td>
</tr>
<tr>
<td>6 h</td>
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<td>6.5±0.1</td>
<td>5.3±0.1</td>
<td>0.43±0.007</td>
<td>203±10</td>
<td>127±5</td>
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<td>7.4±0.1</td>
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<td>0.26±0.001</td>
<td>399±10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15 h</td>
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<td>2.58±0.01</td>
<td>7.4±0.1</td>
<td>8.8±0.1</td>
<td>0.20±0.002</td>
<td>402±10</td>
<td>251±10</td>
<td>&gt; O</td>
</tr>
</tbody>
</table>
Broadly speaking, data in Table 3.2 show that the fibril diameters ($D_{\text{max}}$) are independent of heating time. However, at high concentration (11.4% w/v, Table 3.2b) at pH 2 the fibrils appear to have become thinner after 6 h heating. Actually, at pH 2.0 two regions are observed, at concentrations of 8.4% w/v and below fibrils are ~4 nm thick, while at higher concentration this is almost doubled. This could indicate the formation of two twisted fibrils [55]. When two fibrils are twisted together, the SAXS could probe this twisted structure as an individual fibril (2 h, 6 h heating). As the heating time is increased the monomers are converted to protofibrils or directly to fibrils. Hence, after 6 h heating at 80°C the hydrogen bonding between the protein monomers could be reduced due to prolonged heating at high temperature and hence, a number of monomers could be removed from the peptide chain. The results about the fibril size of 2% w/v gels at pH 2 (~ 4 nm) are in agreement with Jung et al [16] (~ 4.24 nm).

At pH 2.3 (Table 3.2a) irrespective of β-lactoglobulin concentration or heating time the fibril diameter ($D_{\text{max}}$) is approximately 3-4 nm. At pH 0.9 (Table 3.2c) this is increased to approximately 7 nm. Though it should be noted that at 5% w/v β-lactoglobulin and pH 0.9 the fibrils become thinner as the heating time is increased. This could be attributed again to twisted fibrils as described above.

The conversion rate of β-lactoglobulin monomers into fibrils is increased with the heating time and protein concentration [50]. Thus, the mesh length data reported so far in Table 3.2a-b reveal that the mesh length is decreased with the increase of heating time and protein concentration for gels formed at pH 2.0 (Table 3.2b) and 2.3 (Table 3.2a). In contrast, it seems that the mesh length for gels formed at pH 0.9 (Table 3.2c) is independent of heating time and is decreased for concentrations larger than 7% w/v. pH variations also affect the mesh length ($L_{\text{m}}$), since upon decreasing pH the fibril length is decreased and hence, the mesh length within fibril networks formed at pH 2.3 (Table 3.2a) are larger than for gels formed at pH 2.0 (Table 3.2b), whereas pH 0.9 (Table 3.2c) gels exhibit the shortest fragmented-fibril particles. This
could be attributed to the existence of protein degradation at pH 0.9 which results in fragmented fibrils.

The Porod volume for fibrillar β-lactoglobulin gels has received little attention to date in the literature. Onsager [10] investigated the isotropic to nematic phase (I-N) transition of fluids consisting of monodisperse rigid rods. This transition was attributed to the competition between the orientational and translational entropy of the rods. Specifically, before the I-N transition occurs the system is organized in isotropic phase where the orientational entropy dominates relative to the translational entropy. By reaching a critical rod concentration, the total entropy (orientational and translational entropy) is maximized by aligning the rods (the system is trying to get organized in less isotropic/more nematic phase). Above the critical rod concentration the translation entropy is increased and dominates relative to the orientational entropy and in this way the system is transferred from the isotropic to the nematic phase. The larger the Porod volume, the lower the translation entropy [12], since the free volume accessible by the particles is reduced as the Porod volume is increased [56]. Here, it is shown that at pH 2.3 (Table 3.2a) and 2.0 (Table 3.2b) the Porod volume ($V_p$) is increased with the increase of heating time and concentration, while at pH 0.9 (Table 3.2c) the Porod volume is increased with the increase of heating time and concentration only for gel networks with concentrations up to 7% w/v. Generally, the gel networks formed at pH 0.9 exhibit larger Porod volume compared with gel networks formed at pH 2.3 and 2.0 (Fig. 3.14). As the gel network becomes more compact (e.g. pH 0.9 gels as suggested in §2.9.1) and more stable (reduction of free energy), the probability of including the I-N phase transition is reduced. It should be highlighted here that the Porod volume for 11.4% w/v gels formed at pH 2.0 is higher than the Porod volume of the other samples formed at pH 2.0. This may be attributed to a number of factors. Firstly, based on the Kratky plots (Fig. 3.11), the pH 0.9 hydrogels exhibit a larger protein folded domain and as the heating time increases, the size of these becomes smaller. Secondly, it should be taken into account that the Porod volume estimation for pH 0.9 hydrogels and short heating time could be more realistic, since the protein folded domain (integrated area under the peak) is larger.
Based on the data presented above, it is shown that the SAXS method constitutes a useful tool for thermodynamic investigation from the perspective of contributions to entropy (orientational and translational). This implies that in biopolymer networks thermal fluctuations are in a way the source of elasticity (ratio of stress to strain) which can restore forces [57]. For example, when an external force is exerted on an elastic band system, the elastic band is displaced (molecular rearrangements) from its equilibrium position and its entropy is reduced. Reduction of entropy means that the total number of conformations that a polymer can sustain is reduced. At the equilibrium position the entropy of the system is maximum. When the external force is adjusted to zero, the elastic band returns to its initial position and maximizes again its entropy, due to a restoring force. Since the origin of this restoring force are the thermodynamic (entropic) forces, this restoring force is called "entropic force". So it seems that the microscopic states (polymer chains) assist the macroscopic system (elastic band) to maximize its entropy. In other words, the restoring entropic force expresses "how strong is the desire" of the elastic band to maximize its entropy. On the other hand, by exerting an external force on a metal system, like a copper wire, the wire exhibits a displacement from its equilibrium position. By adjusting the external
force to zero, the wire returns to its initial position. The difference between the restoring force of the elastic band system and the restoring force of the copper wire system is that the origin of the restoring forces is different. The restoring force in the system of the elastic band derives from the thermodynamic (entropic) changes and hence, is called "entropic force", while the restoring force in the system of the copper wire derives from the displacement of the atoms and hence, the restoring force in this case is a "mechanical force". By paralleling the elastic band with the β-lactoglobulin fibril systems used in this study, it seems that the fibril systems formed at pH 2.3 and 2.0 behave like elastic band systems, while the pH 0.9 systems behave like copper wire systems. The reason why the pH 0.9 systems behave like copper wire systems is because their large fibril diameter behaves like a "mechanical force" within the systems and shortens the inter-fibril distances. Thus, I suggest that in systems where their stiffness comes from the thickness of the bundles, rather than the thermal fluctuations, mechanics are the main source of network stiffness. In biopolymer networks, like the β-lactoglobulin fibril networks, it is claimed that their microscopic and macroscopic properties derive from the competition between the mechanical and entropic forces that exist within the networks. Under these circumstances, it is suggested here that as the free volume within the fibril protein hydrogels decreases, the translational entropy becomes less important and it is more favourable for the fibrils to adhere together and to self-assemble. Taking into account that pH 0.9 hydrogels have the least free volume and the fewer rod-like particles compared with the pH 2.3 and 2.0 hydrogels, it is proposed that the more rod-like the network, the more favourable it is for the system to organize into a nematic phase. It is also clear that in concentrated or high concentration solutions the inter-fibril interactions trap the system away from the equilibrium phase and prevent a full alignment of the fibrils.

Another significant finding of this study is the MM estimation (Table 3.2) of the protein through the Porod volume [39], since it provides useful information about the oligomeric state. The state of oligomerisation of β-lactoglobulin is affected by pH, temperature, and salts with β-lactoglobulin existing as monomer, dimer or octamer [58-60]. Based on the molecular mass of native β-lactoglobulin which is equal to 18.4
~kDa [61] it is shown in Tables 3.2a-c that β-lactoglobulin exists as monomers and dimers within networks with concentrations up to 8.4% w/v formed at pH 2.3 (Table 3.2a) and 2.0 (Table 3.2b). On the other hand, the monomer-dimer equilibrium is shifted in favour of hexamers, heptamers, and octamers within high concentration networks formed at pH 2.0 (Table 3.2b) and 0.9 (Table 3.2c) with concentrations above 8.4% w/v and 5% w/v, respectively. The presence of two oligomerisation states for β-lactoglobulin at pH 2.3 (Table 3.2a) and 2.0 (Table 3.2b) is in agreement with the different size of oligomers presented in §3.4.1 except the case of pH 0.9 gel networks (Table 3.2c) wherein the presence of oligomers is not obvious for all concentrations. This possibly happens due to the very compact structure at pH 0.9 which could be further investigated using the wide-angle X-ray scattering (WAXS) or because the amino acid sequence of the protein is fragmented into smaller peptides.

The intensity data at the zero scattering-angle (I₀) in Tables 3.2a-c show that as the pH is reduced from 2.3 to 0.9 the intensity of scattered X-rays increases which means that the attractive interactions [20] within the networks become stronger. At pH 2.3 (Table 3.2a) and 2.0 (Table 3.2b) the intensity data are increased with the increase of heating time and concentration, since the particle number per unit volume increases.

3.4.3.1 Cryo-Scanning Electron Microscopy Data Validation Using Small-Angle X-ray Scattering

Taking into account the limitations of the cryo-SEM technique (Chapter 2), which include the need to carefully limit the introduction of artefacts, and 2D image analysis of a 2D projection of a 3D object, SAXS was selected as a complementary method to compare with the cryo-SEM data, since it does not require any sample preparation, i.e. no freezing of liquid. This is the main reason why SAXS was used as a control method. The SAXS method has the potential to provide greater accuracy with respect to (albeit time-averaged) physical characterization of the gels. Here a few representative data sets are compared with the SEM data and the evaluation of the
advantages and limitations of cryo-SEM for fibril and hydrogel characterization is described.

Table 3.3 presents SAXS and cryo-SEM fibril diameter (D) and mesh length (l_m) data for low concentration (2% w/v WPI at pH 2.0 and β-lactoglobulin at pH 2.3 and 2.0) and high concentration samples (8.4% w/v β-lactoglobulin at pH 2.0 and pH 0.9). The SAXS data in Table 3.3 constitute average values derived from time-averaged scattering patterns of molecules as a function of spatial frequency, while the cryo-SEM data present the range of distributions of individual fibrils. Table 3.3 shows that the SAXS data for the fibril diameters and mesh lengths are included in the corresponding value ranges of the cryo-SEM distributions. This means that the SAXS and cryo-SEM data agree each other and this indicates that the propane jet freezing method allows the reliable imaging of the native state of low concentration protein fibril hydrogels.

Particularly interesting are the mesh length data presented in Table 3.3 for 8.4% w/v β-lactoglobulin gels formed at pH 2.0 and 0.9. According to the SAXS data, the mesh length decreases as the protein concentration for β-lactoglobulin at pH 2.0 increases. On the contrary, cryo-SEM data show that the mesh lengths for 8.4% w/v β-lactoglobulin at pH 2.0 and 0.9 are larger compared with the mesh lengths for 2% β-lactoglobulin at pH 2.0. This strengthens my proposition that, although the protein concentration in this case is quite high (8.4% w/v), the protein network does not behave as a complete "cryo-protectant" and ice crystals formation has altered the fibril network resulting in conflicting results, as stated in §2.7 (Fig. 2.10a and 2.16a). This could be a result of the vitrification depth of the propane jet freezing method is too small. This means that a faster freezing method with larger vitrification depth should be used in this case, like the high pressure freezer, in order to freeze samples of larger thickness.
Table 3.3 SAXS data and cryo-SEM distribution ranges for diameters (D) and mesh lengths (I_m) of WPI gels at pH 2.0 and β-lactoglobulin (β-lg) gels at pH 2.3, 2.0, and 0.9 with different concentrations heated at 80°C for 10 h.

<table>
<thead>
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<th>D (nm)</th>
<th>SAXS average values</th>
<th>Cryo-SEM distribution range</th>
<th>I_m (nm)</th>
<th>SAXS average values</th>
<th>Cryo-SEM distribution range</th>
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</thead>
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<td>2% w/v β-lg-pH 2.3</td>
<td>3.5±0.1</td>
<td>3.0-17.0</td>
<td>20.6±0.6</td>
<td>8.5-57.5</td>
<td></td>
</tr>
<tr>
<td>2% w/v WPI-pH 2.0</td>
<td>4.3±0.1</td>
<td>2.0-14.5</td>
<td>9.1±0.9</td>
<td>5.5-30.0</td>
<td></td>
</tr>
<tr>
<td>2% w/v β-lg-pH 2.0</td>
<td>3.1±0.1</td>
<td>3.5-18.5</td>
<td>16.2±0.9</td>
<td>3.5-51.5</td>
<td></td>
</tr>
<tr>
<td>2% w/v β-lg-pH 0.9</td>
<td>7.4±0.1</td>
<td>4.6-36.0</td>
<td>8.8±1.1</td>
<td>4.5-61.5</td>
<td></td>
</tr>
<tr>
<td>8.4% w/v β-lg-pH 2</td>
<td>4.4±0.1</td>
<td>1.0-28.5</td>
<td>6.5±0.9</td>
<td>14.0-85.0</td>
<td></td>
</tr>
<tr>
<td>8.4% w/v β-lg-pH 0.9</td>
<td>7.1±0.1</td>
<td>2.5-28.0</td>
<td>6.9±0.7</td>
<td>9.5-82.0</td>
<td></td>
</tr>
</tbody>
</table>

When comparing the SEM and SAXS data, it should be highlighted that SAXS provides average values, while SEM provides distributions of individual fibrils. Collectively the two methods yield complementary data which together allow fuller understanding of the systems.

