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Massey University, New Zealand

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The MacDiarmid Institute for Advanced Materials and Technology

Microrheological investigations of biopolymer networks

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PhD Thesis

Research conducted at the Institute of Fundamental Sciences, Massey University of Palmerston North, New Zealand

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Co-supervision by: **Dr. Yacine Hemar**

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Abbreviations:

MPT	Multiple Particle Tracking
DWS	Diffusing Wave Spectroscopy
R_{eff}	Ratio of the $[Ca^{2+}]$ quantity over the quantity of the acidic PGA residues which can effectively bind calcium $[COO^-]_{blocks}$
HG	Homogalacturonan
DM	Degree of Methyl-esterification
HM pectin	High-Methoxy pectin
LM pectin	Low-Methoxy pectin
DB	Degree of Blockiness
DB_{abs}	Absolute Degree of Blockiness
RGI	RhamnoGalacturonan I
RGII	RhamnoGalacturonan I
AFM	Atomic Force Microscopy
PME	Pectin Methyl-Esterase
f-PME	Fungal Pectin Methyl-Esterase
p-PME	Plant Pectin Methyl-Esterase
NMR	Nuclear Magnetic Resonance
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
PL	Pectin Lyase
PG	PolyGalacturonase
m_{pectin}	Mass of pectin
$m_{uronic\ acid}$	Mass of the charged galacturonic residues

M_W^{GalA}	Molecular weight of the galacturonic residues
C_p	Polymer concentration
L_p	Persistence length
PGA	PolyGalacturonic Acid
R	Ratio [Ca^{2+}] quantity over the total quantity of the acidic PGA
EBSD	Electron Backscattering Diffraction
σ	Stress
G	Shear modulus
γ	Strain
$\dot{\gamma}$	Strain rate
η	Visocosity
ω	Frequency
σ_0	Stress amplitude
γ_0	Strain amplitude
δ	Out of phase angle of the stress
$G^*(\omega)$	Complex viscoelastic modulus
$G'(\omega)$	Elastic modulus
$G''(\omega)$	Viscous modulus
HF	High frequencies
$\hat{G}(s)$	Laplace transform of the shear modulus
MR	Microrheology
k_B	Boltzmann constant
T	Temperature
$\left\langle \hat{\Delta} r^2(\tau) \right\rangle$	Laplace transform of the MSD

a	Brownain particle radius
DLS	Dynamic Light Scattering
PEG	PolyEthylene Glycol
PEO	PolyEthylene Oxyde
$\langle \Delta r^2(\tau) \rangle$	Mean Square Displacement
τ	Time lag
$g_1(\tau)$	Field autocorrelation function
$g_2(\tau)$	Intensity autocorrelation function
l*	Light mean free path
z_0	Penetration depth
L	DWS sample thickness
$r_\alpha(t)$	Position of the α particle at time t
$D_{rr}(t,\tau)$	Correlated diffusion coefficient
$D_{r\,R}^{\,i}(t,\tau)$	Displacement of the i particle during τ
$D_{r\,R}^{\,j}(t,\tau)$	Displacement of the j particle during τ
R	Distance between 2 particles
$\langle \Delta r^2(\tau) \rangle_{TPMR}$	MSD for the Two-Point MicroRheology

Abstract

Pectin is a major polysaccharide of the plant cell wall which is known to play a role in many mechanical functionalities, especially when a gel is formed in the presence of calcium. Understanding the gelling abilities of pectin is of great interest to the food industry also, since pectin is a widely used as a gelling agent and thickener. The aim of this study was to apply two complementary microrheological techniques to these systems, multiple particle tracking (MPT) and a light scattering technique called diffusing wave spectroscopy (DWS). While the first one provides fundamental information about the homogeneity of the studied gel, the second gives access to the high frequency behaviour, related to the nature of the basic strands of the network.

Firstly, after verifying the validity of the experimental apparati and analysis approaches in a series of careful control experiments on archetypal systems, a regime where pectin gels exhibit the signatures of semi-flexible networks was identified in experiments carried out on gels made of pectin chains pre-engineered by enzymatic deesterification and subsequently assembled with the release of Ca^{2+}. These results were the first showing that polysaccharides networks could be accommodated within the framework of semi-flexible networks, which have become a paradigm for biological gels, such as the well-known F-actin solutions present in the cell cytoskeleton.

However, in the plant cell wall, where calcium is already present, the assembly mechanism could be controlled in a different manner, and a more biologically relevant system was studied where the action of the plant enzyme pectinmethylesterase was used to liberate ion-binding groups in the presence of Ca^{2+}. Gels formed according to this alternative methodology were found to behave as punctually cross-linked flexible networks, strikingly different from the first results. This would be explained by the presence of short blocks of charged residues.

Finally, experiments on pectins carried out with controlled blocky structures showed that a pectin made of short blocks can exhibit both sorts of network, depending on the polymer and Ca^{2+} concentrations. This lead naturally to the construction of a state diagram for the regimes of assembly, with proposed control parameters being the polymer concentration and the ratio of the amount of Ca^{2+} to the quantity of pectic residues which can effectively bind the calcium into cross-links, christened R_{eff}.

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Contents

Chapter I - Background

1. Introduction

'Biopolymers are a class of polymers produced by living organisms' says the Wikipedia definition. They are indeed produced by living organisms, and they provide all the necessary structures and functions of life. Starch, proteins and peptides, DNA, and RNA are all examples of biopolymers, in which the monomer units, respectively, are sugars, amino acids, and nucleic acids respectively. The exact chemical composition and the sequence in which these units are arranged is called the primary structure. Many biopolymers spontaneously fold into characteristic compact shapes (as for example protein folding), which determine their biological functions and depend in a complicated way on their primary structure. The self-assembly of biopolymers in gels and tissues is determined by their fine structure as well as the different environmental conditions (pH, ionic strength…), and controls the mechanical properties of living materials, providing them strength or softness depending of the required functionality. Most mechanical studies on biological materials have been carried out on proteins from animal's cells, while very little has been done about plants [1], especially about the polysaccharides networks which are making the plant cell wall. One of these polysaccharides is called pectin, and is playing a ubiquitous role in many plant cell functionalities.

'Pectin is all around us' says Willats in [2]. Obviously such an affirmation can only come from a member of the small but dynamic pectin community, and saying pectin is an important biopolymer in the plant world will be closer to the truth. It is primarily found in the plant cell wall, where it plays a major role in many of the plant cell functionalities, providing for example stiffness to the cell wall, softness at the tip of the growing pollen tube [3], or inter-cellular adhesion [4, 5]. These smart mechanical properties are possible only due to the high degree of control of the polymeric fine structures. Such fine structure variability can result in different self-assembled polymeric structures in the presence of its main binding agent, calcium cations Ca^{2+}, and therefore the required mechanical properties.

Such utility in the use of a single polymeric component suggests that understanding its use in nature would lead to many interesting biomimetic applications. Pectin is historically known from jam makers for its gelling properties, which are still of an important industrial utility. As a healthy product, it has since obtained a lot interest from the food industry as a protein stabilizer in acid milk drinks for example [6]. Recently, a lot of effort has been done on using pectin as an encapsulation material, to

release drugs or flavours [7, 8]. On a medicinal point of view, pectin is used against constipation and diarrhoea [9]. Very recently, a molecule derived from citrus pectin has been identified as an apoptosis molecule for human cancer cells, even for the androgen-insensitive cells such as prostate cancer cells [10] and multiple myeloma which has developed a multidrug resistance [11].

The chapter that follows is organized as an oriented literature review which will provide an overview of the required knowledge necessary to understand this thesis, as well as motivating this study. It does not intend to be exhaustive, and the specific physics theories used to interpret the experiments or in the analysis of the results will be described more precisely in the respective chapters.

2. Pectin primary structure

Pectin gelling abilities have been used for a long time. The Romans learned from the Greeks that quinces, a pectin rich fruit, slowly cooked with honey would "set" when cooled, though they did not know about pectin. The Roman cookbook attributed to Apicius (4^{th} century AD, [12]) gives a recipe for preserving whole quinces with their stems and leaves attached in a bath of honey diluted with *defrutum*, otherwise called Roman marmalade.

Pectin was first isolated and described by the biologist Henry Braconnot. In his 1825 communication [13], Braconnot reported about an acidic substance he found in all the plants, which was gelling when mixed with sugar. He gave it the name of pectin from the Greek word *Pectis* which means *coagulum*, and he expressed his beliefs both that “it will have many applications in the art of the confiseur” and that pectin is playing an important role in the plant functions. Since then research on pectin has more than validated these two predictions, and even went beyond by adding to pectin a garment of gelling and stabilisation abilities, as well as medicinal functionalities for the treatment of many diseases [14-16]. These varied qualities are possible only due to pectin’s large fine structure variability.

Basic structure

Pectic polysaccharides are assembled in the Golgi apparatus by glycosyl transferases that catalyze the transfer of glycosyl residues from nucleotide sugars to acceptor molecules. The polymers are subsequently transported in vesicles that fuse with the plasma membrane, and are then released and integrated into the pre-existing cell wall. In contrast, cellulose is synthesized by protein complexes located in the plasma

membrane. Cell wall polysaccharides may be modified as the cell grows and develops.