Because of the 2D analysis of 3D objects with respect to the EM images, the possible freezing artefacts introduced during the cryogenic sample preparation, and the lower resolution of cryo-SEM compared with SAXS, I suggest that cryo-EM imaging is a useful method for end-to-end and persistence length estimation for biopolymers. SAXS, due to the requirement of no sample preparation and the higher resolution, is by contrast a more accurate and reliable method for mesh length and fibril size estimation.
3.5 pH Dependence of β-lactoglobulin Networks During Gelation

In Chapter 2 it has been shown that β-lactoglobulin hydrogels heated at 80°C and at different low pH values (2.3, 2.0, and 0.9) form fibrils of different lengths. Here, the surface fractal dimension (D_s, Fig. 3.15a-f) and the inter-atomic interactions of fibril β-lactoglobulin hydrogels of different fibril lengths during the first ~ 2 h of gelation at 80°C are investigated using a short camera length (small length scales). The data presented in Tables 3.4a-c show that the power-law exponent 6-D_s of β-lactoglobulin and WPI gels is decreased with the increase of heating time. As the power-law exponent 6-D_s decreases from 4 to 3, the fibril surface becomes rougher [5-7]. Specifically, the pH 0.9 gel systems (Table 3.4, Fig. 3.15a, b) exhibit particles with the roughest surface in contrast to the WPI networks (Table 3.4, Fig. 3.15f) which exhibit the smoothest particles. As seen in Table 3.4, the purified β-lactoglobulin gels formed at pH 2.0 (Fig. 3.15c) exhibit smoother particle surfaces compared with the β-lactoglobulin gels with added salts (Fig. 3.15d, e). This shows that the decrease of pH from 2.3 to 0.9 increases the surface roughness. This is consistent with an acceleration of monomer conversion to paranuclei and subsequently, to paranucleus self-association, in order to form large non-stretched oligomers [52, 62] just before the protofibril formation, as the heating time is increased.
Fig. 3.15 SAXS data for a) 8.4% w/v and b) 7% w/v at pH 0.9, for c) 8.4% w/v, d) 8.4% w/v with 150 mM NaCl, and e) 8.4% w/v with 60 mM CaCl₂ at pH 2.0 β-lactoglobulin gels and for f) 8.4% w/v at pH 2.0 WPI gels heated all for 46-131 min.
Table 3.4 Comparison of structural parameters of β-lactoglobulin and WPI fibril gel networks upon gelation process at 80°C and pH 0.9 and 2.0. $D_m$ and $D_s$ are the mass and surface fractal dimensions, respectively, and $D_1$ and $D_2$ are the size of oligomers.

<table>
<thead>
<tr>
<th>Samples</th>
<th>6-$D_s$</th>
<th>Peak position $q_{o1}$ (nm$^{-1}$)</th>
<th>FWHM (nm)</th>
<th>Peak position $q_{o2}$ (nm$^{-1}$)</th>
<th>FWHM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH 0.9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4% w/v</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 min</td>
<td>-3.66±0.02</td>
<td>5.27±0.01</td>
<td>3.67</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56 min</td>
<td>-3.29±0.01</td>
<td>5.34±0.01</td>
<td>3.64</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>68 min</td>
<td>-3.13±0.01</td>
<td>5.38±0.01</td>
<td>3.71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>95 min</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>131 min</td>
<td>-2.86±0.01</td>
<td>5.57±0.01</td>
<td>3.51</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7% w/v</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 min</td>
<td>-3.65±0.03</td>
<td>5.26±0.02</td>
<td>3.52</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56 min</td>
<td>-3.23±0.02</td>
<td>5.36±0.01</td>
<td>3.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>68 min</td>
<td>-3.02±0.02</td>
<td>5.42±0.01</td>
<td>3.57</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>95 min</td>
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<td>3.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>131 min</td>
<td>-2.84±0.01</td>
<td>5.52±0.01</td>
<td>3.67</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>pH 2.0</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4% w/v</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>46 min</td>
<td>-4.03±0.04</td>
<td>5.18±0.01</td>
<td>3.36</td>
<td>2.59±0.03</td>
<td>1.57</td>
</tr>
<tr>
<td>56 min</td>
<td>-3.52±0.06</td>
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<td>2.19±0.18</td>
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<td>5.24±0.01</td>
<td>3.44</td>
<td>5.21±2.42</td>
<td>4.02</td>
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<tr>
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<td>5.29±0.01</td>
<td>3.61</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>3.42</td>
<td>-</td>
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</tr>
<tr>
<td><strong>8.4% w/v</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 min</td>
<td>-3.51±0.0</td>
<td>5.21±0.01</td>
<td>3.61</td>
<td>3.84±0.11</td>
<td>1.54</td>
</tr>
<tr>
<td>56 min</td>
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<td>3.69</td>
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<td>-</td>
</tr>
<tr>
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<td>5.37±0.01</td>
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<td>-</td>
</tr>
<tr>
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<td>5.41±0.01</td>
<td>3.59</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>3.81</td>
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</tr>
<tr>
<td><strong>8.4% w/v</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 mM CaCl$_2$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>-3.44±0.02</td>
<td>5.37±0.01</td>
<td>3.54</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>56 min</td>
<td>-3.31±0.01</td>
<td>5.39±0.01</td>
<td>3.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>68 min</td>
<td>-3.27±0.01</td>
<td>5.39±0.01</td>
<td>3.65</td>
<td>-</td>
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</tr>
<tr>
<td>95 min</td>
<td>-3.09±0.01</td>
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<td>3.57</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>131 min</td>
<td>-2.97±0.01</td>
<td>5.47±0.01</td>
<td>3.58</td>
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<td>-</td>
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<tr>
<td><strong>WPI 8.4% w/v</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 min</td>
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<td>3.45</td>
<td>2.59±0.03</td>
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</tr>
<tr>
<td>56 min</td>
<td>-3.98±0.04</td>
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</tr>
<tr>
<td>68 min</td>
<td>-3.81±0.04</td>
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<td>2.63±0.04</td>
<td>1.44</td>
</tr>
<tr>
<td>95 min</td>
<td>-3.58±0.03</td>
<td>5.32±0.01</td>
<td>3.52</td>
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<tr>
<td>131 min</td>
<td>-3.27±0.02</td>
<td>5.41±0.01</td>
<td>3.65</td>
<td>-</td>
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</tr>
</tbody>
</table>
Moving from the Porod volume area to the area of inter-atomic interactions, the oligomer size and structure homogeneity are studied based on the q positions and the FWHM, respectively [53]. There are two sizes of oligomers ($q_{o1}$ and $q_{o2}$) present in Table 3.4. Based on the smaller oligomer size ($q_{o1} \sim 5$ nm$^{-1}$) it is highlighted that the oligomer size is reduced as the heating time is increased. This relation is also present in the β-lactoglobulin networks after the gelation process except for the pH 0.9 networks where the oligomer size is stable. Oligomer size is also dependent on the pH and as it is seen in Table 3.4 the pH 0.9 hydrogels exhibit smaller oligomers compared with those formed at pH 2.0 and with WPI. The presence of salt also affects the oligomer size. The addition of monovalent (NaCl) and divalent (CaCl$_2$) salt results in smaller oligomers being formed at the same pH (pH 2.0) with the divalent salt forming the smallest oligomers.

Based on the FWHM variations [54], where FWHM in Table 3.4 here expresses the network homogeneity for the different β-lactoglobulin systems, the peak positions become broader as the pH is reduced from 2.3 to 0.9. This means that the pH 0.9 systems exhibit the least ordered structures upon gelation compared with the pH 2.3 systems (Fig. 3.16). This system behaviour is similar to that presented after heat treatment (§3.4.2). On the other hand, in the presence of added salt narrower peaks are evident compared with the pH 2.0 gels. The CaCl$_2$ and WPI gel systems are less randomly ordered compared with all the other systems. This is consistent with an increase of attractive interactions [51] due to the addition of salts which would stabilize the systems.
Based on the data above, it is demonstrated that protein fibril hydrogels with high concentrations exhibit the same fibril surface roughness ($D_s$) and degree of periodicity behaviour (FWHM) both during and after the gelation process. This means that the properties of the systems are already defined within the first two hours of the heat treatment.
Conclusions

In this study the relationships between the pH and the network compactness, the surface roughness, the Porod volume, and the degree of structure periodicity during and after the gelation process are established, as summarized in Fig. 3.17 and 3.18, respectively. The structural changes occurring during the gelation process, during the first 2 h of the gelation process were found to involve the conversion of the monomers to oligomers that subsequently form the protofibrils [52]. This results in the protein surface roughness being increased. Fig. 3.17 presents the ascending order of fibril surface roughness and degree of network periodicity for different fibril hydrogels during the gelation process. The WPI hydrogels at pH 2.0 seem to exhibit the smoothest fibril surface, while the β-lactoglobulin hydrogels at pH 0.9 seem to exhibit the smoothest fibril surface. The addition of salts in β-lactoglobulin hydrogels at pH 2.0 also increases the degree of roughness. This is caused by the salts decreasing the repulsive interactions and hence, accelerating the self-assembly process [51]. On the other hand, it is inferred from Fig. 3.17 that the salts also affect the degree of order within the protein structures with β-lactoglobulin gels with added salts exhibit the most random structure (Fig. 3.17) consistent with trapping by the increased assembly route, while the β-lactoglobulin gels formed at pH 2.0 exhibit the more well-ordered structures.

Fig. 3.17 Summary of structural parameter changes during the gelation process for purified β-lactoglobulin (β-lg) gels at pH 2.0 (with or without NaCl or CaCl₂) and pH 0.9 and for WPI gels at pH 2.0.
Fig. 3.18 shows the ascending order of network compactness, fibril surface roughness, degree of network periodicity, and Porod volume for different fibril hydrogels after the gelation process. Investigating the systems after the heat treatment it is also demonstrated that the surface roughness and structure compactness are increased with reduced pH. β-lactoglobulin hydrogels at pH 2.3 exhibit the highest degree of periodicity, the minimum network compactness, and the lowest fibril surface roughness. On the other hand, β-lactoglobulin hydrogels at pH 0.9 exhibit the lowest degree of periodicity and the highest network compactness and fibril surface roughness. Another interesting point in this case, which actually constitutes the main difference with the parameters defined upon the gelation process, is that pH 0.9 gel networks with concentration above 7% w/v (Table 3.1 and 3.3) exhibit similar surface roughness and structure compactness. Another significant difference in this case is that the surface roughness and structure compactness for pH 0.9 gels with concentrations above 7% w/v are independent of heating time.

Fig. 3.18 Summary of structural parameter changes after the gelation process for purified β-lactoglobulin (β-lg) gels at pH 2.3, 2.0, and 0.9.
Another finding of this study is the Porod volume (Fig. 3.18) for $\beta$-lactoglobulin gels of different length scales. Taking into account that in biopolymer networks thermal fluctuations [57] are a source of elasticity, I propose that as the fibril length scales are decreased, systems become thermodynamically more stable (reduction of Gibbs energy). The Porod volume reveals a competition between the orientational and translational entropy [10] and it is increased with increasing heating time and protein concentration where the change in orientational entropy exceeds the translational entropy. As the fibril length and size are decreased, the Porod volume is increased. It is apparent from Fig. 3.18 that the pH 0.9 gels are thermodynamically more stable with more independent macroscopic properties of structural parameters, such as fibril surface roughness and network compactness, degree of periodicity in structures, and Porod volume, while the pH 2.3 gels are the least thermodynamically stable networks.

The more rod-like the protein system is, the more favourable for the system is to be organized into a nematic-like phase, where the translational entropy dominates relative to orientational entropy. In other words, less rod-like the protein system is, the more favourable for the system is to be organized into an isotropic-like phase, where the orientational entropy dominates relative to the translational entropy. Isotropic phases are presented in the pH 0.9 hydrogels where the free volume is less compared with the free volume within pH 2.0 and 2.3 hydrogels. Hence, it is proposed that as the free volume decreases, the orientational entropy increases relative to the translational entropy which favours the isotropic phase and hence, the fibrils bind together more easily and undergo self-assembly.

Additionally, it was shown that the fibril size increases for gels with concentrations greater than 8.4% w/v and as the heating time is increased the diameters of the fibrils increase. The fragmented-fibrils formed at pH 0.9 (shortest fibrils) are thicker compared with the fibrils formed at pH 2.3 and 2.0 and independent of heating time and protein concentrations. On the contrary, the fibril size is roughly the same for gels formed at pH 2.3 (longest fibrils) and 2.0 and it is independent of the heating time and protein concentration up to 8.4% w/v.
The mesh length of β-lactoglobulin gels at pH 2.3 and 2.0 is decreased upon increasing heating time and concentration, since the conversion rate from monomers into peptides and the number of cross-links are increased, respectively. The mesh lengths within pH 0.9 gels exhibit negligible changes with increasing heating time and protein concentrations up to 7% w/v. The mesh lengths start to decrease for concentrations higher than 7% w/v, but they remain independent of heating time. This may be attributed to the occurrence of protein degradation.

SAXS data focused on the structural characterization of the gels were compared with the cryo-SEM data. Even with good sample preparation for cryo-SEM imaging, 2D analysis instead of 3D analysis of the 3D fibril gels and the resolution able to be achieved (combination of instrument limitations and sample characteristics) constitute two limitations for the proper imaging of the architecture of biopolymers by SEM. Hence, even with optimization of the sample preparation, cryo-SEM potentially exaggerates the values for the fibril characteristics compared with SAXS data. Generally, SAXS is a reliable method for fibril diameter investigation, compared with cryo-SEM which is more appropriate for fibril length scale investigation.