Three different pectic components have been isolated from the plant cell wall: homogalacturonan, rhamnogalacturonan I and II. Homogalacturonan (HG) is a linear copolymer composed of 1,4-linked α-D-galactosyluronic acid residues with its methylated counterpart. HG may account for up to 60% of the pectin in a primary wall, and some 90% of extracted commercially available samples. The relative quantity of methyl-esterified residues is called Degree of Methyl-esterification (DM), and conventionally High Methyl-Esterified pectins (HM-pectins) will have a DM larger than 50%, while Low Methyl-Esterified pectins (LM-pectins) will have a DM smaller than 50%.

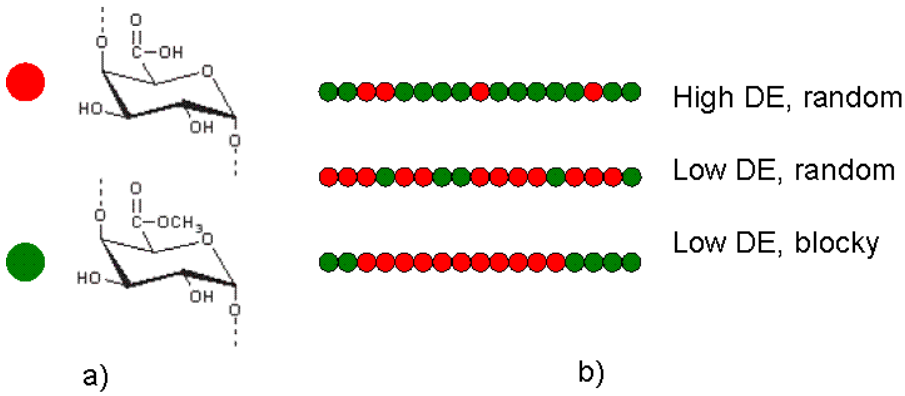

Figure1: a) Homogalacturanan is composed of α-D-galactosyluronic acid residues and its methylated counterpart – b) Pectin has a large variety of possible fine structures

In the plan cell wall, one of the main ion is the divalent cations Ca^{2+} which is supposed to play a wide range of functions. Notably, it is a favoured binding agent of pectins, and a gel will form when Ca^{2+} is release in a pectin solution, assuming the used polymers are sufficiently charged. Understandably, the DM (related to the number of available binding zones) is strongly linked with the gelling properties of pectin. Another quantity called Degree of Blockiness (DB) has been evocated in [17], quantifying if the pectin pattern is made of long strings of acidic residues or if these later are randomly distributed. A change in DB is linked to important changes in the ionic binding properties of pectins. But even if the DB is a good qualitative indication of the blockiness, it has no quantitative value in determining the gelling abilities of pectin. Thus, the term of DB will not be used in this study, and the pattern of the used pectins will be described literally for the different pectins.

As mentioned previously, two other pectic molecules are present in the plant cell wall [2]. Rhamongalacturan I (RGI) backbone consists of the repeating disaccharide galacturonic acid – rhamnose, to which a variety of sides chains are attached to the rhamnose residues, these side chains being made mostly of arabinan and galactan residues. The confusingly named Rhamongalacturan II (RGII) is made of HG backbone, with complex side chains attached to the galacturonic acid residues, these side chains being made of 11 different mono-saccharides. For a long time, it was accepted that the HG and RG domains where making the pectic polymer backbone, but recently an alternative structure has been proposed in [18], where HG is a long side chain of RGI as depicted in figure 2 - B.

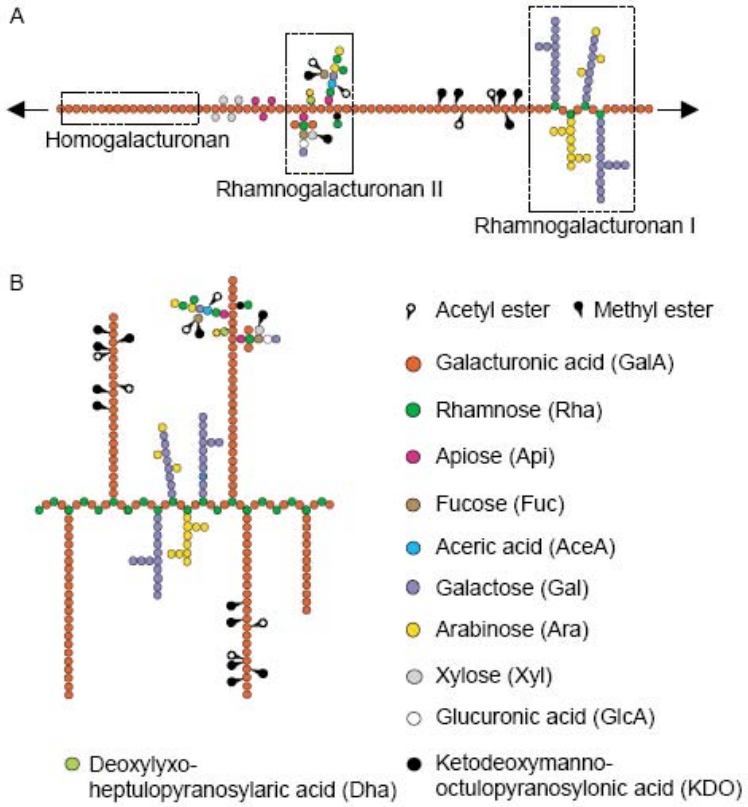

Figure 2: The basic structure of pectin, from [2]. A) is the conventional structure of pectin, while B) represents the alternative model proposed by [18].

The pectin macromolecule is arguably one of the most complex polysaccharide, if not the "most complex polymer in nature" [18]. Its functionalities are strongly related to this complex structure, which can vary largely inside a plant species as well as between species. In order to render a physical approach viable, only the HG domain will be studied here as it is often the case in food science. This approach is further motivated by the fact that the many biological functionalities are linked to the HG domain [3, 4], whereas no functionality has been clearly assigned to the RG domain yet [19, 20].

Synthesis and fine structure modification

Extraction from the plant

Three steps are required to extract pectins from the plants: aqueous extraction from the plant material, and purification and isolation of the extracted pectin from the solution. Although the two last steps are important in their own right, the first step plays the most important role for the extracted product and has the most variability in its process. The classical way for the food industry is to perform the operation in hot mineral acid (70-80 Celcius, pH = 2) which separates pectin from the other plant polymers [21]. This step tends to reduce the quantity of neutral sugars of the extracted product, this later being composed mostly of linear HG domain. This is probably the most controlled/simplest structure which has the most well-known gelling abilities, and subsequently is the basis of all the commercial pectins. It is also the best model system for studying the structure-properties relationship of the pectin gels as the fine structure characteristics are tractable. A full understanding of the structure inside the plant cell wall will require softer ways of extraction and subsequent analysis, for example by AFM [22] or by chromatography [23].

Modification of the fine structure

Pectin extracted from apple or citrus typically have a DM value of 75-80%. As its gelling behaviour as well as other functionalities are strongly linked to its fine structure, pectin's structure is often modified and tuned regarding both the DM and the pattern of the acidic residues. In that aim, pectins can be deesterified by different ways leading to different patterns. Chemical methods include mostly working under alkali conditions where the methyl groups can be liberated, while biochemical tools implies the use of enzymes extracted from the plant cell itself or from bacterial or fungal pathogens, the Pectin MethylEsterases (PMEs). The inverse process is also possible using chemical methods, the methyl-esterification being usually carried out under acidic conditions (Sulphuric acid or organic acids) and in the presence of methanol [24]. By playing with these different techniques, one can imagine to engineer a huge variety of pectin patterns.

In the plants, pectin is synthesized with a DM of 75-80% [25], and subsequently de-methylesterified in the cell wall by PMEs, in order to change its assembly properties to allow the required physiological changes. Their reaction with pectin has been classified on the basis of the three traditional modes of action [26]: the single-chain or proccessive mechanism is when the enzyme converts all the consecutive residues after binding to its substrate, while the multiple-chain mechanism means the enzyme

converts only one residue before dissociating from its substrate, and the multiple-attack mechanism describes when the enzyme transforms a limited number of residues. Until recently, the fungal PME (f-PME) has been characterised with a multiple-chain mode of action, and the plants PMEs (p-PME) with a proccessive one. While the first one induces a random deesterification of the pectins, the second one is leading to pectins with a highly blocky distribution of their galacturonic acid residues, which has been reported for p-PMEs from apple [27], orange peel [28] or tomato [29], all under neutral conditions. But this mode of action is currently questioned for the p-PME [30, 31]. A new and intermediate mode of action has been proposed: a multi-attacks mechanism is followed by a proccessive phase, where the p-PME attacks preferentially on a former zone of action. Thus, for low decrease of DM, the pattern would be made of randomly distributed short blocks of acidic residues, when for a higher deesterification long blocks would be formed. This has been called variable processivity in [31]. According to NMR studies [32], the length of the initial short blocks is on average 10 residues.