In addition to the limitations referred to above for the cryo-SEM imaging, the resolution of the SEM setup used in this study does not allow the easy examination of high viscous hydrogels, because the hydrogel features, like pore sizes and fibril end-to-end lengths, are smaller. Based on the cryo-SEM and SAXS data for high concentration samples and specifically on 8.4% w/v β-lactoglobulin hydrogels formed at pH 2.0 and 0.9 (§2.9, Table 2.2), it seems that the small vitrification depth of the propane jet freezing method does not always allow the appropriate freezing of high concentration samples. The high pressure freezer method is a more accurate method for the freezing of high concentration samples, although it is quite expensive, and currently of limited availability.
References


CHAPTER 4

Macroscopic Mechanical Responses of Protein Amyloid Fibril Gels

Abstract

Here, the relationship between the architecture and the mechanical responses of β-lactoglobulin fibril networks is investigated comprised of fibrils of different structural features such as length, stiffness, and diameter. As reported in Chapter 2, heat-induced β-lactoglobulin fibril formation is affected by pH. Fibrils are found to be progressively shorter when formed at reduced pH values from 2.3 to 0.9. Dynamic oscillatory tests were applied to characterize the protein gel networks during and after gelation. Time sweep tests (sample scanned over a range of time) were applied on β-lactoglobulin fibril networks measuring the gelation time, the rate of the elastic modulus (G') increase, and the G' variation upon increasing protein concentration (2-11.4% w/v) or changing pH (0.9-2.3). Frequency and strain sweep tests were also used for the structural characterization of β-lactoglobulin fibril gels post gelation. Here, it is shown that protein hydrogel networks, consisting of the longest fibrils form the most slowly and exhibit the stiffest structural response compared with these networks comprised of shorter fibrils. The linear viscoelastic region of β-lactoglobulin fibril networks, as seen in strain sweep tests, becomes more restricted as the fibril length shortens. Furthermore, it was found that the maximum rate of G' increase is higher as the β-lactoglobulin concentration is increased.

Fractal models fitted to elastic modulus and critical strain versus concentration plots allow the fractal dimension of the gels to be estimated and compared with the value obtained from SAXS analysis. The results indicate that the number of inter-fibril cross-links, the strength of the electrostatic and hydrophobic interactions, and chain stiffness and thickness within the protein gel networks are crucial elements which
define the relationship between the protein gel architecture and its macroscopic properties.

4.1 Introduction

Rheology investigations are widely applied in the study of hydrogel structures, particularly in order to characterize their macroscopic mechanical properties. Hydrogels are deformed when a force is applied to them and controlled deformation allows properties, like the degree of cross-linking and network stiffness, to be determined using dynamic oscillatory tests [1]. There are several different types of oscillatory tests, including for example the measurement of the storage and loss moduli over time, which provides information on the assembly of a hydrogel upon gelation, and frequency and strain sweep tests which are typically used to characterize the mechanical properties after the gelation process [2].

For a stress-controlled rheometer a sinusoidal stress wave function, \( \tau(t) = \tau_0 \sin(\omega t) \), is applied to the sample and the resulting strain wave function \( \gamma(t) = \gamma_0 \sin(\omega t + \delta) \) is measured. The angle phase difference, \( \delta \), between the stress and strain wave functions varies from \( 0^\circ \) to \( 90^\circ \) for viscoelastic materials. For perfect elastic materials \( \delta=0^\circ \) and for perfect viscous materials \( \delta=90^\circ \). The resulting elastic modulus (\( G' \)) which expresses the in phase solid-like behaviour of the sample and the viscous modulus (\( G'' \)) which expresses the out of phase liquid-like behaviour are recorded against time [3-5]. The gel state of hydrogels is generally characterized by the relation \( G' > G'' \), where the solid-like behaviour dominates over the fluid-like behaviour [3].

Following the protocols published in literature rheological measurements were conducted on in-situ gelled systems rather than gelling them ex-situ [6-8] and then loading onto the rheometer. An unknown pre-shear history may be introduced into the sample in the case of ex-situ gelation that can make it difficult to compare replicate
samples. Special care should also be taken for hydrogels that gel upon heating at high temperatures as is the case here. In this case, restricted evaporation of the solvent is critical. Kavanagh et al. [6], Gosal et al. [7], and Loveday et al. [8] are three examples of studies in which rheological measurements have been applied to hydrogels by in-situ gelation at high temperatures. Sealing was achieved in each of these cases by applying paraffin or silicone oil around the exposed surface of the sample.

One important characteristic of the mechanical character of hydrogel systems is the linear viscoelastic (LVE) region. The LVE is the region in strain/stress sweep tests where $G'$ forms a constant plateau signifying a constant modulus with varying strain or stress. The high end of this strain/stress plateau determines the strain/stress-amplitude above which the hydrogel network exhibits non-linear behaviour (the so-called critical strain/stress) [3, 9]. The non-linear behaviour can reflect the non-linearity of the constituent strands of the network or non-affine reorientations of anisotropic fibers or the disruption of the gel structure [3, 9, 10].

There is little published information regarding how the inherent lengths of the fibrils that form the protein fibril networks affect the macroscopic mechanical properties of the networks as probed by rheological investigation. Although a number of investigations on the structural and kinetic parameters of heat-induced β-lactoglobulin gels at pH 2.0 have been conducted using a combination of experimental methods. For example, Bolder et al. [11] described the dependence of whey protein isolate (WPI) conversion from monomers to fibrils on heating time and protein concentration using centrifugal filtration, TEM, high performance liquid chromatography (HPLC), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) methods. It should be highlighted here that WPI comprises a variety of globular proteins with the most abundant being β-lactoglobulin and α-lactalbumin. β-lactoglobulin is the only protein within WPI that forms fibrils after prolonged heating at pH 2. In addition, Bolder at al. [12] also proposed, based on rheo-optical and
centrifugal filtration methods, a model for fibril formation based on fibril activation, nucleation, growth, and a termination step.

Further studies have used rheology to examine the structural and kinetic changes that occur in heat-induced β-lactoglobulin gels formed at pH 2.0 upon addition of salt. Divalent salts, as compared with monovalent salts, speed up the gelation process and rather than long semiflexible fibrils that are formed in salt-free environments curly fibrils are produced [8, 14]. Temperature is another factor that affects fibril morphology, but apparently not the fibril self-assembly process. According to Loveday et al. [14], through a rheology, TEM, and fluorescence investigation, β-lactoglobulin fibril gels heated at 100°C and 110°C at pH 2.0 exhibit slightly shorter fibrils compared with the long semiflexible fibrils formed at 80°C. Fibrils formed at 120°C and pH 2.0 are predominantly short and are actually comprised of the products of larger fibril fragmentation.

Several studies have also applied fractal analysis to colloidal gels. These studies consider colloidal gels to be formed by overlapping aggregates (flocs), which constitute fractal objects. Hence, fractal dimensions allow the investigation of the internal structure of fractal aggregates within the hydrogels. Eleya et al. [15] highlights that there are several studies in the literature estimating fractal dimensions of aggregates using a variety of experimental tools, like scattering, microscopy, and rheology. All these experimental tools exhibit advantages and disadvantages. Specifically, scattering methods [15-16] do not allow fractal dimension estimation of high concentration samples or highly-viscous samples with accuracy. On the other hand, microscopy [15, 17], although allowing direct estimation of fractal dimension from the images, requires sample preparation during which artefacts could be introduced within the native state of the sample. Rheology allows the fractal dimension to be estimated for low and high concentration samples. This is important, because it allows the relationship between the fractal dimensions and the macroscopic properties of a structure to be investigated. Hence, in this study rheology is used to determine how the different hydrogel architectures affect macroscopic properties.
Several studies [6-7, 15-19] have focused on the application of different scaling and fractal models to protein hydrogels using rheology. Eleya et al. [16] presents a useful overview of the differences presented by these different models. These models actually constitute theories for the description of the elastic behaviour of gel structures. Specifically, these models determine the elastic behaviour (G’) of the gels in connection with the volume fraction \( \varphi \) of the aggregates. Bremer et al. [20-21] and Mellema et al. [22] present two scaling models widely reported in the literature for the fractal dimension estimation within protein hydrogels, but both exhibit some limitations. The main disadvantage of these models is that they require prior knowledge of the nature of the strands composing the hydrogels (e.g. if the strands are stretched or bent). This is the reason why these models require the use of other tools as complementary methods, like microscopy. Shih et al. [23-24] and Wu and Morbideli [25] present two alternative models which allow fractal dimension estimation without the use of any complementary method. The main difference between the Shih et al. [23-24] and Wu and Morbideli [25] models is that the first is only valid above the gelation threshold and the critical gel concentration \( \varphi_c \) is assumed to be zero [14], while the latter exceeds these limitations (see for details §4.3.2).

Additional alternative models based on other statistical methods, like the percolation theory [26] or the theory of branching processes [27-28], have also been applied for the description of the relation between the elastic modulus and the protein concentration. Branching cascade theory allows the critical concentration of the gel to be estimated [29-30], but it does not allow the fractal dimensionality to be determined [14], as the fractal model does. In contrast, percolation theory [17] premises the recognition of the critical percolation threshold concentration \( \varphi_c \) (where \( \varphi_c=0 \) for fractal models) which depends on the morphology of the monomers within the gel network and the strength of interactions between them [31, 17]. This constitutes the recognition of a reality not included in the fractal model.
The study presented here addresses gaps in the literature and aims to relate the macroscopic mechanical properties of protein amyloid fibril hydrogels of different fibril lengths to the different network architecture presented by these hydrogels. It is also considered how the fibril length variations are correlated with structural and kinetic changes during the self-assembly process.
**Materials and Methods**

4.2 Preparation of β-Lactoglobulin Hydrogels

Lyophilised β-lactoglobulin protein (see §2.2.1) was used for the production of β-lactoglobulin hydrogels, as described in §2.2.2. It should be highlighted that for the rheological experiments conducted here the filtered solutions were placed in eppendorf tubes of 1.5 mL, degassed under vacuum for 10 min, and finally, centrifuged within the eppendorf tubes (with open caps) for 2 min using Sigma Aldrich micro centrifuge model SD with speed 6,000 rpm. The degassed protein solutions were used immediately after degassing, in order to avoid bubble formation during the heat treatment.

4.3 Bulk Rheology

4.3.1 Dynamic Oscillatory Measurements

In-situ gelation, as reported in Gosal et al. [7], was used here since in this method care can be taken so as not to introduce an unknown pre-shear history into the sample. Rheological measurements were performed using a stress-controlled AR 2000 rheometer (TA instruments, New Castle, DE, USA). The fitted geometry was a 4° cone with diameter of 40 mm and truncation gap of 125 μm. The reason why cone geometry (Appendix C) was selected in this study is because there are studies [7-8] already reported in the literature using the same in-situ gelation methodology successfully. In addition, other geometries, such concentric cylinder, are more expensive and require a larger volume of sample. A cover plate also used combined with a peltier plate for temperature control (see Appendix C). The cone geometry and the cover plate were made of 316 stainless steel (316 SST is resistant to corrosion and is required due to the low pH values used here). Geometries fabricated from 316 SST are appropriate for use with samples with pH adjusted to 2.0 and 2.3. For the samples pH adjusted to pH 0.9, a 4° cone with 40 mm diameter and 125 μm truncation gap, and a cover plate, both made from polyether ether ketone (PEEK), were used. Experiments were also carried out using protein solutions adjusted to pH 2.0 using the
PEEK geometry, in order to confirm that no artefacts were introduced due to the use of different geometries.

The sample solutions were loaded using a pipette onto the cover plates preheated at 56°C and then, the rheometer cone geometry, which applies stress on the sample (see Appendix C), was lowered onto the sample. Any excess solution was removed. Fibril formation occurs upon heating at temperatures above 65°C at pH ~2.0 [14, 32]. Using plates pre-heated to a temperature of 56°C ensures avoidance of fibril formation at this stage of the process, but reduces the required heating time after loading. A layer of silicone oil (Rotitherm M220, Carl Roth) was applied with a pipette around the exposed sample, in order to prevent evaporation. Silicone oil was introduced into the cone geometry’s solvent trap and a solvent trap cover was placed (see Fig. C1 in Appendix C) over the sample with its edges dipped into the silicone oil, in order to form a better vapour barrier. Silicone paste (Dow Corning 340, heat sink compound) was also applied between the peltier and cover plate, in order to optimize the heat transfer [32]. The zero gap setting was performed at 80°C and the cone and cover plate expansion due to heating was taken into account.

A series of oscillation tests were applied on the samples using a Rheology Advantage Instrument Control AR based on Loveday et al. [8]. Specifically, as shown in Fig. 4.1, the series of oscillation tests consists of 1) a temperature ramp step from 60°C to 80°C with ramp rate 15°C/min, 2) a time sweep step of duration 10 h at a temperature 80°C, 3) a temperature step from 80°C to 20°C using a ramp rate of 8°C/min, 4) a frequency sweep test from 10^3 Hz to 100 Hz at a temperature of 20°C, and finally, 5) a strain sweep test from 0.01 to 1000 again at a temperature 20°C. The strain and angular frequency were set at 0.1 and 1 rad/s, respectively, where required (see Fig. 4.1).
4.3.2 Fractal Models

Fractal models are used in this study, in order to estimate the fractal dimensions $d_f$ for different β-lactoglobulin hydrogels. These models are preferred as the extracted fractal dimensions can be directly compared with values extracted from other characterization methods. The fractal dimensionality in this study expresses the degree of the structure compactness of β-lactoglobulin hydrogels. The first step before the fractal dimension $d_f$ estimation was to estimate the slopes for the elastic modulus ($G'$) vs protein concentration (C) plot ($G'\sim C^m$) and the critical strain ($\gamma_c$, limit of hydrogel’s viscoelastic behaviour) vs protein concentration (C) plot ($\gamma_c\sim C^n$) based on the rheological data (see Fig. 4.7). In this way, the power-law exponents m and n are determined for β-lactoglobulin gels over a range of concentrations at pH 2.3, 2.0, and 0.9. The second step was to estimate the fractal dimensions for the same β-lactoglobulin gels based on Shih et al. model (eq. 1-4) [23-24] and on the Wu and Morbidelli model (eq. 5-6) [25]. By substituting the power-law exponents in eq. 1-6 (see below) with the corresponding slopes obtained from the $G'\sim C^m$ and $\gamma_c\sim C^n$ plots, the fractal dimensions for the β-lactoglobulin gels reported above can be estimated.
\[ G' \sim \varphi^{\frac{3+x}{3-d_f}} \]  
\[ \gamma_c \sim \varphi^{\frac{-(1+x)}{3-d_f}} \] 
\[ G' \sim \varphi^{\frac{1}{3-d_f}} \]  
\[ \gamma_c \sim \varphi^{\frac{1}{3-d_f}} \] 
\[ G' \sim \varphi^{\frac{\beta}{3-d_f}} \]  
\[ \gamma_c \sim \varphi^{n=\frac{2-\beta}{3-d_f}} \] 

\( d_f \) in eq. 1-6 is the fractal dimension of the flocs (fibril aggregates), \( x \) (\( 1 \leq x < d_f \)) is the fractal dimension of the fibril backbones, \( \alpha \) is a constant, and \( \beta = (d-2) + (2+x)(1-\alpha) \). The value 3 in eq. 1-6 expresses the three-dimensional Euclidean space.

The model of Shih et al. [23] takes into account the inter-particle bonding energies within a gel network and clarified gelation regimes within the gel network as those with strong-links or weak-links. The strong-link regimes are characterized by the domination of inter-floc links as compared with the intra-floc links, while in the weak-link regimes the intra-floc links are stronger. Hence, the elasticity of the gel networks is determined by the inter-floc links for the strong-link regimes and by the intra-floc links for the weak-link regimes [33]. Wu and Morbidelli [25] extended the model of Shih et al. [23] to the regimes between the strong-link and weak-link regimes by adding a third regime, the so-called transition regime, where both intra- and inter-floc links contribute to the network elasticity formation. In other words, from a mathematical point of view the constant \( \alpha \) expresses the presence of two limits; one limit refers to the strong regimes where \( \alpha=0 \) and \( \beta \rightarrow d+x \) (Shih et al. model) and the other to weak regimes where \( \alpha=1 \) and \( \beta \rightarrow d-2 \) (Shih et al. model), while for the transition regime \( 0 < \alpha < 1 \). On the other hand, the constant \( \alpha \) does
have a physical meaning, expressing the contribution of inter- and intra-floc links in three regimes.

**Results and Discussion**

β-lactoglobulin fibril hydrogels were prepared spanning a range of protein concentration and for different pH values. The first part of the experimental section refers to the investigation of the kinetics of the gel formation and structural characterization of the resulting networks using time sweep tests (sample is scanned over a wide range of time), while the second part refers to the structural characterization of the networks post gelation using frequency and strain sweep tests. The final part of the experimental section describes the dependence of the elastic behaviour (G') and critical strain (γ_c) of β-lactoglobulin fibril networks on protein concentration. In addition, it presents the estimation of the fractal dimensions within these β-lactoglobulin networks and a comparison with their fractal dimensions estimated by SAXS.

**4.4 Gelation Time and G' Modulus Determination**

The macroscopic mechanical properties of fibrillar β-lactoglobulin hydrogels under acidic conditions were investigated using a stress-controlled rheometer, as described in §4.3.1. Time sweep measurements were applied on 2%, 5%, 8.4%, and 11.4% w/v protein fibril gel structures formed by in-situ gelation at a temperature of 80°C for a 10 h heating period with pH at 0.9, 2.0, or 2.3 (which comprise different fibril lengths as characterized by SAXS and microscopy). It was not possible to get good results for the 2% w/v samples at pH 2.0 and 2.3, since these samples exhibit low viscosity even after prolonged heating; hence the data had poor signal-to-noise. For each sample, the time sweep test was repeated three times, in order to verify the results. In this study the results derived from one of the three replicates are presented as representative of the behaviour. The replicates were in good agreement with each other. Fibril length is known to vary across this range of concentrations and pH values based on the cryo-SEM investigation described in Chapter 2, where fibrils formed at pH 2.0 are shorter
(range of the fibril end-to-end length distribution: 500-1650 nm) than those formed at pH 2.3 (range of the fibril end-to-end length distribution: 1050-3300 nm). In contrast, at pH 0.9 fragment-like fibril particles are present; hence these samples have the shortest fibril length scale (range of the fibril end-to-end length distribution: 150-750 nm).

There are several different ways to express the gelation time ($t_c$): the transition from the liquid to solid state, described in the literature. Tung and Dynes [26] suggested that the crossover point of the elastic ($G'$) and viscous ($G''$) moduli constitutes the gelation time. Winter and Chambon [19-20] highlighted that in the Tung and Dynes [26] method, the dependence of the gelation process on the frequency of the oscillation tests is not taken into account ($\tan \delta = G''(\omega)/G'(\omega)$); hence they suggested an alternative method which is well cited with a fundamental criterion of the independency of gelation time on frequency ($G'(\omega) = G''(\omega), \delta=45^\circ$) [34]. The Tung and Dynes [26] definition is a special case of the Winter and Chambon definition [26, 29].

As reported by Ross-Murphy [30] there are other (less widely-used) ways to estimate the gelation time. These include reference to the points at which the recorded signal from the gelation process becomes higher than the background noise or the elastic behaviour of the polymer networks becomes higher than a chosen threshold value. Both of these two methods exhibit some disadvantages. The first one does not take into account that background noise depends on the instrument’s torque sensitivity which varies from rheometer to rheometer, while the second one assumes a knowledge of gel strength.

For all gel investigations the well-cited Winter and Chambon [27] method could not be used here, because the $G'$ values were always greater than the $G''$ values and hence no inter-section occurs. The gelation times of $\beta$-lactoglobulin gels were estimated based on Gosal et al. [7]. Specifically, the gel-cure time curves ($G'$ vs heating time) of
these β-lactoglobulin fibril networks over a range of concentrations at pH 2.3, 2.0, and 0.9, were plotted as presented in Fig. 4.3a, b, and c, respectively. Fig. 4.3a-c show a steep increase of $G'$ after a few seconds of heating until it reaches a plateau. It is noticed that the steep increase of $G'$ occurs at different heating times for each system. This indicates that the rate of structural changes during gelation is not the same for all systems. The changes during the gelation process can be described by the gelation time, the rate of $G'$ change, and the magnitude of $G'$, as presented in Table 4.1a, b, and c, respectively. The gelation time was determined from the steep slope change (point of inflection) in a log-log time-$G'$ plot, as presented in Fig. 4.2.

![Fig. 4.2 Estimation of gelation time for 11.4% w/v fibrillar β-lactoglobulin gels formed at pH 2.3 and heated at 80°C for 10 h.](image)

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Fig. 4.3 Rheological time sweep mechanical responses of β-lactoglobulin fibril gel networks formed at a) pH 2.3, b) pH 2.0, and pH 0.9 and heated at 80°C for 10 h.
The data presented in Table 4.1a show that β-lactoglobulin fibril networks that exhibit different fibril lengths in scattering and microscopy exhibit different gelation times. Gel networks formed at pH 2.3 exhibit the slowest gelation process, while gel networks formed at pH 0.9 exhibit the fastest gelation process for a given protein concentration. There is one exception, the 11.4% w/v gel systems at pH 2.3 seems to gel faster (752 s) than the gel systems at pH 2.0 (1114 s). This is consistent with a total volume change caused by adjustment of the pH with small volumes of HCl or NaOH.

**Table 4.1**: a) Gelation time, b) maximum rate of G’ increase (dG’/dt)\textsubscript{max}, and c) maximum experimental G’\textsubscript{Exp} and extrapolated G’\textsubscript{∞} modulus for β-lactoglobulin fibril gel networks of different concentrations heated at 80°C for 10 h at pH 2.3, 2.0, and 0.9. These kinetic parameters refer to measurements shown in Fig. 4.2.

<table>
<thead>
<tr>
<th>Conc. (% w/v)</th>
<th>pH 2.3</th>
<th>pH 2.0</th>
<th>pH 0.9</th>
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<tbody>
<tr>
<td>11.4</td>
<td>750±2</td>
<td>1110±4</td>
<td>-</td>
</tr>
<tr>
<td>8.4</td>
<td>5520±5</td>
<td>3110±8</td>
<td>120±2</td>
</tr>
<tr>
<td>7.0</td>
<td>6850±4</td>
<td>5520±5</td>
<td>190±26</td>
</tr>
<tr>
<td>5.0</td>
<td>12289±1</td>
<td>7870±3</td>
<td>229±1</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>300±24</td>
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<th>pH 2.3</th>
<th>pH 2.0</th>
<th>pH 0.9</th>
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<tbody>
<tr>
<td>11.4</td>
<td>1.03</td>
<td>0.75</td>
</tr>
<tr>
<td>8.4</td>
<td>0.14</td>
<td>0.53</td>
</tr>
<tr>
<td>7.0</td>
<td>0.05</td>
<td>0.08</td>
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<tr>
<td>5.0</td>
<td>0.02</td>
<td>0.04</td>
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<tr>
<td>2.0</td>
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<th>Conc. (% w/v)</th>
<th>pH 2.3</th>
<th>pH 2.0</th>
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<tbody>
<tr>
<td>11.4</td>
<td>6020±1</td>
<td>8169±1</td>
<td>-</td>
</tr>
<tr>
<td>8.4</td>
<td>2900±1</td>
<td>6609±1</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>1412±2</td>
<td>3210±6</td>
<td>56</td>
</tr>
<tr>
<td>5.0</td>
<td>580±4</td>
<td>1460±4</td>
<td>60</td>
</tr>
<tr>
<td>2.0</td>
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<th>pH 2.3</th>
<th>pH 2.0</th>
<th>pH 0.9</th>
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<tbody>
<tr>
<td>11.4</td>
<td>9280±3</td>
<td>9808±8</td>
</tr>
<tr>
<td>8.4</td>
<td>3880±6</td>
<td>7000±1</td>
</tr>
<tr>
<td>7.0</td>
<td>2740±1</td>
<td>5619±1</td>
</tr>
<tr>
<td>5.0</td>
<td>1070±4</td>
<td>2670±6</td>
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<td>2.0</td>
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<th>pH 2.3</th>
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It is suggested that different length scales in combination with the variation of electrostatic interactions upon pH changes affect the gelation time. The gelation time results for 5% w/v β-lactoglobulin fibril networks formed at pH 2.0 (7873 s, Table 4.1a) were compared with the results of Loveday et al [8]. Loveday et al. [8] estimated a gelation time of ~4620-5598 s for 5% w/w WPI gels formed at pH 2.0. As is expected, the gelation process of the β-lactoglobulin networks is slower compared with the WPI networks, due to the presence of salts in the WPI networks which increase the ionic strength and decrease the inter-molecular interactions [8] within the network.

Another point gleaned from the data is the reduction of gelation time (Table 4.1a) with the increase of protein concentration which is attributed to the increase of the number of cross-links within the networks which results in the inter-molecular distance decrease as the protein concentration increases.

Using the empirical models developed by Scott Blair and Burnett [35] (eq. 7-11) allows the time sweep data to be further analysed and the empirical parameter B to be estimated [8]. $G'_{\infty}$ is $G'$ at $t=\infty$ and the empirical parameter B [8] expresses the time taken for $G'$ to reach the value $G'_{\infty}/e$ (see eq. 7). The value of $G'$ at infinite time is an important characteristic for the estimation of fractal dimensions which derive from the description of the $G'$-concentration relationship (§4.7). $G'$ increases with the increase of heating time until it reaches a plateau. It was found [6] that the time required for the $G'$ of low concentration samples to reach a plateau was longer than the time required for high concentration samples. All in all, the $G'$ estimation at infinite time determines when the $G'$ plateau is reached for samples where experimental monitoring is impractical as discussed in detail in Clark [36]. Many studies have applied the Scott Blair and Burnett models successfully on globular proteins, including β-lactoglobulin, such as Kavanagh et al. [6], Gosal et al. [7], and Loveday et al. [8].
Gosal et al. [7] conclude that to obtain an estimate of the fractal dimension (§4.7) the final $G'$ modulus ($G'_{\infty}$) is needed, but sometimes that is impractical. This is the reason why I have to extrapolate to estimate the $G'$ modulus at very long heating times ($G'_{\infty}$). Practically, this assumes that $G'_{\infty}$ derives from a cross-linking mechanism within the gel network that remains unchanged over time. Gosal et al. [7] have also highlighted that the extrapolated $G'_{\infty}$ data can be sometimes overestimated in the case that $G'_{\text{Exp}}$ (final $G'$ experimental values) values exhibit a reduction at long heating times. Specifically, if $G'_{\text{Exp}}$ values decrease at long heating times, this possibly constitutes an artefact derived from sample shrinkage. Nevertheless, it is stated in the Gosal et al. study that the extrapolation method, performed here, is the most objective method for the $G'_{\infty}$ estimation.

In order to estimate $G'_{\infty}$, based on eq. 7, fits of the Scott and Blair model [35] are performed to the cure curves shown in Fig. 4.3. Specifically, $\ln G'$ versus $1/t$ plots were created based on eq. 8 where $B$ is the slope and $\ln G'_{\infty}$ is the y-intercept. Eq. 8 was applied only to the range of data points over which the plot is linear [7-8, 35]. The maximum rate of $G'$ increase ($\left(\frac{dG'}{dt}\right)_{\text{max}}$) in eq. 11 was estimated by firstly differentiating eq. 7 with respect to time which yields eq. 9. Differentiation of eq. 9 yields eq. 10. From this, when $t=B/2$ corresponds to the maximum rate. Finally, by substituting the time with $t=B/2$ in eq. 9 the final form of the maximum rate of $G'$ increase is given by eq. 11. The estimation of the maximum rate of $G'$ increase is an important factor for the investigation of kinetics during the gelation process. It reveals the optimal rate under which the structural changes can occur.

\[
G' = G'_{\infty} e^{-\frac{B}{t}} \quad (7)
\]

\[
\ln G' = \ln G'_{\infty} - \frac{B}{t} \quad (8)
\]

\[
\frac{dG'}{dt} = G'_{\infty} \frac{B}{t^2} e^{-\frac{B}{t}} \quad (9)
\]

\[
\frac{d^2G'}{dt^2} = \left(G'_{\infty} \frac{B}{t^2} e^{-\frac{B}{t}}\right)' = 0 \quad (10)
\]

\[
\left(\frac{dG'}{dt}\right)_{\text{max}} = \frac{4G'_{\infty}}{B} e^{-2} \quad (11)
\]
These data are presented in Table 4.1b and c. Here the $G'_\infty$ values derived from the empirical Scott Blair and Burnett model [35] are compared with the final experimental values $G'_{\text{Exp}}$, which correspond to the $G'$ values of the last seconds of the 10 h heating time (Table 4.1c).