The temperature, pH and salts all play an important role on the activity of these enzymes. Not only the speed but also the mode of action can be strongly affected, as found in [27] where p-PME working at pH 4.5 instead of its ‘optimal’ of pH 7 provides a pattern made of short blocks of about 30 acidic residues (resolved by NMR).

Fine structure characterisation

Pectins gelling abilities, medicinal functions and *in-vivo* functionalities are strongly linked to the basic structure of the used pectin. Consequently, precise analytical methods are needed to obtain precisely the fine structure of the linear HG chains, and quantify the DM as well as describing the pattern of the acidic residues. Ideally, polysaccharides sequencing will be possible as for the proteins, but despite of some recent publications on the subject [33, 34], and despite being the dream of many plant biologists, such sequencing is very challenging for many reasons, the main one being the intrinsic polydispersity of polysaccharides. Nevertheless, in the last decade, a handful of molecular tools have been developed for pectins structural analysis, leading to some tremendous advances on its basic structure understanding and its structure-function relationship in the plant cell wall. Industrially, DMs of commercial pectins are determined by base titration. The average DM has been determined as well by chemical methods [35], gas chromatography [36], Fourier transform infra-red spectroscopy [37], Capillary Electrophoresis [38], and Nuclear Magnetic Resonance (NMR) spectroscopy [39], this later giving some information on the pattern at the

same time. But all these techniques lack to give information about how these polygalacturonic acid residues are distributed, which is essential to understand better how the pectin chains are interacting with their cross-linking agent in order to from networks, and will give many insights in how the enzymes are processing to decrease the DM.

Anti-bodies

Monoclonal antibodies are antibodies that are identical because they are produced by one type of immune cell that are all clones of a single parent cell. Given (almost) any substance, it is possible to create monoclonal antibodies that specifically bind to that substance, and are used as a marker. They are widely used for pectin in plant science, because they allow defined structural patterns to be precisely localized in the context of the intact plant cell wall. They started recently to be used in the food industry in order to probe the pectin location *in-situ* in complex heterogeneous structures. Antibodies can be used as markers for fluorescent microscopy as well as for electron microscopy techniques TEM and SEM. A few antibodies are now well characterized, and can probe specifically different pectin patterns in the HG domains, from partially methyl-esterified chains (LM7, JIM5, JIM7, [40]) to long strands of acidic residues (PMA1, [41]), as well as calcium junctions zones (2F4, [42]). Many others can recognize sections specifically in the RG I or RG II domains. They are fantastic tools for the plant scientist for characterising the pectin *in-vivo*, but the limited number of fully characterised antibodies is still too small to obtain a very precise characterisation of the HG pattern.

Enzymatic fingerprinting

Enzymatic fingerprinting is a rapid and powerful means of analysing a specific polysaccharide component, in the plant cell wall or on a home-made gel. It involves the enzymatic digestion of pectin using lyase or polygalacturonase enzymes with well defined cleavage specificities. The subsequent analysis of the resulting oligomers can be made by different techniques, including chromatography or mass spectrometry [43, 44], allowing the determination of a Degree of Blockiness DB. Recently electrophoretic methods have been reported, including gel electrophoresis (PACE) [45] and Capillary Electrophoresis (CE) using UV detection of the oligomers [46]. This latest methods has been improved since [47] and has been revealed a very promising way of quantifying both the DM and the DB, as well as giving the intermolecular polydispersity [48]. This is the method which has been used to characterise all the commercial and home-made pectins used in this study.

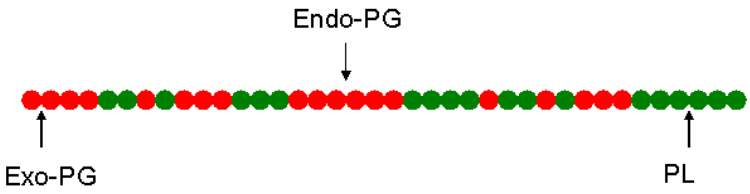

Figure 3: Attack sites of pectin degrading enzymes.

The digestion of the pectin chains can be made by different enzymes, such as pectin lyases (PL) or polygalacturonases (PG). While the first ones cleaves the HG backbone via beta-elimination in methyl-esterified regions, PGs cleave the pectin chain between un-esterified residues, with endo-PGs attacking at random places of the chain and exo-PGs cleaves from the reducing end (figure 3). EndoPGII has been extensively used to elucidate the intra-molecular distribution of the un-esterified residues [43, 48], due to its absolute requirement for the site of attack to be a polygalacturonic acid sugar, providing qualitatively similar digestion products for any pectin. The pattern and degree of polymerisation of the resulting pectic oligomers have been characterised by electrospray ionisation tandem mass spectroscopy. The main pectic oligomers are represented on figure 4.

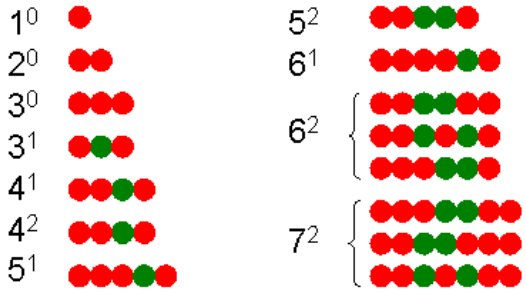

Figure 4: Remaining galacturonic oligomers from an endo-PGII digests of pectin. Small amounts of longer oligomers can be also detected [43]

A definition for the DB has first been first given in [17]. Pectin has been deesterified via three different ways (alkali, f-PME and p-PME), and found that the subsequent digestion of the p-PME deesterified pectins by Endo-PG produced a much larger amount of completely un-esterified oligomers compared to the f-PME and alkali deesterified pectins. As the p-PME is known to have a blocky action, contrary to the two others ways, they proposed the following definition for the DB:

$$DB = \frac{\left(1\times 1^0 + 2\times 2^0 + 3\times 3^0\right)\times M_w^{GalA}}{\left(1 - DM/100\right)\times m_{pectin}\times\left(m_{uronicacid}/m_{pectin}\right)}\times 100 \qquad (1.1)$$

where 1^0, 2^0, 3^0 are the molar amount of the monomer, dimer and trimer of galacturonic acid residues resulting from the action of endoPGII, M_w^{GalA} is the molar mass of the GalA residues, m_{pectin} the mass of digested pectin, and $m_{uronicacid}$ the fraction of uronic acid present in the pectin sample. In other words, the DB is the quantity the un-esterified GalA residues which is digestible by the endoPGII. An alternative degree of blockiness has been proposed in [49] and is related to the total amount of galacturonate residues, including the methyl-esterified ones. It is called absolute DB, and its expression is:

$$DB_{abs} = \frac{\left(1\times 1^0 + 2\times 2^0 + 3\times 3^0\right)\times M_w^{GalA}}{m_{pectin}\times\left(m_{uronicacid}/m_{pectin}\right)}\times 100 \qquad (1.2)$$

The difference between the DB and the DB_{abs} is especially noticeable for very blocky pectin.

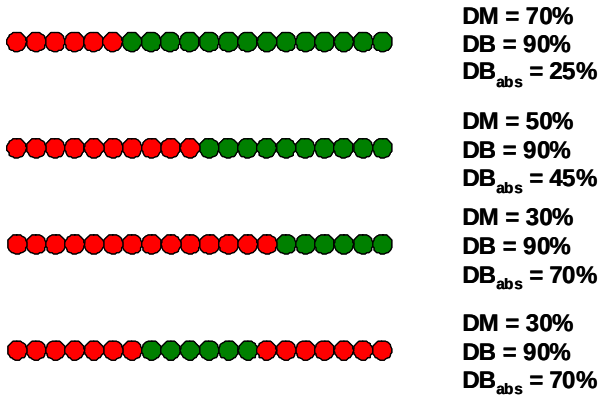

Figure 5: illustration of the differences between DB and DB_{abs} on blocky pectins

It has to be noticed that both the DB and the DB_{abs} of two blocky pectins with a similar DM but with different average block length will be the same (figure 5), and in that sense they lack of precision. Nevertheless, the presence and the importance of other oligomers than 1^0, 2^0, 3^0 provides further information. For example, the presence of short and partly esterified oligomers is a sign of a blocky structure made of short blocks. Important information could arise from the normalized percentage of the mono, di and trimers, as suggested by [50]. But unfortunately, the difference should be noticeable only for shorter blocks than eight residues as suggested by simulations [51]. In the present work we will describe qualitatively the digest patterns of the studied pectins, and link the results obtained from CE pre and post digestion with the micro-rhelogical results and the existing literature.

Pectin chains characteristics

Polyelectrolytes are polymers whose repeating units bear an electrolyte group. These groups will dissociate in aqueous solutions, making the polymers charged. Polyelectrolytes have thus properties similar to both electrolytes and polymers. Like salts, their solutions are electrically conductive. Like polymers, their solutions are visco-elastic. Charged molecular chains, commonly present in soft matter systems, play a fundamental role in determining structure, stability and the interactions of various molecular assemblies. Theoretical approaches to describe their statistical properties differ profoundly from those of their electrically neutral counterparts, while their unique properties are being exploited in a wide range of technological and industrial fields. Their major fundamental roles, however, seems to be the one played in biology and biochemistry.