The data presented in Table 4.1c show that gels at pH 2.3 and 2.0 are still evolving after 10 h of heating. Their fully gelled properties are characterized by the $G'_\infty$. On the contrary, pH 0.9 hydrogels seem to be almost fully gelled. This is consistent with differences with the flexibility of the fibrils at the three pHs studied here. The pH 2.3 and pH 2.0 networks exhibited the most flexible fibrils and the pH 0.9 networks exhibited the least flexible fibrils, as is reported in Chapter 2. Hence, pH 0.9 gels are less able to contribute to entropic restoring forces. On the other hand, hydrogels formed at pH 2.3 are slightly softer compared with the pH 2.0 gels, while the gels formed at pH 0.9 are the softest. The explanation for this result is that the fibrils formed at pH 0.9 are quite thick and "open" up the pores, as described in §2.7.2. For this reason the pH 0.9 hydrogels could exhibit the lowest number of cross-links which could explain the lowest $G'$ values that the pH 0.9 hydrogels exhibit. Generally, comparing the $G'_\infty$ and $G'_{\text{Exp}}$ data for pH 2.0 gels derived from this study with Loveday et al. [8] and Kavanagh et al. [6] data, it is obvious that the data do not totally agree with each other, although the trends are convincing. This possibly is attributed to 1) the sample shrinkage caused after the prolonged heating time which could affect the final $G'$ values, 2) the different rheometers that are used in each study (different torque sensitivity), and 3) the different geometries.

$G'_\infty$ values are larger than $G'_{\text{Exp}}$ values for pH 2.0 and 2.3 (Table 4.1c). This means that after 10 h heating the macroscopic mechanical properties of the gel networks have not been stabilized. It is important to note that stabilisation is achieved more quickly as protein concentration is increased. By contrast, for gels formed of at pH 0.9, the $G'_{\text{Exp}}$ values more closely correspond to the $G'_\infty$ values. This happens because the structural changes for pH 0.9 samples occur rapidly within the first ~300 s. The pH 2.0 and 2.3 samples undergo structural changes over the first 10 h of heating.
These kinetics are related to the SAXS investigation based on the relationship between the Porod volume and the orientational entropy (§3.4.3, Fig. 3.13) as described in Chapter 3. According to the SAXS investigation, it was found that the less rod-like the particles, the greater the Porod volume and the orientational entropy increases [37-38] at the expense of the translational entropy. This results in an increased probability of the particles sticking together via a self-assembly process with structural changes occurring rapidly. Many studies [39-41] focused on DNA gels highlighting the dynamics of DNA strands. It is stated that the shorter the DNA strands in length, the faster they move through the gel.

The maximum rate of $G'$ increase (Table 4.1b) expresses the maximum rate of structural changes that occur within the samples. The maximum rate of $G'$ ($\frac{dG'}{dt})_{max}$ is approximately the same for pH 2.0 and 2.3 gels, which is relatively very slow compared with that for the pH 0.9 gels. This means that the structural changes during heating for pH 2.0 and 2.3 gels occur with roughly the same rate compared with the pH 0.9 networks where the structural changes occur with the fastest rate. This difference in the kinetics of gelation could once again be interpreted based on the orientational entropy prevalence [42-43] related to the translational entropy within the pH 0.9 hydrogels. Furthermore, the maximum rate of $G'$ increase increases with increasing protein concentration and hence, the structural changes associated with gelation occur faster.

One crucial characteristic of these rheological experiments was the use of silicone oil for sample sealing to avoid evaporation upon heat treatment. As was noted in Fig. 4.3a and b, the $G'$ values for the gels formed at pH 2.0 and 2.3 at short times are higher (~10 Pa) compared with the $G'$ values ($\leq$0.02 Pa) for the gels formed at pH 0.9. This difference in the $G'$ values for the first seconds of heat treatment for the gels formed at pH 2.0 and 2.3 is a result of artefacts introduced by the application of the silicone oil around the exposed sample. This was confirmed by recording $G'$ data (Fig. 4.4) for $\beta$-lactoglobulin solutions in the presence and absence of silicone oil for the first seconds of heat treatment. It was found that the addition of silicone oil increased
the recorded $G'$ from $\leq 0.01$ Pa to $\sim 10$ Pa. In this case, the silicone oil does not behave as a plasticizer; contrariwise, the samples at pH 2.0 and 2.3 at the first seconds of the heat treatment exhibit low viscosity and this allows the silicone oil to creep under the rheometer cone geometry; hence $G'$ increases from 0.01 Pa to 10 Pa. Despite this, it should be noted that the presence of silicone oil does not affect the minimum $G'$ values reached for the pH 0.9 gels. In order to explain this, it should be taken into account that gelation for pH 0.9 gels is much faster compared with the gelation for pH 2.0 and 2.3 gels. This means that samples at pH 0.9 already exhibit high viscosity at the first seconds of heat treatment and this prevents any creep of the silicone oil under the rheometer cone geometry and hence, any $G'$ changes are prevented. When silicone oil is added around for example the exposed 5% w/v sample at pH 0.9, the protein solution takes just $\sim 230$ s (Table 4.1a) to form a high viscous gel, while the 5% w/v samples at pH 2.0 and 2.3 require more than $\sim 8000$ s and 12000 s, respectively, (Table 4.1a) to form a soft gel. Hence, the 5% w/v samples at pH 2.0 and 2.3 exhibit a liquid-like behaviour for an extended period ($\sim 8000$ s), i.e. slow kinetic changes take place prior to the start of gelation and during this time the results could be affected by the presence of the silicone oil. This is in contrast to the 5% w/v samples at pH 0.9 which exhibit gel behaviour within $\sim 300$ s.

![Graph](image)

**Fig. 4.4** Elastic modulus, $G'$, changes within the first 1000 s of heating at 80°C for 10% w/v β-lactoglobulin fibril gel networks formed at pH 2.0 in the presence and absence of silicone oil used to prevent evaporation during the extended heating period.
4.5 Frequency Dependence of Elastic Modulus $G'$

Fig. 4.5 presents the frequency dependence of the elastic modulus, $G'$, for β-lactoglobulin fibril networks of different concentrations at pH 2.3, 2.0, and 0.9. Frequency sweep tests were carried out at 20°C following gel formation at 80°C for 10 h and show that $G'$ is largely insensitive to frequency for the fibril networks. Frequency sweep tests applied on these hydrogels provide information about the degree of cross-linking and the gel strength (rigidity). $G'$ for cross-linked polymers is larger than $G''$ and exhibits a constant limiting value in the low-frequency region. When the constant limiting value of $G'$ extends from low (0.001 rad/s in Fig. 4.5) to high (1000 rad/s in Fig. 4.5) frequencies, the greater the extent of cross-linking in the gel [3]. Furthermore, the higher the constant limiting $G'$ value, the stiffer the gel structure.
**Fig. 4.5** Frequency sweep tests at 20°C of β-lactoglobulin fibril gel networks formed at a) pH 2.3, b) pH 2.0, and c) pH 0.9 and heated at 80°C for 10 h.
Generally, the data presented in Fig. 4.5 shows that the different fibril β-lactoglobulin hydrogels with different protein concentrations all exhibit the characteristics of highly cross-linked stiff gel structures, since $G' > G''$ and the $G'$ modulus is independent of frequency (for at least some of the frequency range investigated). Gels formed at pH 2.3 exhibit slightly lower rigidity compared with gels formed at pH 2.0, while gels formed at pH 0.9 exhibit the lowest rigidity.

Generally speaking, based on the investigation presented in this thesis, there are two types of hydrogels; the pH 0.9 and the pH 2.3/2.0 hydrogels. The pH 0.9 hydrogels exhibit short and rigid fibrils with β-sheet stacks parallel to the fibril axis (§2.7.1) which result in soft gels, as presented in time sweep tests (Fig. 4.3), while pH 2.3/2 hydrogels exhibit long and flexible fibrils with β-sheet stacks perpendicular to the fibril axis (§2.7.1) that yield stiff gels (Fig. 4.3). In the frequency sweep tests presented here (Fig. 4.5) there are also two kind of behaviours presented by the hydrogels; the behaviour of the hydrogels formed at pH 2.3 and pH 2.0 where $G'$ and $G''$ modulus exhibit almost a constant plateau from low to high frequencies, and the behaviour presented by the hydrogels formed at pH 0.9 where $G'$ exhibits a slight reduction and a rapid increase of $G''$ at high frequencies. This could indicate structural changes occurring within the pH 0.9 hydrogels at high frequency regions, due to the increase of cross-linking interactions between the fragmented-particle fibrils. Specifically, this could mean that an internal stress rises within the networks making these networks more brittle. In other words the pH 0.9 hydrogels crack easily compared with the other two hydrogels, since the number of cross-links within the pH 0.9 hydrogels is low. Possibly, this is the reason why stronger gels (higher $G'$) exhibit lower critical strain, as it is shown in §4.6 in strain sweep tests.

Besides, the $G'$ and $G''$ modulus for pH 0.9 hydrogels is about two orders of magnitude lower compared with the pH 2.3 and pH 2.0 hydrogels. The same trend is followed by the $G'$ behaviour for these three different types of hydrogels presented in the time sweep tests (Fig. 4.3). These low $G'$ values presented by the pH 0.9 hydrogels are possibly attributed to the large fibril diameters exhibited by these hydrogels which
"open" up the pores resulting in the formation of a low number of cross-links within these networks. This possibly makes the pH 0.9 hydrogels softer compared with the pH 2.3 and pH 2.0 hydrogels (G' for pH 0.9 hydrogels lower than G' for pH 2.0 and 2.3 hydrogels).

The difference between G' and G" (Fig. 4.5a and b) increases as the concentration increases for the fibril networks formed at pH 2.0 and 2.3 with concentrations lower than 8.4% w/v. The G' increase upon increasing the protein concentration is a consequence of the strengthening of the gel structure. It is evident that the liquid-like behaviour (G") remains roughly constant, while the solid-like behaviour (G') is enhanced with increasing the protein concentration. In other words, as the protein concentration increases, the number of cross-links increases and hence, G' increases. In contrast, the gap between G' and G" is approximately constant for the fibril networks formed at pH 0.9 (Fig. 4.5c) with the G" increasing more at high frequencies compared with the networks formed at pH 2.3 and 2.0 (Fig. 4.5a and b).

Frequency sweep systems, applied on the different β-lactoglobulin hydrogels described above, do not provide any novel information, rather confirming that the hydrogels formed at pH 2.3 are the most highly cross-linked gel structures. They exhibit the highest rigidity and slightly higher compared with the hydrogels formed at pH 2.0, while those formed at pH 0.9 exhibit the least cross-linked gel structure. This is consistent with the increase of ionic strength when the pH is decreased which results in a reduction in the electrostatic interactions between the amino and carboxyl groups with the polypeptide chains [44] that is accompanied by a decrease of the number of cross-links. Additionally, given that the fragmented-fibrils formed at pH 0.9 are thicker than the thin fibrils formed at pH 2.0 and 2.3, it is likely that the structures and inter-fibril interactions are very different.
4.6 Strain Dependence of Elastic Modulus G'  

Strain sweep tests (the sample is scanned over a wide range of strains) were also performed on the same set of gels. Based on Fig. 4.6, the critical strain, $\gamma_c$, could be estimated for each of the fibril networks. Below the critical strain $\gamma_c$ the fibril network response is linear (rheological properties, like G', are independent of strain), while after the critical strain the network response is non-linear. The critical strain was defined based on the Mezger [3] method. The critical strain was determined as the strain at which G' (see Fig. 4.6a) starts to decrease by 5% from its maximum value and expresses the breaking of bonds within the network. The results are presented in Table 4.2 and show that pH 2.0 samples exhibit the highest critical strains, while the pH 0.9 samples exhibit the lowest critical strains. This is in agreement with the results found in §4.4 (Table 4.1c) where the G’ values for the pH 2.0 gels are higher than those for pH 0.9 gels. The critical strains seem also to decrease as the protein concentration increases, although the hydrogels become stiffer with an increase in the protein concentration. This possibly means that an internal stress rises within the networks, as it is reported above, and this is the reason why the high concentration gels or the gels formed at pH 0.9 become more brittle and crack easily.
Fig. 4.6 Strain sweep tests at 20°C of β-lg fibril gel networks formed at a) pH 2.3, b) pH 2.0, and c) pH 0.9 heated at 80°C for 10 h.
Table 4.2 Critical strain $\gamma_c$ of $\beta$-lactoglobulin fibril gel networks of different concentrations heated at 80°C for 10 h at different pH extracted from strain sweep tests.

<table>
<thead>
<tr>
<th>Conc. (% w/v)</th>
<th>pH 2.3</th>
<th>pH 2.0</th>
<th>pH 0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.4</td>
<td>0.16±0.01</td>
<td>0.40±0.08</td>
<td>-</td>
</tr>
<tr>
<td>8.4</td>
<td>0.21±0.01</td>
<td>0.63±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.24±0.02</td>
<td>0.63±0.01</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.40±0.01</td>
<td>1.10±0.01</td>
<td>0.20±0.05</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>0.40±0.01</td>
</tr>
</tbody>
</table>

Another main characteristic of strain sweep tests for biopolymer networks is strain-stiffening behaviour. In the literature there are several studies reporting the strain-stiffening behaviour for polysaccharides [42], like pectin, and proteins, especially for those composed of semiflexible chains, like fibrin and collagen [43]. This study suggests that proteins, like $\beta$-lactoglobulin, which are peptide-based with low molecular weight do not exhibit strain-stiffening behaviour.

Licup et al. [45] have investigated the mechanism of non-linear strain-stiffening behaviour of athermal networks consisting of straight filaments. A few prior studies highlighted what happens in non-linear network stiffening during the transition from bending to stretching of fibrils. This work established that fibril bend-dominated behaviour still exhibits non-linear network stiffening ($G' \sim \sigma$, where $\sigma$ is the shear stress), while stretch-dominated behaviour exhibits different stiffening-behaviour ($G' \sim \sigma^{1/2}$) and usually appears when the network stiffness has increased by more than an order of magnitude. Licup et al. [45] also suggested that non-linear network stiffening is controlled by the applied stress rather than the thermal fluctuations and is independent of protein concentration changes. This suggestion applies only on systems assuming that the networks are athermal, bend dominated, and their geometry is similar at different concentrations, like the collagen networks. On the contrary, in
my study the β-lactoglobulin gels exhibit geometry that is not similar at different concentrations, since they are peptide-based with low molecular weight.

Here, a series of rheological experiments on β-lactoglobulin fibril networks is undertaken, in order to investigate strain-stiffening, but strain-stiffening behaviour was not detected. There is also a noticeable lack of studies in the literature on β-lactoglobulin strain-stiffening behaviour. Generally, it seems that peptide-based low molecular weight gelators do not strain stiffen. It should also be highlighted that when different strains are applied to other networks, the fibrils reorient, bend and straighten, and the cross-links undergo a displacement [45]. This causes changes in the fibril length and curvature and also in the elastic energy stored in the fibrils. Hence, the fibril network undergoes stiffening [46]. In my study, this does not happen. This means that possibly the bonds between the β-sheet peptides, which constitute the fundamental building blocks of amyloid fibrils, break and hence, the fibrils break instead of being stretched or bent, as different strains are applied. This explanation does however require further detailed investigation, in order to provide evidence for my assumptions and it could constitute a promising future investigation. Besides, this assumption also explains why there is little difference in the plots in Fig. 4.6. This assumption could also be quite interesting as an application in microrheology. Such β-sheet fibrils do show a $\frac{3}{4}$ power law exponent ($G'/G'' \sim \omega^{3/4}$) which is a sign for the existence of semiflexible fibrils [47], but in large deformation the fibrils are pulled apart.

### 4.7 Concentration Dependence of Extrapolated Modulus $G'_\infty$ and Critical Strain

Kavanagh et al. [6] has applied cascade theory on β-lactoglobulin gel systems formed at different pH values and has shown that cascade models are more accurate for the description of the elastic modulus vs protein concentration relationship compared with the fractal model [20-21]. Although branching cascade and percolation theories are broadly used for the characterization of the elastic modulus vs gel concentration relationship and are broadly applicable compared with fractal models, here fractal
models are applied. I prefer the application of fractal models on my β-lactoglobulin gel systems, since they provide the opportunity to estimate the fractal dimensions which other models, like cascade theory, are not able to provide. The estimation of fractal dimensions allows the characterization of the structural differences between different β-lactoglobulin gel systems and can be compared with the fractal dimensions estimated by SAXS (§3.4.2).

Firstly, the relationship between the elastic modulus \( G'_{\infty} \) and the protein concentration \( C \) is investigated, which follows a power law behaviour \( G'_{\infty} \sim C^m \), Fig. 4.7a) and the relationship between the critical strain \( \gamma_c \) and the protein concentration which also follows a power law behaviour \( \gamma_c \sim C^n \), Fig. 4.7b). Based on the power-law exponents derived from the power-laws reported above, I apply the Shih et al. [23-24] (eq. 1-2 in §4.4) and Wu and Morbidelli [25] (eq. 5-6 in §4.4) models, in order to estimate the fractal dimensions \( d_f \) for our gel systems. The power-law exponents \( n \) and \( m \) for the power law relationship between the elastic modulus \( G'_{\infty} \) and the critical strain \( \gamma_c \) with the protein concentration, respectively, are presented in Table 4.3. Since \( m<0 \), we consider that the gelation regimes in my gel systems are strong-link regimes and hence, I use equations 1-2 for the Shih et al. model. Using the resulting \( n \) and \( m \) values in equations 1-2 (Shih et al. [23-24] model, strong-link regime) and in equations 5-6 (Wu and Morbidelli [25] model), I estimate the fractal dimensions \( d_f \) and the constants \( x \) (\( x>0 \) represents the fractal dimensions of the backbone of colloidal aggregates) [15]. In the Wu and Morbidelli [25] model the constant \( \alpha \) is characterized by the equation \( \beta = (d - 2) + (2 + x)(1 - \alpha) \) (see §4.4 for details) which allows characterization of the inter- vs intra-floc contribution within the elastic behaviour of the hydrogels. As determined in the literature [25], \( x \) is set equal to 1 and 1.3, in order to correspond to these situations. These \( x \) values were derived from the application of fractal models on colloidal aggregates.
Fig. 4.7 Log-log concentration versus a) $G'$ at infinity ($G'_{\infty}$) and b) critical strain ($\gamma_L$) of $\beta$-lactoglobulin fibril gel networks at different pH values heated at 80°C for 10 h. Power law models were fitted to low concentrations where $n$ is the power law exponent.

Table 4.3 Power law exponent estimation for the relationship between protein concentration $C$ with $G'$ at $t=\infty$ ($G'_{\infty}$) and critical strain ($\gamma_L$) for $\beta$-lactoglobulin fibril gel networks of heated at 80°C for 10 h at different pH 2.3, 2.0, and 0.9

<table>
<thead>
<tr>
<th>pH</th>
<th>$m$</th>
<th>$n$</th>
<th>$d_i$</th>
<th>$d_i$</th>
<th>$\alpha$ (for $x=1$)</th>
<th>$\alpha$ (for $x=1.3$)</th>
<th>Gelation Regime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$(G'_{\infty}/C)$</td>
<td>$(\gamma_L/C)$</td>
<td>(Shih et al.)</td>
<td>(Wu &amp; Morbidelli)</td>
<td>(Wu &amp; Morbidelli)</td>
<td>(Wu &amp; Morbidelli)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Model</td>
<td>Model</td>
<td></td>
<td>Model</td>
<td>Model</td>
<td>Model</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>2.90±0.02</td>
<td>-1.12±0.01</td>
<td>1.90±0.02</td>
<td>1.88±0.01</td>
<td>0.25±0.01</td>
<td>0.32±0.02</td>
<td>Transition Regime</td>
</tr>
<tr>
<td>2.0</td>
<td>2.60±0.02</td>
<td>-1.14±0.04</td>
<td>1.70±0.04</td>
<td>1.63±0.01</td>
<td>0.48±0.01</td>
<td>0.52±0.07</td>
<td>Transition Regime</td>
</tr>
<tr>
<td>0.9</td>
<td>2.52±0.01</td>
<td>-0.90±0.02</td>
<td>2.24±0.02</td>
<td>1.76±0.01</td>
<td>0.10±0.01</td>
<td>0.17±0.01</td>
<td>Transition Regime</td>
</tr>
</tbody>
</table>
The power-law exponents $m$ and $n$ (Fig. 4.7a, b and Table 4.3) seem to be roughly the same for the $\beta$-lactoglobulin hydrogels formed at pH 2.3 and 2.0, while the power-law exponents $m$ and $n$ for hydrogels formed at pH 0.9 are smaller. This means that there are no apparent differences between the structures of hydrogels formed at pH 2.3 and 2.0.

The $m$ and $n$ values estimated from this study (Table 4.3) differ from those reported in the literature. In addition, $m$ and $n$ values reported in different studies in the literature do not agree with each other. Gosal et al. [7] estimated the power-law exponent ($m$) for the power-law behaviour which connects the elastic modulus with the protein concentration of $\beta$-lactoglobulin hydrogels formed at pH 2 to be 5.1-5.7. Wu and Morbidelli [25] refers that $m$ values vary from -3.4-5.3 depending on the nature of the protein. Sagis et al. [17] highlight that the power-law exponent $m$ for $\beta$-lactoglobulin hydrogels with different ionic strength varies from 3 to 11.5. The difference between my experimental results and those reported in the literature is possibly attributed to the different protein concentration range used for the application of the fractal model in each case and the different $\beta$-lactoglobulin batches used in each study. The variations in the power-law exponent $\alpha$ variation could be attributed to the different ionic strengths that the systems exhibit which affect the fibril length and the structures of aggregations or to the different range of concentrations that the models were applied.

Results derived from the Shih et al. model [23-24] and presented in Table 4.3 show that the networks formed at pH 0.9 exhibit the highest fractal dimension $d_f$ (2.2±0.02). This means that these systems exhibit the highest degree of aggregation, whereas the networks formed at pH 2.3 and 2.0 exhibit a similar degree of aggregation (1.9±0.02 and 1.7±0.04 respectively). This is expected, since the networks formed at pH 0.9 exhibit the strongest hydrophobic interactions which accelerate the self-assembly process and hence, the protein particles within the hydrogel occupy more the free volume (water volume that is not already occupied by other protein particles), in order to become interconnected and form a network. It should be highlighted here, that
hydrogels formed at pH 2.3 seem to exhibit more compact structure compared with the hydrogels formed at pH 2.0.

The Wu and Morbidelli model was also applied on β-lactoglobulin hydrogels formed at pH 2.3, 2.0, and 0.9 hydrogels and the results are presented in Table 4.3. The fractal dimensions $d_f$ derived from the application of the Shih et al. [23-24] and Wu and Morbidelli [25] models for the hydrogels formed at pH 2.3 and 2.0 are roughly the same. On the other hand, it seems that the fractal dimension derived from the Shih et al. [23-24] model for hydrogels formed at pH 0.9 is higher compared with the fractal dimension derived from the Wu and Morbidelli [25] model. Possibly this difference could be interpreted if I focus on the constant $\alpha$ estimated from the Wu and Morbidelli [25] model. It seems that the constant $\alpha$ is close to zero for hydrogels formed at pH 0.9 ($\alpha \sim 0.1-0.17$). It should be reported again here that when $0 < \alpha < 1$, the gelation regime is a transition regime where the inter- and intra-floc links contribute to the elastic modulus of these hydrogels, while when $\alpha$ is close to zero the gelation regime is a strong-link regime where the inter-floc links dominate. This means that the gels formed at pH 0.9 are within the transition regime, but the inter-floc links appear to be more dominant than the intra-floc links and hence, the gelation regime to be converted to strong-link from transition regime.

Taking into account that the Shih et al. [23-24] model is reliable only when $\alpha=0$ (strong-link regime) and $\alpha=1$ (weak-link regime), whereas the Wu and Morbidelli model [25] is valid only for $0 < \alpha < 1$, I assume that the Shih et al. model is more appropriate for hydrogels formed at pH 0.9, while the Wu and Morbidelli [25] model is more appropriate for hydrogels formed at pH 2.3 and 2.0, since in a considerable number of studies the Shih et al. model gives a negative value for $x$ (fractal dimension of the backbone of colloidal aggregates) in eq.1 and 2 which is unrealistic. However, the estimated fractal dimensions $d_f$ for my β-lactoglobulin systems seem to be similar to those reported in the literature. The fractal dimensions for protein gels vary from 1.5 to 2.8. Specifically, Kavanagh et al. [6] estimated $d_f=2.7$ for β-lactoglobulin gels formed at pH 2.0.
In addition to the mass fractal dimensions estimated from the rheology data, I also estimated mass fractal dimensions for the same systems using SAXS (§3.4.2, Table 3.1 \(D_m\)) estimated from the power-law relationship between the SAXS intensity and the wave vector \(q\) \(\left(I \sim q^{-D_m}\right)\). In the SAXS study I estimated the degree of compactness through the value of \(D_m\) (mass fractal dimension) which expresses the distribution of the inter-atomic distances [48]. The main difference in this case is that the SAXS data for a \(\beta\)-lactoglobulin hydrogel are derived from the averaged scattering patterns of fibrils within the hydrogel as a function of spatial frequency; hence the fractal dimensions for a sample derived from SAXS data constitute average values of a single sample. On the other hand, estimation of fractal dimensions within a \(\beta\)-lactoglobulin hydrogel using rheology data are derived from rheological measurements applied over a range of concentrations. Generally, in the SAXS study the fractal dimensions estimated for the pH 2.3 and 2.0 \(\beta\)-lactoglobulin hydrogels are slightly smaller than 1, while for the pH 0.9 hydrogels are \(~1.2\).

On the other hand, the application of fractal models in rheology requires a wide range of sample concentrations (from low to high concentrations). It is also noticed that carrying out rheological experiments for low concentration samples is difficult due to the prolonged heating (~10 h) of the samples. However, the fractal models in rheology take into account that the critical gel concentration equals to 0; hence fractal models assume that the power-law exponent \(m\) is stable [7] for the same sample with different concentrations, while in reality \(m\) changes when it reaches the critical concentration gel. On the contrary, SAXS allows \(m\) value estimation for each sample separately.

The fractal dimension data using rheology exhibit higher values compared with the corresponding SAXS data. This happens, because these two techniques do not refer to the same length scales. Specifically, rheology refers to a wider range of length scales, covering larger scales compared with the SAXS method. The SAXS method refers to a limited \(q\) range which results in the investigation of length scales <200 nm.
All in all, the estimation of mass fractal dimension using either SAXS or rheology is not straightforward, but by using these two methods as complementary it can be shown which trend follows the behaviour of each different gel. SAXS allows estimation of the mass fractal dimension over the fibril distribution for each sample separately covering small length scales (<200 nm), while rheology averages the mass fractal dimension data over a wide range of length scales (>mm). The SAXS method allows the estimation of the surface fractal dimension, which is not possible using rheology, and constitutes an appropriate method for the estimation of mass fractal dimension for low concentration samples. On the other hand, rheology constitutes an appropriate method for the estimation of fractal dimension covering a wide range of length scales.
Conclusions

In this study changes in the macroscopic properties for different β-lactoglobulin fibril-like networks were investigated by rheology. Rheology gives the opportunity to investigate the structural and kinetic changes on a macroscopic level (>mm), which is not achieved with the other experimental tools used in this work; cryo-SEM and SAXS. It was found that structural and kinetic changes for pH 2.3 and 2.0 β-lactoglobulin hydrogels are roughly the same in contrast to the pH 0.9 hydrogels which exhibit faster kinetics (inter-fibril interactions) and lower elastic mechanical properties.