Pectin is a weak polyelectrolyte with a pKa of about 3.5 (depending of the DM and degree of dissociation), and is not fully charged in solution. Its fractional charge can be modified by changing the solution pH, counterion concentration, or the ionic strength, and this charge influences the conformation of the polymer in aqueous solution: the pectin being more extented when charged, i.e. at pH above its pKa. Subsequently, the relative charge quantity (DM) and distribution will affect the persistence length as well. Depending on the author and the used method, pectin has been reported to behave from a rod-like polymer [52] to a semi-flexible coil of different stiffness [53, 54], with the most recent studies giving a persistence length Lp ranging from 6.7 to 9nm [55, 56], obtained by experimental viscosimetry. Some Monte Carlo studies confirmed these values for chains made of polygalacturonic acid residues [56].

Some authors have reported that the persistence length of the pectin chains could depend on the DM [58]. This hypothesis has been checked in [59] where the persistence length has been determined for pectins with a DM going from 28 to 73%. Viscosity measurements gave values ranging from 5.9 to 12.6nm, while small angle neutron scattering leaded to values ranging from 4.5 to 7.5nm. In the same paper, molecular dynamic simulation agreed well with the experiments, ending with Lp=13.5nm for HM pectins. More recently, a new simulation method [60] gave a persistence length of 10nm for uncharged chains and 30nm for pure polygalacturonic acid (PGA), fully charged. While the first value agrees very well with the experiments, the second could seem sensible due to the high present repulsion, but is unfortunately unverifiable experimentally, the PGA precipitating in water. Anyway, as a general conclusion, we can not consider pectin as semi-flexible rod like polymers

because it has not a huge persistence length, but they are definitely a bit semi-flexible with a persistence length of the order of 10 residues. We could refer pectin as a semi-flexible coil like polymer.

3. Calcium induced pectin gels – Structure and mechanical properties

Calcium induced gels and the egg box model

Due to its wide industrial applications, pectin gels have been studied and made via many different ways. Gels have been made from HM and LM pectin, from mixtures of both, from interaction with its cousin polysaccharide alginate, or from amidated pectins. While most of them were made by adding binding cations, it has been shown that acid induced gels could be made of LM pectins with a blocky pattern. Amongst all this jungle, comparably little is known about the structure-function relationship of these gels, even for the simplest structures.

Calcium cations Ca^{2+} are the main ions in the plants, where their interaction with the plant cell wall pectin governs the functionality of *in-vivo* gels. Ca^{2+} has been used a lot by the food industry as well, as being one of the most powerful pectic cross-linkers, surpassed only by other divalent ions such as copper or zinc. While this strong interaction with metallic ions is of important as a means to purify industrial waste water and detoxify living organisms from heavy metals [61], the use of these heavy ions is obviously not relevant for the food applications. The affinity of pectin for monovalent ions, such as potassium or sodium, has also been studied, and is generally not believed to be able to induce a gel. Nevertheless, blocky pectin gels have been shown to be influenced by the type of the monovalent ion used [62], and even some gels have been obtained recently using really low DM pectins with a highly blocky pattern [62, 63]. Acid induced gels have been made of the same kind of pectins [64, 65], where the gelation is imputed to weak hydrogen bonds.

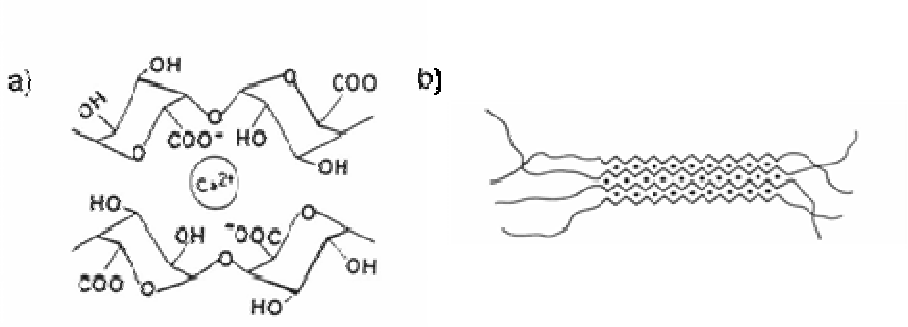

Figure 6: the egg-box model applied to the pectin gelation

We decided in the present thesis to limit the problem to the most studied binding ions, i.e. Ca^{2+}. The calcium-induced gelation of pectin has been described by the 'egg-box' model, which was originally described for the alginate gelation. Indeed X-ray diffraction data of desiccated alginate gels are indicating that the junction zones are formed from the pairing of 2/1 helical chains of guluronate sequences, providing cavities accommodating Ca^{2+} [66, 67]. As pectin can almost be seen as the image of alginate in a mirror, the same egg-box model has been accepted for the pectin gels as depicted in figure 6 [68-70], but its validity has not been corroborated by direct structural information [71]. A lot of similarities have been found between pectin and alginate for their calcium-binding abilities, as for example a cooperative effect in Ca^{2+} binding for both at chain length above 20 residues [71, 72]. And subsequently pectin and alginate have been modelled upon the same assumptions, such as the charge annihilation and reversal mechanism to interpret the binding of calcium [73], and a general theoretical framework has been used by [74] to describe the chain-chain association. But molecular dynamics simulations [71] pointed out significant differences: the 'egg-box' model was applicable to the calcium binding of alginate chains, but was not the most energetically favourable conformation for the association of pectin chains, the anti-parallel one being the most favourable. [75] proposed the name of the 'shifted egg-box' model for the description of calcium-induced pectin gels. However, this conformation still has to be verified experimentally.

Calcium-induced self-assemblies of pectin chains

It is believed that a minimum number of consecutive charged residues is required to from a stable calcium-junction zone. It has been found experimentally that this number is ranging from 8 [76] to 16 [77]. This is of a major importance for the gel formation, as shorter strands of acidic residues of the pectin chains will not be able to bind calcium with a stable fashion. Assuming this, one can easily guess that it will be easier to make a gel with a blocky distribution of the acidic group, and with pectins with a lower DM, but as we will see this is a subtle interplay between block length and number of blocks. It is widely accepted that it is not possible to make a calcium gel from HM pectins, i.e. DM > 50%, at least for random pectins. The minimum length to form a stable junction zone is about all what is known about the self-assemblies of pectin chains in gels.

Again we can draw a parallel between pectin and alginate. As for pectin, most of the literature on the calcium-induced gelation is relative to the semi-dilute regime, and could not conclude on the binding process of the alginate or pectin chains in presence of Ca^{2+}. Studying the dilute regime was necessary, and has been done on alginate

very recently in [78]. In this paper, by combining isothermal titration calorimetry, Ca^{2+} selective potentiometry and viscometry, it is concluded that there are three distinctive steps in the binding of calcium to alginate depending on the Ca^{2+} concentration. As shown on figure7, the first step (called monocomplexation) is the interaction of Ca^{2+} with a single charged residue, which is agreeing with the charge mechanism proposed for pectin and alginate in [73]. This initial step is followed by the formation of dimmers made of stable egg-box junction zones for higher calcium concentration, and subsequently lateral dimer association when Ca^{2+} is in excess. These two later steps are agreeing with some small angle X-rays scattering experiments performed by [69] on solutions of alginate with a slow release of Ca^{2+}, where an initial egg-box dimers association was found, followed by a lateral association for higher calcium concentrations.

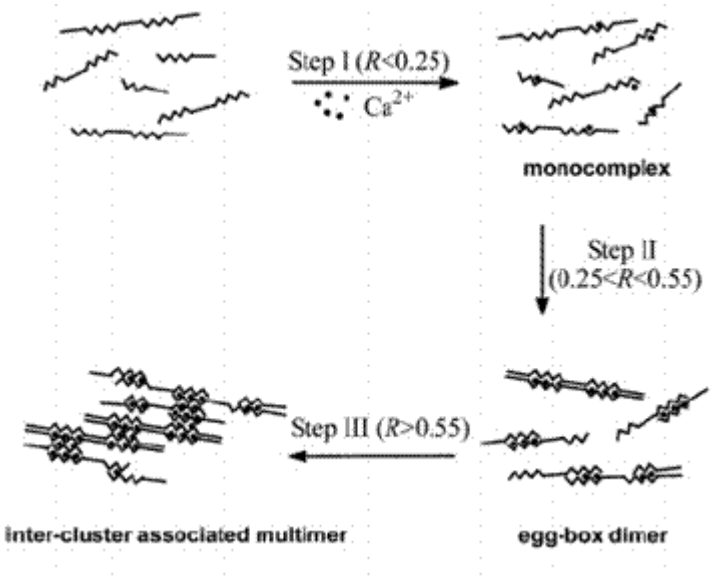

Fig.7: multiple-steps of the binding of calcium to alginate (from [78])

The same people applied the same methodology to pectin in order to compare its calcium binding processes with the ones of alginate [74], but unfortunately they compared alginate with amidated pectins which have some positively charged residues, and thus have intrinsically different polyelectrolytic properties. Their main and obvious conclusion is that the observed different behaviours are due to the fact that the used alginate has a blocky distribution of its charged residues, the pectins being the random kind. We would hypothesise that with a non-amidated and blocky pectin the observed behaviour might be similar to that of alginate. However, the experimental approach is very interesting for the study of ionic binding to charged

biopolymers, which is of importance to understand the network formation in ionic gels.