A combination of factors contributes to the determination of the macroscopic properties of such hydrogels and these are 1) the electrostatic and hydrophobic interactions and 2) the chain stiffness (entropic elasticity) and thickness (enthalpic rods). Specifically, as the pH is reduced from 2.3 to 0.9, the fibril lengths are reduced and the self-assembly and gelation processes are sped-up, due to the decrease of repulsive electrostatic interactions and the increase of hydrophobic interactions between the solute and the water. This results in the faster self-assembly process and hence, in the increase of the number of cross-links and the reduction of the fibril length. As the pH reduces, the particles become less rod-like. The more rod-like the protein system is, the more favourable it is to organize the system into a nematic-like phase. In other words, as the free volume decreases, the orientational entropy increases against the translational entropy (§3.4.3) which favours the isotropic phase and hence, the fibrils stick together and undergo self-assemble. On the other hand, the long length scale networks (pH 2.3 networks) exhibit more flexible structure compared with the short length scale particle networks (pH 0.9 networks), since thermal fluctuations constitute the only source of elasticity within the pH 2.3 networks. I suggest that when a system is athermally controlled, as for the pH 2.3 and pH 2.0 β-lactoglobulin hydrogels, and the fibrils are able to be bent or stretched then, the system exhibits a flexible structure with high modulus.
Lastly, the mass fractal dimensions within β-lactoglobulin fibril hydrogels were estimated by rheology and compared with the corresponding SAXS data, but generally, the mass fractal estimation by either using the one or the other method is not straightforward. It seems that SAXS and rheology constitute complementary methods for the fractal dimension estimation. Fractal dimension data derived from rheological experiments exhibit higher values compared with the corresponding data derived from SAXS. This happens, because rheology covers a wide range of length scales (>mm), while SAXS refers to smaller length scales (<200 nm). It seems that SAXS is a more appropriate tool than rheology for the estimation of the fractal dimensions for low concentration samples. SAXS provides average values of fractal dimensions for each single sample, while rheology provides average values covering a wide range of samples with different concentrations and hence, different length scales. Although rheology can be applied on a wide range of protein concentrations, rheological measurements with high accuracy on very low (<2% w/v) concentrations cannot be applied, since signal-to-noise ratio is low.
References


Chapter 5

General Conclusions

The novelty of this study concerns the development of new β-lactoglobulin hydrogels formed at pH 0.9, the investigation of the effects of different freezing methods used in cryo-SEM imaging on the native state of hydrogels, and the control of mesoscale. Novel β-lactoglobulin hydrogels formed at pH 0.9 were developed which exhibit completely different properties compared with the already well studied [1-10] β-lactoglobulin hydrogels formed at pH 2.0 and 2.3. Another significant finding of this study refers to the major challenge in cryo-SEM which is the ice crystal formation during the cryo-fixation of high-water content samples. Many cryo-fixed structures damaged by the ice crystal formation are reported in the literature as being the representative structures of the native state of fibrillar hydrogels. Here, I show that by selecting the appropriate freezing method, the cryo-SEM imaging of the native state of high-water content samples is feasible. Lastly, this study adds to the literature the factors (entropy, chain stiffness and thickness, electrostatic interactions, inter-fibril distances, and the number of cross-links) that control the mesoscale where the self-assembly process occurs and were until now uninvestigated. This could be very useful for the industry, since the different β-lactoglobulin fibril systems presented in this project could be used by the industry, in order to manipulate and define the properties of biomaterials that already exist in the market, like WPI, and improve their macroscopic properties or create new biomaterials with novel properties. For example, pH 0.9 hydrogels could be introduced into the market with applications in different fields, like in pharmaceuticals where acidic drugs can be absorbed better compared with the lipid-based drugs.

Recent literature investigating hierarchical structures in self-assembled biopolymer networks has focused on specific length scales with few addressing all length scales with the exception of, for example, some studies on fibrin [11-14]. The macroscopic properties (here the so-called top) of self-assembled protein networks are well studied
using a variety of different experimental methods, like rheology, to probe mechanical behaviour. Microscopic properties (here the so-called bottom) are also well studied using methods, like scattering methods and single-molecule techniques. On the contrary, the mesoscale, the scale between the macroscale and microscale is not yet well understood. This study attempts to build links through the hierarchical scales by acquiring knowledge on how the structural and kinetic control of the mesoscale is achieved and how it connects the top to the bottom (top-bottom approach) by using a combination of techniques; rheology (macroscopic level), cryo-SEM (mesoscopic level), and SAXS (microscopic level) (Fig. 5.1). Using different techniques together allows me to build a picture that, individually, each technique cannot furnish.

![Diagram of the hierarchical scales](image)

**Fig. 5.1** General scheme depicting the approach used in this dissertation. Top-bottom approach, using bulk rheology, cryo-SEM, and SAXS. The characteristic factors of the mesoscale that connect the top to the bottom include; entropy, repulsive forces, and thermal bending fluctuations.

The protein studied in this dissertation was β-lactoglobulin heated at 80°C for different time periods under acidic conditions (pH 2.3, 2.0, and 0.9). The interesting point regarding β-lactoglobulin hydrogels formed at 80°C and acidic pH is that the protein unfolds and then self-assembles to yield fibrils with different characteristics. In order to determine these fibril characteristics, the first step used here was to undertake a cryo-SEM investigation (Chapter 2). Cryo-SEM allowed the
determination of fibril characteristics, like fibril size (diameter), end-to-end fibril length, etc. β-lactoglobulin fibrils at pH 2.3 and 2 are already well studied [1-10], in contrast to β-lactoglobulin hydrogels at very acidic conditions, like pH 0.9. In addition, the investigation of the self-assembly process of such hydrogels which results in a variety of hierarchical levels on the mesoscale is not widely reported in the literature and this is where I add knowledge.

The cryo-SEM data, focused on β-lactoglobulin hydrogels formed at pH 2.3, 2.0, and 0.9, revealed that β-lactoglobulin fibrils formed at pH 2.3 exhibit the longest end-to-end fibril lengths and slightly thinner but essentially the same diameters as fibrils formed at pH 2.0. The novel finding derived from this study was the thick and short particles generated from the β-lactoglobulin assembly at pH 0.9. The β-lactoglobulin hydrogels formed at pH 0.9, which have not previously been reported in the literature, exhibit completely different structure and macroscopic properties compared with the standard β-lactoglobulin hydrogels formed at pH 2.3 and pH 2.0. It was shown here that β-lactoglobulin hydrogels formed at pH 0.9 exhibit fragmented-fibril particles with the shortest end-to-end fibril lengths and the largest diameters. Another important point of the cryo-SEM study is that it allows the characterization of the fibril flexibility by comparing the end-to-end fibril lengths with the persistence lengths which constitutes a measure of the fibril flexibility. Cryo-SEM data showed that the fibrils formed at pH 0.9 are the least flexible, while the fibrils formed at pH 2.3 are the most flexible.

Furthermore, the cryo-SEM study makes a particular contribution in understanding the application of cryo-SEM in investigating network structures. To date many of the cryo-SEM images of high water content biopolymer networks reported in the literature, with or without the use of cryo-protectants, actually present altered networks due to freezing artefacts, instead of reflecting the network’s native state. Although Dubochet’s work [15] on the phase transition of water upon freezing, presented in a seminal paper in 1984, constitutes the origin of cryo-SEM, and while further studies have been done on the amorphous state of frozen water, these
investigations do not appear to have been widely appreciated and there are numerous literature results containing images of water freezing artefacts with no elucidation of the native state of the system.

The freezing rate and the sample thickness are two main factors affecting successful cryo-SEM sample preparation. Essentially, the thinner the sample, the faster its freezing with minor introduction of ice crystal formation. Specifically, in this study it has been shown that by using boiling liquid nitrogen, which exhibits a slow freezing rate, the native state of β-lactoglobulin hydrogels is altered, due to the introduction of ice crystal formation within the hydrogel structure due to the slow freezing of the sample. This results in the hydrogel structure exhibiting a honeycomb-like structure. The slower the freezing rate is, the more time the ice crystals have to grow. Thus, by using the propane jet freezing method, which exhibits a faster freezing method, SEM imaging of β-lactoglobulin fibril networks is achieved with the minimum introduction of freezing artefacts, thereby ensuring the avoidance of the disruption of the native state of the fibril networks.

It should be highlighted here that hydrogels formed at pH 0.9 exhibit a highly viscous behaviour. This means that it is difficult to load a quite thin layer of sample into the sample holder, which will allow a faster and hence, more homogeneous freezing of the whole layer of sample. This is one of the reasons that high concentration hydrogels and generally, highly viscous hydrogels, like those formed at pH 0.9, cannot be imaged easily by cryo-SEM. A high pressure freezing method is a more appropriate method in this case, but it was not readily available during the current study. The high pressure freezer method is an expensive experimental set up and is provided only by a few facilities internationally.

SAXS (Chapter 3) is another method that can be used as a complementary method to cryo-SEM mainly because it constitutes a non-invasive method. Namely it does not require any specific sample preparation. SAXS data constitute time-averaged values
derived from averaged scattering patterns of molecules as a function of spatial frequency, while cryo-SEM data present the range of distributions of individual fibrils. Importantly SAXS data allows the mesh-lengths of the networks to be estimated which can then be compared with the corresponding cryo-SEM data. This allows me to understand if any ice crystal formation in the cryo-SEM study has altered the mesh lengths within the frozen hydrogel structure. Based on my cryo-SEM and SAXS results (Table 2.2, §2.9) on mesh lengths, it has been shown that the propane jet freezing method allows reliable imaging of low concentration protein fibril hydrogel structures. On the other hand, it should be highlighted that the comparison between the mesh length data derived from cryo-SEM and SAXS experiments on high concentration β-lactoglobulin hydrogels shows that the propane jet freezing method is not the best option for high concentration samples, since the data do not always agree. This indicates that the high pressure freezing method should be used to explore its applicability in investigating high concentration samples which is one of the biggest disadvantages for the propane jet freezing method.

In connection with the cryo-SEM method, it should be highlighted that, in addition to the introduction of freezing artefacts, 2D analysis of 3D fibril in the cryo-SEM images constitutes an additional limitation to the quantitative analysis of the EM images. Taking into account the limitations in EM imaging introduced by the freezing artefacts, the 2D analysis of the 3D objects, and the low resolution of the used cryo-SEM experimental set up, it is suggested that cryo-EM imaging is a useful method for end-to-end and persistence length estimation for biopolymers. SAXS, due to the requirement of no sample preparation and the higher resolution, is by contrast a more accurate and reliable method for mesh length and fibril size estimation.

The production of images from low concentration samples prepared using the propane jet freezing method which are free or almost free from freezing artefacts is a considerable contribution to the literature. Here, it is shown not only that images representing the native structure can be produced but that they can then be used for qualitative and quantitative analysis of fibril and gel characteristics.
Another widely-used method reported in the literature is the use of cryo-protectants [16-19] for cryo-EM imaging. The use of cryo-protectants in biopolymer hydrogels delays ice crystal formation during freezing and hence, the distortion of the fibril networks is avoided. In this work, it is demonstrated that, while the use of cryo-protectants for cryo-EM imaging does reduce water freezing artefacts, they alter the native state of high-water content samples due to ionic bonding changes and thus, it does not allow the nature structure of the gel to be imaged. My work shows that cryo-protectants, like sucrose, cause shrinkage of the network structure, changes to the curl of the fibrils, and sometimes introduces large void spaces due to sublimation problems.

Focusing now on the use of SANS to investigate the hydrogels, it is highlighted one important advantage of this method; it does not require any additions or changes to the sample preparation. Furthermore, SANS allows the study of the structural changes on different hierarchical levels. These different hierarchical levels refer to the gel’s structure compactness, the fibril surface roughness, and the fibril diameter. My SANS study has shown that hydrogels formed at pH 0.9 exhibit the highest compact structure and the highest fibril surface roughness. On the other hand, hydrogels formed at pH 2.3 exhibit the least compact structure and the lowest fibril surface roughness. This can possibly be attributed to the smaller inter-fibril distances that the hydrogels at pH 0.9 exhibit (cryo-SEM study) compared with the inter-fibril distances within the hydrogels formed at pH 2.3 which could force the cross-links to undergo a displacement and change the fibril persistence length.

Rheology (Chapter 4) was used as a supplementary method to SANS for the characterisation of the structure compactness. The structure compactness was evaluated by estimating the mass fractal dimension. The higher the mass fractal dimension, the more compact the structure. By using the two methods, it has been shown that the mass fractal dimension estimation is not straightforward. By using rheology, average values of fractal dimensions can be estimated covering a wide range of sample concentrations (from low to high concentrations) and hence, length
scales. On the contrary, SAXS refers to smaller length scales (<200 nm) and provides average values for the fractal dimension that can be derived from each single sample. SAXS also provides the opportunity for estimation of the surface fractal dimension which is not feasible using rheology. On the other hand, there are some disadvantages also exhibited by these two methods. Specifically, carrying out rheological experiments on low concentration samples (<2% w/v) is difficult due to the prolonged heating (in-situ gelation), since the gels’ viscosity is low and hence, the data produced are noisy. It also seems that it is more difficult using SAXS compared with rheology to estimate fractal dimensions for hydrogels with high concentrations. This happens, because it is difficult to see if the scattering comes from individual rod-like (fibrils) particles or from a bundle of particles.

Another interesting point derived from my SAXS study is the thermodynamic (entropy) behaviour of the β-lactoglobulin hydrogels. Until now most of the studies [1-10, 20] in the literature are focused on the structural changes within the protein networks. My study goes one step beyond this moving from the study of structural changes within protein systems to the study of thermodynamics (entropic changes) within these systems. Consideration of the SAXS data was based on the conceptual framework of Onsager’s [21] study for the I-N phase transition for self-assembled hard rod systems here, in order to interpret the kinetics of the self-assembly process for protein fibril hydrogels on the mesoscale. Onsager [21] reported that this phase transition is caused by the competition between the orientational entropy which favours the isotropic phase and the translational entropy (excluded volume entropy) which favours the nematic phase.