Microscopy techniques

A more direct way to understand how networks are organized is to simply image them by microscopy. Actually, some model biopolymeric systems have been selected on the ability to visually describe precisely the properties of their constitutive filaments and how they self-assemble in networks. One such system is F-actin solution whose huge and rigid proteinous filaments can be seen by fluorescent microscopy, rendering them the best model system for proving the recently developed theories for semi-flexible networks [79, 80].

For polysaccharide gels, the network filaments are much smaller and less easy to mark for imaging purposes. In fact, very high magnification is necessary, and most of the published results on polysaccharide are done with electron microscopes TEM or SEM, or by atomic force microscopy (AFM). These techniques have been mainly used on polysaccharide systems by the structural plant biologist in order to understand the complex structure of the plant cell wall, and require a complex sample preparation which could alter the network characteristics. They have been translated to the study of polysaccharides gels for food applications, and there are a few papers dealing with the structure of *in-vitro* calcium induced networks.

The first AFM images of a pectin network have been done by [81], where the image of a pectin solution under butanol on mica is shown. Of course this as little relevance for the aqueous pectin solution as the solvent is rather different, but the resolution of the images demonstrates the ability of AFM to see pectin and pectin networks at the molecular scale. Although its potential for probing networks, most of the AFM work on pectin has been done for single molecule imaging, such as in [81] where it is possible to asset the nature of the side branches of pectin chains or as in [82] where the adsorption and the desorption of a single molecule is studied. To our knowledge, only one study has been done about calcium induced gels [83], where they found that for low Ca^{2+} concentration a gel was forming, and saw a second step when they increased this concentration, resulting in a secondary aggregation. This can obviously be linked with the results found by [78] as discussed in I-2, where this second aggregation would correspond to the third step of calcium binding, i.e. a lateral aggregation. Furthermore, they analysed the enzyme digested product of pectins and were able to see the digested short blocks, even if the quality of the images is questionable.

More recently, transmission electron microscopy (TEM) has been used to image pure pectin networks [84], and mixed pectin gels [85]. The main challenge of TEM on aqueous samples is the sample preparation, where the water has to be substituted by something dry as a resin. For pectin, a method is modified from classics biological sample preparation: after chemical fixation of the network, the sample is dried by substituting slowly the water by ethanol, this later being replaced by a specific resin. Another sample preparation technique is freeze fracture [86]. Despite on many controversy of the validity of TEM on aqueous biological samples, with possible artefacts coming from the change of solvent or the ice crystal growth notably, this is currently the only way to see directly a pectic network.

Electron backscattering (EBSD) with a scanning electron microscope (SEM) is sensitive to metallic elements, including calcium. It has been used to probe the repartition of calcium in pectin micro-beads made of a pectin calcium induced gel [87]. The repartition of the fixed calcium can be probed, although the resulting image is of a low resolution due to the low calcium concentration. However, EBSD should find some applications in plant biology to map the fixed calcium by the pectic network in the plant cell wall for example, and might be useful for *in-vitro* gels.

As a short conclusion, microscopy techniques are challenging to use on pectin gels, as it is generally the case for biological applications where the validity of the resulting images is often questioned. The sample preparation is always complex, and can induce many possible artefacts on these particularly delicate networks. Nevertheless, efforts in this direction should be continued as it is the only way to see directly biopolymer networks. The solution will probably be the use/development of biological SEM or TEM, where aqueous samples can be imaged *in-situ*.

Mechanical properties

It has been described previously what is known about the pectin basic structure and how these polymers self-assemble in presence of the most important binding ions, Ca^{2+}. In order to understand the structure-function relationship, the study of the subsequent function needs to be done. While biological or food functions could be of different natures (chemical, texture, porosity…), the present thesis will pursue a fundamental understanding of the mechanical properties of pectin gels, and by linking these to what we know about the micro-structure of these gels, a better understanding of how these mechanical properties are linked to the gelling conditions. Determining the visco-elastic properties of soft materials is the domain of the science of deformation and flow.

Rheometry

The reference rheological tool is the rheometer, which is of an important use for both the industry and the fundamental research. This is due to its versatility and precision, as the modern rheometers are a reliable tool to measure the flowing behaviour of soft materials under various constrains, ranging from small amplitude oscillations to continuous shear tests. By combining these different constrains, the possible applications are manyflod, and the following description will be oriented toward the scope of this thesis, which we will limit to the linear behaviour which is well understood from a theoretical point of view, and thus will give more information about the networks' structure.

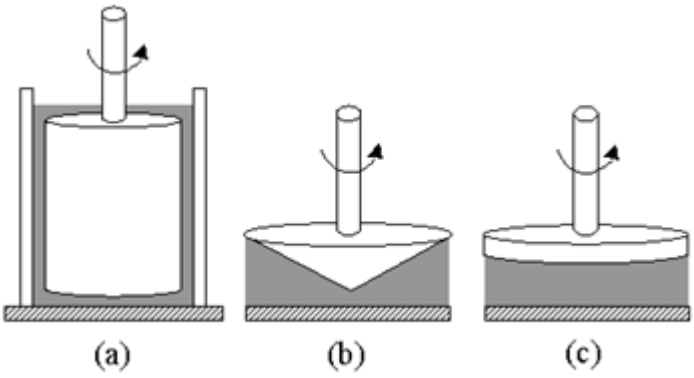

Figure 8: different configurations of rheometers a) concentric cylinder used for low viscosity fluids, b) cone-plate and c) plate-plate, both used manly for higher viscosity materials. The sampled material is represented in grey.

In the simple shear geometry, the ratio between the deformation of a material along an axis and the size of this sample in this direction is called the strain γ (no units), and the ratio of the applied force and the area is the stress σ (in Pa or N/m^2). For a purely elastic material, the stress is directly proportional to the strain, as defined by the Hooke's law:

$$\sigma = G \times \gamma \qquad (1.3)$$

where G is the shear modulus (in Pa), a constant for a given material. For a purely viscous material, the stress will be proportional to the shear rate or deformation rate:

$$\sigma = \eta \times \dot{\gamma} \qquad (1.4)$$

where η (Pa.s) is the well known viscosity of the liquid. Most soft materials are visco-elastic and exhibit both properties. In order to accommodate this mathematically, a possibility is to consider the response to the application of small oscillatory deformations:

$$\gamma(t) = \gamma_0 \times \sin(\omega \cdot t) \qquad (1.5)$$

where γ_0 is the strain amplitude and ω the angular frequency (rad/s). This can be easily recorded and the resulting stress will be recorded and is assumed to have the following form:

$$\sigma(t) = \sigma_0 \times \sin(\omega \cdot t + \delta) \qquad (1.6)$$

where σ_0 is the stress amplitude and δ the out of phase angle of the stress. A phase shift of 0^o is representative of a perfect elastic material, while a liquid will exhibit a shift of 90^o. The stress curve for a visco-elastic material can be written as the sum of an elastic component and a viscous component:

$$\sigma(t) = \gamma_0[G'(\omega)\sin(\omega t) + G''(\omega)\cos(\omega t)] \qquad (1.7)$$

which can be translated in the complex space by:

$$\sigma(t) = [G'(\omega) + iG''(\omega)] \times \gamma(t) = G^*(\omega) \times \gamma(t) \qquad (1.8)$$

G(ω)* is the complex visco-elastic modulus, whose the real part is called conservative or elastic modulus *G'(ω)*, and the imaginary part *G''(ω)* is the dissipative or viscous modulus.

Small oscillatory deformation rheometry has been widely used to study the linear behaviour of all sort of polymeric systems, and in particular of pectin gels for the food industry. The influence of many factors on the rheological properties of pectin has been studied. An increasing content of neutral sugars in the pectic chain gives weaker gels [88, 89], while the strength of a pectin gel is increasing with the molecular weight M_w [55]. But the actual influence of the pectin fine structure on the mechanical properties has been studied only recently [65], where the influences of the DM, DB and DB_{abs} on the rheology of calcium induced pectin gels are meticulously studied. They found that the elastic modulus *G'* was increasing with a decrease of DM, or with an increase of one of the DBs, which was expected. The main result is that *G'* was fitting linearly with a slope close to 1 to the factor DB_{abs} multiplied by the calcium concentration.

The use of a rheometer is essential due to its versatility, as able to determine precisely the functionality of a material (texture, melting…), and as able to perform complex mechanical tests necessary to study all sort of phenomena such as stress stiffening [90]. It has even been recently used for the study of living cells mechanical properties [91], where a kinematic hardening behaviour has been found, strangely similar to the behaviour of metals! But by working with a relative amount of sample, local information about heterogeneous networks is missing. And the accessible frequency range is limited at the moment to the low frequencies over 2 or 3 decades (limited by the inertia of the physical parts of a rheometer), where the access to the high

frequencies (HF) would allow knowing more about the basic structure of the networks. Thus some alternative way of determining the mechanical properties were necessary such as micro-rhelogy (MR). However, recently a mechanical way of determining the HF rheology of soft materials has been developped by [92] and [93], where the mechanical vibrations are applied by piezoelectric vibrators. The accessible frequencies are going up to 3.10^5 Hz, and the resulting results have been compared successfully with high frequency MR techniques [97]. Combining these HF macro-rheological methods with the classic rheometry ones is a very promising way of characterising materials where MR is not applicable, i.e. stiffer gels or opaque materials.

Microrheology

In the present thesis, we used the MR techniques Multiple Particle Tracking (MPT) and Diffusing Wave Spectroscopy (DWS), which are only two techniques amongst a jungle of other techniques, including AFM, optical tweezers and magnetic tweezers. MPT and DWS will be fully described in Chapter II, thus the following text will only introduce MR and its techniques, and motivate the choices of DWS and MPT for studying polysaccharides gels.

MR is the study of the motion of a micro-particle embedded in a material, and aim to determine the micro-structure or the mechanical properties of this material. When only the thermal self-motion of the particle is studied, the term of passive MR is used, contrary to active MR when the motion of the probe particle is driven. Passive MR will help to characterise the linear behaviour or intrinsic mechanical properties of a given material, while a driven motion should get more information on other properties such as strain-stiffening or stress relaxation. The aim of the present work being to understand better the micro-structure of pectin gels, we will limit ourselves to non-destructive passive MR methods. The reader can get more information about active MR in the excellent reviews [95] and [96].

Einstein was the first to suggest the idea of using a Brownian particle to probe the visco-elastic properties of complex fluids [97]. He described it as “the buoy in the pudding”, and one can easily understand it by thinking that a micro-probe will move faster when embedded in a weak material than in a stronger one. In a liquid, the link between the particle’s motion and the viscosity is given by the famous Stokes-Einstein equation, which link the viscosity to the mean square displacement (MSD) of the spherical probe. In order to render it applicable to visco-elastic solution, a generalized Stokes-Einstein has been derived [98]:

$$\hat{G}(s) = \frac{k_B T}{\pi a s \left\langle \hat{\Delta} r^2(s) \right\rangle} \qquad (1.8)$$

where $\hat{G}(s)$ is shear modulus in the Laplace space, a the radius of the Brownian particle and $\left\langle \hat{\Delta} r^2(s) \right\rangle$ the Laplace transform of the MSD. The complex modulus $G^*(\omega)$ is obtained by a Fourier transform of $\hat{G}(s)$. Although numerical Fourier transforms [99] are useful tools for data with a lot a features, numerical methods with analytical continuation [98, 100] are often preferred for the usually limited data sets, providing less noisy results.

Now the visco-elastic properties of a material are linked to the MSD of a Brownian probe embedded in a material, ways of determining experimentally this self-motion are required. The first precise tracks of Brownian particles were recorded by Jean Perrin [101], and show to be consistent with Einstein's theory [97] subsequently leading to the acceptance of the existence of the atom. But it took years to Perrin to isolate mono-disperse pollen particles sets and to record their motion in water, with a light microscope. At this time, the Brownian motion was only a "curiosity of limited interest. But it became since the basis of all the life sciences" [102].

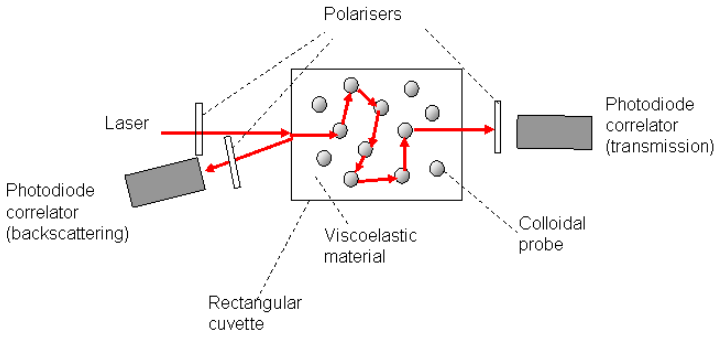

Figure 9: DWS principle

For MR, the limiting factor was the ability to measure precisely very small displacements of sub-micron sized probes. The first methods to allow this where light scattering techniques, in particular dynamic light scattering (DLS) and diffusing wave spectroscopy (DWS) [103, 104]. DLS probes the scattering of only a limited amount of probes, and subsequently can probe efficiently the MSD only at long times. On the contrary, DWS is a multiple scattering technique and probes the motion of thousands of particles, and provides an excellent averaged MSD [94] over a large frequency range, up to 1MHz which is the upper limit of passive MR. As depicted in Figure 9, DWS can probe the decorrelation of either the transmitted or the backscattered light. The first one is favoured in the present study due to the fact that every photon is strongly scattered, which is one of the main requirements for DWS. The method to

determine the MSD from the recorded correlation function of the light is described in details in the experimental section of Chapter 2. At higher frequency, the inertia of the micro-sphere will become important, and the complex fluid itself will not be the only constraining factor for the probe's motion [98]. Nevertheless, this high frequency limit allows getting essential information about the nature of a network basic building strands. DWS can even probe non-ergodic systems [105], by adding in series a second ergodic cell which renders the transmitted light ergodic. The ergodicity condition is primordial for all the light scattering techniques for their validity. Despite of being less stringent for the DWS configuration than for classic light scattering techniques (due to its excellent spatial resolution), the ergodicity has to be verified in any studied system. The ergodicity limits for the DWS were defined in [106], and all the results presented in this thesis were chosen to stay in these boundaries.

The inherent drawback of DWS is its inability to probe spatially inhomogeneous samples, and the homogeneity of the samples must be checked in order to insure the validity of the light scattering results. This can be done using multiple particle tracking (MPT), where the motion of many fluorescent microspheres is recorded simply by fluorescent microscopy with a CCD camera. The use of MPT as a way of measuring tiny motions was possible only through the use of algorithms which can determine the position of a sphere with a precision of 1/10 of a pixel [107], i.e. 10nm. The frequency range is limited by the speed of the camera, usually 30Hz for the upper limit (eq. 30 frames per second), but very fast cameras has been used recently for studying the HF behaviour of F-actin networks [108]. The upper frequency was 3700Hz, and the frequency range was enough to identify the HF scaling of the MSD of these huge semi-flexible filaments. In the case of a polymer with a smaller persistence length such as wormlike micelles, the high frequency behaviour is shifted to higher frequencies [107], rendering the use of DWS mandatory to get a precise characterisation of these very short time scales.

Nevertheless, MPT is most useful due to its ability to probe the motion of individual particles. Thus the heterogeneity of the samples can be probed, at the microscopic level between two particles as well as a larger scale by moving the microscope slide. By simply observing the tracks, one could see jumps of particles between different cages [109, 110] or if the particle is moving more randomly. This can be quantified by many statistical tools like plotting a Van Hove correlation of the motion, giving some information about each particle local micro-environnement, which is of a high interest for probing highly heterogeneous samples such as a living cell [111, 112] where some particles are behaving in a liquid, while the motion of others is restricted

by the cytoskeleton network. These statistical tools will be described and tested on pectin gels in Annexe I.

An alternative way of tracking individual beads is to use a laser detection scheme, similar to an optical tweezers setup [113, 114], but with an optical force much smaller than the Brownian force kT. The MSD of the tracer is determined from the laser deflection, which gives an excellent spatio-temporal resolution with a resolution of 1nm up to 50 kHz. This allow to study the HF behaviour of a single bead motion and then the single filament properties [113, 115], but on the other hand the inability of probing a large amount of particles prevent from good statistics at lower frequency, rending the study of the plateau modulus more difficult. Figure10 summarizes the different frequency and modulus ranges of the different MR techniques.

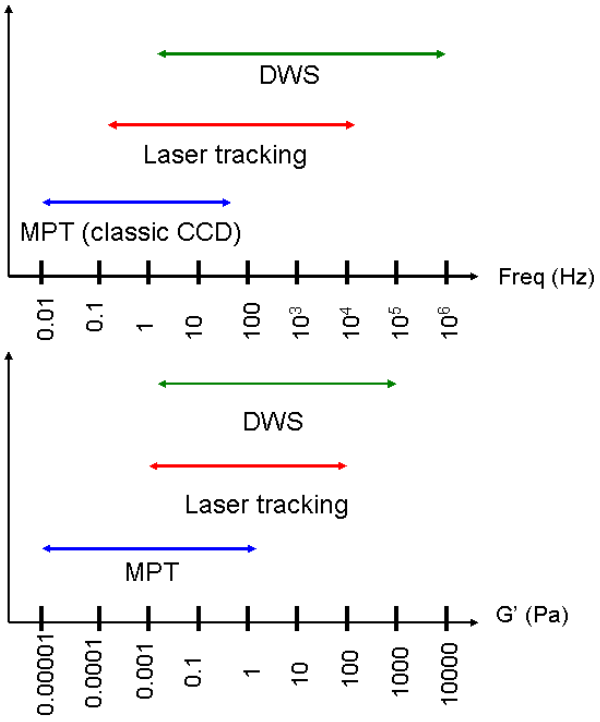

Figure 10: frequency and modulus ranges of the passive MR methods.