Thermal fluctuations contribute as a source for elasticity in semiflexible biopolymer networks and forces applied on them can be restored due to entropy [22], in contrast to rigid biopolymer networks where forces applied can be mechanically restored. Under these circumstances, I suggest, based on my SAXS investigation, that as the free volume decreases in self-assembled semiflexible fibril gels, the entropy becomes less important and it is more favourable for the fibrils to stick together and to self-
assemble (Fig. 5.2). Taking into account that the fragmented-fibril particle networks formed at pH 0.9 have the least free volume (§3.4.3, Table 3.2c) compared with the fibril networks formed at pH 2.3 and 2.0 (§3.4.3, Table 3.2a, b), I suggest that the more rod-like the network, the more favourable it is for the system to organize into the nematic phase. While the anisotropy might be qualitatively discerned from images, it is clear that in concentrated solutions the inter-fibril interactions trap the system away from the equilibrium phase.

**How fibril properties determine architecture**
1) less free volume or stiffer fibrils=> entropy is of less importance=> favours fibril self-assembly
2) more rod-like particles=> favours nematic phase (fibril orientation in one direction) formation

3) less rod-like or more flexible particles=> favours the isotropic phase (random orientation)=> higher degree of multiple inter-fibril connections
4)↑ orientational entropy & isotropic phase=> β-sheet planes stack laterally to the fibril axis=> fibril diameter increases
5)↑ transitional entropy & nematic phase=> β-sheet planes stack perpendicularly to the fibril axis=> fibril length increases

**How fibril properties relate to rheology**
1) ↑Js => stiffer gels
2) apply strain to rigid fibrils=> breakage

**How molecular properties affect the fibrils**
1) less interparticle interactions=> stiffens fibrils → I_p (molecular to fibril scale)

**Fig. 5.2** Summary of the impact of orientational and transitional entropy, inter-fibril forces, and thermal bending fluctuations on the structural and kinetic characteristics of β-lactoglobulin fibril networks on the mesoscale.

Based on my cryo-SEM and SAXS investigation, I claim that the orientational versus the translational entropy competition as the free volume varies in self-assembled semiflexible fibril gels could be connected with the degree of aggregation (mass fractal, §3.4.2, Table 3.1). I suggest, that the more free volume decreases, the more favourable it is for semiflexible fibril self-assemblies to exhibit a higher degree of aggregation, like the hydrogels formed at pH 0.9.
Electrostatic interactions and entropy changes also have an impact on self-assembly forces, as it is noticed from the cryo-SEM investigation (§2.7). As the pH drops, the repulsive electrostatic interactions decrease and the protein system becomes less rod-like. The more rod-like the protein system is, the more favourable it is to organize the system into a nematic-like phase (non-random fibril orientation). In other words, as the free volume decreases, the orientational entropy increases against the translational entropy which favours the isotropic phase (random fibril orientation) and hence, the fibrils stick together and undergo self-assemble. In the latter case parallel β-sheet planes stack laterally [23] to the fibril axis with a small possibility of arrangement of the planes along the fibril axis (Fig. 2.11). This lateral β-sheet plane stacking results in the increase of fibril thickness. On the other hand, as the transitional entropy and nematic phase (fibril orientation in one direction) become more important, the more rod-like and thinner fibrils are more favourable to organize in parallel β-sheet plane stacks perpendicular [24-25] to the fibril axis (Fig. 5.2). This perpendicular β-sheet plane stacking results in the increase of fibril elongation. This phenomenon and/or the presence of protein degradation could be a mechanism for the reduction of the fibril-like particle length scales and allow interpretation of the fibril changes upon pH variation from 2.3 to 0.9 in my study.

Rheology is used in this work for the mechanical characterization (G’ and G” estimation, §4.5, Table 4.1c) of β-lactoglobulin hydrogels on a macroscopic level (>mm) which is not possible for the other two techniques (cryo-SEM and SAXS) used here. Based on the fibril characteristics determined by cryo-SEM and SAXS, how the fibril network architecture is related with the macroscopic properties was investigated. Specifically, it was found that hydrogels at pH 2.0 exhibit the stiffest macroscopic behaviour which is slightly stiffer than the hydrogel networks formed at pH 2.0. On the other hand, hydrogel networks formed at pH 0.9 exhibit the softest macroscopic behaviour. Based on my cryo-SEM investigation, it should be expected that the hydrogels formed at pH 2.3 should exhibit the least stiff macroscopic behaviour and the hydrogels formed at pH 0.9 the stiffest, because the fibrils formed at pH 2.3 exhibit the longest fibril lengths, while the fragmented-particle fibrils at pH 0.9 exhibit the shortest fibril lengths. This means that pH 2.3 hydrogels should form fibril
networks with the smallest number of cross-links, while the pH 0.9 hydrogels should form fragmented-fibril networks with the largest number of cross-links. While my experimental results for hydrogels formed at pH 2.3 agree with my theoretical assumption based on the cryo-SEM investigation, the experimental results for hydrogels formed at pH 0.9 do not agree with my assumption. This possibly is attributed to the thick fibrils and small inter-fibril distances which cause the fibrils to "open" up the pores. This shows that the characteristics of individual fibrils could result in a macroscopic behaviour of the whole fibril network that can be completely different from the individual fibril properties. This results from the self-assembly processes that occur on the mesoscale; the scale between the macroscale (macroscopic behaviour of fibril networks) and the microscale (behaviour of individual fibrils).

Additional information provided by rheology that the other two techniques (cryo-SEM and SAXS) cannot provide is the gelation time for each hydrogel. It is shown that the hydrogels formed at pH 2.3 exhibit the slowest gelation process, while the hydrogels formed at pH 0.9 exhibit the fastest gelation process. This is attributed to the number of cross-links and the electrostatic interactions. Specifically, the hydrogels formed at pH 0.9 exhibit the largest number of cross-links and it seems that the repulsion electrostatic interactions decrease and possibly the hydrophobic interactions increase so that it is more favourable for the fragmented fibrils to stick together and self-assemble. This explanation also agrees with the explanation described above based on the entropy changes derived from the SAXS investigation (as the free volume decreases in self-assembled semiflexible fibril gels, the entropy becomes less important and it is more favourable for the fibrils to stick together and to self-assemble).

It should also be highlighted that $G''$, for fibril networks formed at pH 2.0 and pH 0.9 (Fig. 4.5b-c), begins to increase slightly at high frequencies, while $G'$ for fibril networks formed at pH 0.9 begins to slightly decrease at high frequencies. The use of microrheology would be an ideal option in this case, where the recording of $G'$ and $G''$ values at high frequencies (>100 rad/sec) can be performed. Specifically, Corrigan
and Donald [26] have used particle tracking microrheology (PMT) for the investigation of β-lactoglobulin fibril networks with concentration 3% w/v formed at pH 2.0. This technique is based on tracking the motion of micron-sized particles and compared with the bulk rheology, used in this study, allows the investigation of fibril networks without the need of application of oscillatory shear force which can disrupt the network structure. On the other hand, it allows the investigation only of low concentration hydrogels. Using this technique, frequency ranges up to 2000 rad/s (shorter-time regimes) can be investigated compared with bulk rheology and hence, the area around the gelation point (gelation time) for low concentration fibril hydrogels can be investigated with higher accuracy.

All in all, while most studies to date refer to the structural investigation of β-lactoglobulin fibril systems on a macroscopic or microscopic level, in this work I address the factors that control the self-assembly process which occurs on the mesoscale and determines the architecture and the macroscopic properties of protein fibril hydrogels. Specifically, I address that the thermodynamics (entropy), chain stiffness (entropic elasticity) and thickness, electrostatic interactions, inter-fibril distances, orientation of the β-sheet stacks, and the number of cross-links constitute the basic factors on the mesoscale that affect the self-assembly process of the fibril networks and connect their architecture with their macroscopic properties. This is the reason why macroscopic properties cannot be determined based only on the characteristics of individual fibrils.

Since the characteristics of individual fibrils do not immediately define the macroscopic properties of hydrogels, the self assembly process constitutes the crucial "key" point for the industry to manipulate, in order to create biomaterials with different novel macroscopic properties using biomolecules already used in the industry and trusted by the market.
In this study WPI hydrogel systems with β-lactoglobulin hydrogel systems are also compared. WPI systems are widely used in industrial applications in contrast to β-lactoglobulin systems which are more expensive. Here, it was found that the properties of purified β-lactoglobulin hydrogels do not exhibit significant differences compared with the properties of WPI hydrogels at pH 2. Hence, it may be possible for industry to use my β-lactoglobulin study, in order to manipulate and define WPI biomaterials.

The novel system developed during my study and of potential interest for industrial applications is β-lactoglobulin hydrogels formed at pH 0.9. These hydrogels, compared to those formed at pH 2.0 and pH 2.3, exhibit completely different behaviour, as presented in Table 5.1. Specifically, pH 0.9 hydrogels exhibit short and rigid fibrils with β-sheet stacks parallel to the fibril axis which result in soft gels with the fastest gelation time and the strongest inter-particle interactions. On the other hand, pH 2.3 and 2 hydrogels exhibit long and flexible fibrils with β-sheet stacks perpendicular to the fibril axis that yields stiff gels with slow gelation time. From the perspective of thermodynamics (entropy), it is known that the larger the Porod volume (excluded volume of the hydrated particles) [27], the lower the translational entropy (nematic phase). According to Onsager [28], the I-N transition of fluids consisting of monodisperse rigid rods is attributed to the competition between the orientational (isotropic phase) and translational (nematic phase) entropy of the rods where above a critical concentration the translational entropy dominates over the orientational entropy. Here, the pH 0.9 hydrogels present larger Porod volume compared with the pH 2.3 and pH 2.0 hydrogels hence, the possibility of I-N transition within pH 0.9 hydrogels is lower. It is suggested that the more rod-like the protein system is, the more favourable it is to organize the system into a nematic-like phase. In other words, as the free volume decreases, the orientational entropy increases against the translational entropy which favours the isotropic phase and hence, the fibrils stick together and undergo self-assemble.
Table 5.1 Characteristics of hydrogels formed at pH 2.3, 2.0, and 0.9.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hydrogels at pH 2.3/2.0</th>
<th>Hydrogels at pH 0.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril length</td>
<td>long</td>
<td>short</td>
</tr>
<tr>
<td>Fibril flexibility/rigidity</td>
<td>flexible</td>
<td>rigid</td>
</tr>
<tr>
<td>β-sheet strands orientation</td>
<td>perpendicular to fibril axis</td>
<td>parallel to fibril axis</td>
</tr>
<tr>
<td>Inter-particle interactions</td>
<td>repulsive interactions</td>
<td>reduction of repulsive interactions/increase of hydrophobic interactions</td>
</tr>
<tr>
<td>Gelation time</td>
<td>slow</td>
<td>fast</td>
</tr>
<tr>
<td>Network elasticity</td>
<td>stiff</td>
<td>soft</td>
</tr>
<tr>
<td>Thermodynamics (entropy)</td>
<td>strong nematic phase/high possibility for I-N transition</td>
<td>strong isotropic phase/low possibility for I-N phase</td>
</tr>
</tbody>
</table>

Moving beyond that described here it is highlighted that there are many additional areas for further research. The mechanical properties of single fibrils constitute one of these fields that need further investigation. There are only a few studies in the literature [29-32] focused on the mechanical properties of single-fibrils. Optical tweezers (OT) constitute one of the experimental tools that could accelerate such an investigation [30]. OT are able to measure tiny forces of the order of pN that are applied on single filaments and their displacements with sub-nanometre accuracy and sub-millisecond time resolution. Although OT are an innovative experimental tool, sophisticated optical preparation is required, in order for the laser to generate a force of pN [30]. This constitutes one of the major limitations of this tool that constitutes a challenge to be overcome.

Another research field that requires further investigation is the field of simulation models. So far only a few simulation models have been developed, like Usov and
Mezzenga model [33] and Meakin and Jullien model [34] that allow the determination of structural parameters, like fibril size, fibril mesh length, fractal dimension, etc., of fibril biopolymer networks. On the other hand, these models do not allow prediction of the macroscopic properties of such networks based on the single-fibril mechanical properties from which they are constituted. The development of simulation models that could predict the macroscopic properties of assembled networks using parameters defined by single-fibril (OT) study could constitute a novel simulation model within the field of biopolymer networks. This kind of simulation model could contribute to the experimental investigation of fibril biopolymer networks, like that presented in this dissertation, by using cryo-SEM, SAXS, and rheology, in order to investigate further the relationship between the biopolymer network architecture and their macroscopic properties.

Lastly, I strongly believe that in this way physical sciences, including my work can contribute to New Zealand agricultural food sectors by changing the way in which soft materials and biopolymer engineering is performed and by taking biomaterials from commodities to specialties by adding knowledge. Generally speaking, gels are commonly used in everyday life, and they have a wide range of applications. Gel systems could contribute to human health improvement by allowing functional foods to be formulated, such as aerated gels already in the market which increase the feeling of satiety [35].
References


Appendix A. Cryo-Scanning Electron Microscopy Experimental Instrumentation

The cryo-SEM instrumentation presented here was used for the current dissertation in the School of Chemical and Physical Sciences at VUW, NZ.

Fig. A1 a) Gatan Alto 2500 cryo-unit fitted onto the b) JEOL JSM-6500F Field Emission Scanning Electron Microscope.
**Fig. A2** Sample holders for cryogenic sample preparation: a) stub with 1 mm wide slot [1] and b) two copper plates (specimen sandwich).

**Fig. A3** Freezing methods for cryo-SEM: a) slushy nitrogen and b) propane get freezing method [2].
References


Appendix B. Australian Synchrotron Small-Angle X-ray Scattering Experimental Instrumentation

Fig. B1 a) Australian synchrotron SAXS/WAXS experimental setup [1] and b) moving Dectris-Pilatus 1M detector.
Fig. B2 a) Stainless steel sample holder plates (316 SST) of thickness 2 mm appropriate for low pH conditions covered with Mica windows used for the heat treated gels. Mica windows were secured in place with Scotch duct green and black tape. b) Protein solutions were loaded into capillaries placed in temperature controlled sample mount.

Reference

[1] Australian Synchrotron SASX/WAXS.

Appendix C. Rheology Experimental Instrumentation

Fig. C1 a) Stress-controlled AR 2000 rheometer. b) Silicone oil was applied in the geometry’s solvent trap, in order to prevent evaporation. A solvent trap cover was also placed over the sample with its edges dipped into the silicone oil, in order to form a better vapour barrier.