The principal drawback of tracking individual particles is the high sensitivity of the calculated MSD to the interactions between the probes and the studied network [116]. The bio-chemical way to circumvent this problem is to use probes with a surface chemistry preventing any interactions with the studied network. A wide range of micro-spheres with controlled surface chemistry is available, and one can further tune these surface properties in order to reach the desired properties, by for example covering the beads by specific polymers such as PEG to prevent protein depletion at their surface [116].

A more physical solution was developed by [117], where the correlated motion between two Brownian particles is used to determine the average mechanical properties between these two particles. Subsequently, assuming the distance between the two probes is much larger than the surface interaction length, the effect of the surface properties become negligible comparing to the mechanical properties. This technique is called two-point MR, and is usable only with techniques were the tracking of individual spheres is done, i.e. MPT [108] or laser detection [115]. However, this elegant technique has practically the disadvantage to increase greatly the data noise, due to the smaller MSD measured. It needs more statistics to decrease the noise, reducing the already limited frequency range by about one decade.

4. Aims of the thesis

How the mechanical properties of soft biomaterials are linked to their micro-structure is still poorly understood. Micro-rheology (MR) techniques are very powerful tools in that aim, probing the mechanical properties at the network intrinsic scale. MR techniques by their large frequency range allowed the verification of semi-flexible theories [118, 119] on cross-linked solutions of F-actin, whose huge filaments render this network visible by microscopy, and thus the best model system as a semi-flexible network. The main idea of this thesis is to apply two complementary techniques (Diffusing Wave Spectroscopy DWS and Multiple Particle Tracking MPT) to these calcium induced gels of pectin, a ubiquitous polysaccharide of the plant cell wall, and to understand how the polymer chains self-assemble depending of the polymer concentration, the Ca^{2+} concentration, and the pectin fine structure. While MPT determines necessary information about the homogeneity of the materials, DWS should allow the determination of the elementary strands building the network by giving access to the high frequency behaviour.

The first purpose of this work was to set up these techniques as well as checking their validity and complementarities on systems increasing in complexity, starting basics purely viscous solutions to more complex and heterogeneous visco-elastic biopolymeric systems. This will be the object of Chapter II. Being confident in the validity of our experimental apparatus, MR will be firstly applied to pectin gels in the aim of finding a regime where pectins' gels behave as the widespread [120] semi-flexible networks. Indeed, calcium induced gels are linked to many functionalities in the plants, and some of them might require the presence of semi-flexible networks due to their essential strain hardening properties. This will be achieved by enzymatically designing blocky pectins which should self-assemble as stiff filaments, after a subsequent release of Ca^{2+}. (Chapter III)

But semi-flexible networks are probably not the only sort of network used by nature, especially in the plant cell wall where the strain hardening requirements may be already assumed by the rigid cellulosic network. In order to find the utility of the pectic network in the plant cell wall, the second purpose is to copy nature by making gels where the pectin chains are enzymatically modified in presence of its binding ion Ca^{2+}. MR will carefully be performed on these networks in order to have some clues about the structure of these biomimetics networks (Chapter IV). Last but not least, a more general scheme will be proposed, by drawing a state diagram of the different pectic networks regimes depending of the polymer and ion binding concentrations (Chapter V).

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Chapter II – Experimental details and set up

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Abstract

Microrheological measurements have been carried out on a series of model systems of increasing structural and temporal complexity using multiple particle tracking (MPT). Purely viscous media, entangled polymeric solutions, and subsequently a biologically relevant polysaccharide gel have all been studied. In addition, the gelled sample has been mechanically disrupted in order to induce a transient heterogeneous microstructure. For the polysaccharide sample MPT results are analysed in order to yield both one- and two-point microrheological measurements and these are compared with those obtained by additional experiments carried out using diffusing wave spectroscopy (DWS) and those obtained using a conventional rheometer.

Results from glycerol / water mixtures and polyethylene oxide solutions agree with expectations, providing confidence both in the techniques used and the analysis employed. Moreover, a calcium-induced gel of the polysaccharide pectin yields similar results to those published recently on actin, with the elastic and viscous moduli being frequency independent at low frequency, and exhibiting a high frequency scaling of $\omega^{3/4}$. Furthermore, preliminary results reported here indicate that these techniques are ideally suited to the study of the temporal evolution of the spatial distribution of mechanical properties in such samples, for example during gelation or digestion.

1. Introduction

It is well known that a plethora of biologically relevant soft materials exhibit heterogeneous hierarchical architectures, that arise naturally from the propensity of constituent biopolymers to self-assemble and aggregate. Furthermore, owing to the presence of such structures, biopolymer solutions typically exhibit large deviations from Newtonian behaviour. Thus, understanding how to probe the viscoelastic properties of such systems is expected to have wide relevance, both from fundamental and industrial view-points. Microrheology, an extension of rheology that is concerned

with how materials store and dissipate energy as a function of length scale when exposed to stresses, is ideally suited to this task.

Since its conception microrheology has continually offered unprecedented new ways to examine the structures of soft materials [1]. Advances in passive microrheology, where the thermally activated motion of tracer particles is monitored, have included improvements and innovations in the measurement of the mean square displacement (MSD) of embedded tracers [2-5], and in the interpretation of these MSDs. Once obtained, the MSD data can be transformed to reveal the viscoelastic properties of the material. Several routes exist in order to perform this transformation, all based on equivalent representations of a generalised Stokes-Einstein (GSER) equation. This inverse problem can be tackled by direct Fourier transformation, Laplace transformation and analytic continuation, or numerical methods based on assuming a power law form of the MSD. Such transforms are well-documented [1, 6].

Initially microrheology might be considered as a technique enabling measurements of rheological properties, akin to those carried out on a conventional mechanical rheometer, on minute amounts of sample. Indeed, this simple idea is particularly appealing in the biophysical arena and to researchers interested in biopolymeric structure-function relationships, where it is often the case that only small amounts of material with controlled molecular architectures are available. However, driven largely by the development of DWS [2, 7, 8] that allowed small particle displacements to be measured that previously would have been undetectable, microrheology's ability to extend the frequency range of rheological characterisation was immediately appreciated. In contrast to conventional rheometry, the small mass of the tracer particles ensures that inertial affects can be ignored in microrheolgical investigations up to frequencies of some 10^6 Hz, allowing the rich dynamics of soft condensed matter systems to be probed over a hitherto impossible frequency range of some eight orders of magnitude.

The development of real-space particle tracking, first using single [9-10], and later multiple [11-14] beads, as a mechanism for extracting the MSD afforded the opportunity to examine the variation of mechanical properties in the spatial as well as temporal dimension. While other, scattering based, techniques typically average the system's behaviour over several millimetres, as many as a hundred individual fluorescent beads can be tracked within areas of some tens of microns squared. By moving the position of the observation window and taking ensemble averages of the MSD the degree of spatial heterogeneity existing at this scale can easily be ascertained, as has been elegantly demonstrated in living cells [15, 16]. However,

structures existing in biopolymer gels, biomaterials and tissues are well known to exist on length-scales substantially smaller than this, so that in order to obtain the maximum information single particles would ideally be tracked. Furthermore, when a tracer particle is introduced into a sample it is possible that there is a surface chemistry dependent depletion zone induced around the particle [17]. Both these effects, the existence of naturally existing structures that vary the local mechanical properties of soft materials sampled by tracers, for example pore-like structures [13], and particle induced pockets of different local concentration mean that for systems of this type the rheological properties sampled by the motion of tracers are not likely to reflect the behaviour of the bulk mechanical properties. However, rather than being a disadvantage, such cases simply highlight the fact that having measurements of a sample's rheology at different length scales provides additional information regarding its internal structure.

While tracking single particles is a worthwhile goal, the extended observation time required in order to ensure that the statistics are good enough to achieve ergodicity means that, in general, the MSD of each individual track in MTP is not equivalent to the ensemble averaged MSD. One approach that has been taken in order to circumvent this problem is the construction of the van Hove correlation functions from multiple particle tracking data giving, at a fixed time, the distribution among paths of the mean square distance travelled [13]. A statistical test can then be applied in order to determine if this distribution is consistent with the statistical expectation.

Considerations of such possible heterogeneity and how the local behaviour is related to the bulk rheological properties led to the development of two-point microrheology [11, 18, 19]. Two-point microrheology is based on cross-correlating the motion of two tracer particles in order to examine the rheological properties on the length-scale of their separation. For homogeneous materials it can be shown that scaling these contributions back to the tracer size successfully yields data that compare favourably with that measured using one point microrheology. However, if there are extra contributions to the particle's motion at the scale of its size, for example motion in a more compliant depletion layer, or relatively unconstrained motion in a pore, then the one and two-point microrheology are not expected to map onto each other. Furthermore, owing to the extended scale of the rheological measurement of two-point data it is expected to agree more closely with bulk rheological measurements, and it is independent of the size or shape of the particle and of the coupling between the tracer and the medium. In cases where observed differences between one and two-point microrheology arise from the presence of a depletion layer, an expression has been derived that is able to map the two datasets onto one another, with the depletion

zone thickness as an extractable parameter [19]. This was successfully demonstrated for solutions of DNA [14]. In terms of systems that are indeed believed to be locally inhomogenous *per-se* the methodology has been successfully tested on biopolymeric solutions of guar, where the two-point method was shown to extract rheological behaviour close to the bulk behaviour measured by a conventional rheometer, in contrast to the one-point data [18]. A study using worm-like micelles also found reasonable agreement between all techniques over a large frequency range [20], while in contrast, it is apparent that actin solutions have a scale-dependent rheology [18].

We are particularly interested in biopolymers that assemble into networks and in attempting to understand the spatial arrangement and the dynamics of such systems over relevant length- and time-scales. While a considerable amount of microrheological work has been carried out on actin, an important proteinaceous component of the cytoskeleton, we sought another model system with a view to investigating the applicability of the polymer physics models being applied to actin in describing polysaccharide gels. We selected pectin, an important gelling polysaccharide that has a crucial role in governing the properties of plant cell walls [21]. En route, we also present results from purely viscous and flexible polymer systems (glycerol / water mixtures and polyethylene oxide solutions respectively) in order to validate our experimental set-up and analysis techniques, and finally we describe the results of preliminary experiments designed to investigate the potential of using these techniques for studying the evolution of spatial heterogeneity in polysaccharide systems.

2. Materials and Methods

Materials

Glycerol (99.5%) was purchased from AJAX Chemicals, Auburn, NSW, Australia, and used without further purification.

PEO (M_w ~900 kDa) was purchased from ACROS Organics (New Jersey, USA) and used without further purification. Stock solutions of 6.2% and 10% w/w were prepared by dissolving the PEO powder in Milli-Q water (water purified with a Milli-Q apparatus, Millipore Corp., Bedford, MA, with a minimum resistivity of 18.2 MΩ) and stirring gently for 24 hours at room temperature.

Pectin (M_w ~150 kDa), extracted from citrus peel, with a 35% degree of methyl-esterification and a galacturonic content of 90%, was kindly supplied by CP Kelco ApS, DK 4623 Lille Skensved, Denmark. A stock solution of 3% w/w was prepared

by dissolving the pectin powder in Milli-Q water and stirring overnight. The pH was adjusted to 5.6 with a solution of 1M NaOH, in order to ensure that the unmethylated galacturonic residues were fully charged (pK_a ~ 3.5). The final concentration was 2.94% w/w.

$CaCO_3$ powder with a mean particle diameter of 1 μm was kindly provided by Provencale s.a., Avenue Frédéric Mistral, 83172 Brignoles Cedex, France.

Glucono-δ-lactone (GDL) was purchased from Fisher Scientific, Bishop Meadow Rd, Loughbourough, LE11 5RG, UK.

Latex particles of diameters 202 and 465 nm (2.62% v/w stock solutions) and fluorescent particles of diameter 541 nm (Fluoresbrite plain YG, 2.64% stock solution) were purchased from Polyscience Inc. (Warrington, PA).

Sample Preparation.

Glycerol / Water Mixtures. Glycerol / water mixtures of the required ratio were prepared simply by mixing with Milli-Q water.

PEO Solutions. Samples of different PEO concentrations and 1% v/w latex particles (DWS) or 0.02% v/w fluorescent particles (MPT) were prepared by mixing appropriate amounts of stock solutions and Milli-Q water and were stirred overnight. Three different PEO concentrations (2.2, 3.9, and 6% w/w) were studied, all between 10 and 45 times the overlap concentration, [22] corresponding to the semi-dilute regime. At these concentrations, the polymer solution mesh size [22] is the order of nm, which is significantly smaller than the latex particle diameter.

Pectin Gels. Ionotropic pectin gels were obtained by slowly releasing calcium ions into a 1% w/w pectin solution [23] containing 1% v/w of latex beads (DWS) or 0.02% v/w of fluorescent beads (MPT). The appropriate amounts of pectin and bead stock solutions were mixed and stirred, and immediately prior to loading into appropriate test cells a salt solution was added, the final mixing of which achieves the final desired concentration of all components of the system. This aqueous salt solution was composed of $CaCO_3$ and GDL. The GDL hydrolyses with time, releasing protons that solubilise the Ca ions from the $CaCO_3$. These components were introduced as powders into water, quickly mixed, and added to the pectin / bead solution as quickly as possible, in order to avoid significant calcium release before mixing with pectin. The quantity of $CaCO_3$ added determines the R value, R=2*[Ca2+]/[COO-] which can be varied to tune the elasticity of the material, and a stoichiometric ratio of GDL [GDL]=2*[Ca2+] is used in order to maintain the pH of

the solution [23]. After the addition of the salts the final prepared solution was stirred for a few minutes, and the samples were loaded on the appropriate test cell and left overnight. After 24 hours the mechanical properties of the resulting gel did not undergo any further significant evolution (as checked by conventional rheometry at 1Hz). Precautions were taken in order to avoid the dehydration of the sample during the gelling phase. Available microscopy evidence suggests that the mesh size in such gels is of the order of 100 nm [24].

Biphasic Gels. Mechanically heterogeneous biphasic samples were made by superposing two layers of pectin gel with two different R values. The bottom half of a DWS cell was loaded with an R=0.4 gelling solution, and subsequently once the gel had stopped evolving, an R=0.2 gelling solution was introduced directly on top and left overnight. The interface formed was not macroscopically straight owing to a meniscus in the cell, but was marked on the cell before addition of the second layer. For the MPT samples, a cavity microscope slide was filled with an R=0.4 gelling solution, and left overnight with a cover slip in place. Subsequently, the cover slip was removed and half of the gel removed using a scalpel. The resulting space was then filled with an R=0.2 gelling solution. The cover slip was then reintroduced and the sample left overnight. Thus, model macroscopically biphasic samples were designed.

Mechanically Disrupted Heterogeneous Systems. Preformed pectin gels (R=0.2) were introduced into a sonic bath for 2 minutes in order to introduce large scale heterogeneities into the sample.

Microrheology

As described in the introduction the aim of microrheology is to extract the rheological properties of soft materials from the motion of probe particles immersed in the material [1]. Passive microrheology, contrary to active microrheology, is the simple study of the particle's thermal motion. It is a non-destructive technique and recovers the linear response of the material, in contrast to active microrheology. (Far from this distinction being a disadvantage the potential complementarity of the techniques offers the extraction of more information, akin to performing conventional small and large deformation experiments [25]).

For a viscoelastic fluid, the MSD of a probe particle will vary as a local power law $\left\langle \Delta r^2(\tau) \right\rangle \sim \tau^{\alpha}$ with $0 \le \alpha \le 1$ depending on the nature of the medium, and τ is the observation time. In order to link the measured MSD to the viscoelastic properties, a generalized Stokes-Einstein relation (GSER) [6] can be used as described in the

introduction, with the caveats that the fluid is incompressible and the boundary non-slip.

$$\hat{G}(s) = \frac{k_B T}{\pi a s \left\langle \hat{\Delta} r^2(s) \right\rangle}, \qquad (2.1)$$

with $\hat{G}(s)$ the shear modulus in the Laplace space, s the Laplace frequency and $\left\langle \hat{\Delta} r^2(s) \right\rangle$ the Laplace transform of the MSD. The complex shear modulus $G^*(\omega)$ is the Fourier transform of $G(t)$, with ω the Fourier frequency. A numerical method for determining the elastic (G') and viscous (G'') shear moduli from the MSD has been detailed by Mason [6]. The storage and loss moduli are given as a function of the frequency ω by:

$$G(\omega) = \frac{k_B T}{\pi a \left\langle \Delta r^2(1/\omega) \right\rangle \Gamma[1+\alpha(\omega)]} \qquad (2.2)$$

$$G'(\omega) = G(\omega)\cos[\pi a(\omega)/2] \qquad (2.3)$$

$$G''(\omega) = G(\omega)\sin[\pi a(\omega)/2] \qquad (2.4)$$

$\left\langle \Delta r^2(1/\omega) \right\rangle$ is the MSD at time $\tau = 1/\omega$, $\alpha(\omega) = \left| \partial \ln \left\langle \Delta r^2(\tau) \right\rangle / \partial \ln \tau \right|_{\tau = 1/\omega}$ and Γ is the gamma function. We used this method and an extension in which second order derivatives are taken in order to better characterise the local power laws [6, 22].

DWS Experimental Arrangement.

The DWS apparatus used in this study has been fully described previously [26]. The samples were contained in glass cells of width 10 mm, height 50 mm, and path length L of 4 mm, and were illuminated with a 35 mW HeNe Melles-Griot laser operating at wavelength λ=633nm. The laser beam was expanded to approximately 8 mm on the surface of the cell. The transmitted scattered light was detected using a single-mode optical fibre (P1-3224-PC-5, Thorlabs Inc., Germany). The optical fibre was connected to a Hamamatsu HC120-08 PMT photomultiplier tube module, and the intensity autocorrelation functions of the scattered light were obtained using a Malvern 7132 correlator. Tests were run for 20 minutes to ensure low noise intensity autocorrelation functions.

For an expanded beam mode, the field autocorrelation function is $g_1(t)$ is obtained from the measured intensity autocorrelation function $g_2(t)$ using the Siegert equation $g_2(t) = 1 + \beta g_1^{\,2}(t)$, where β is a constant depending on the instrument. In the transmission geometry, the field autocorrelation function is given by [27]

$$g_1(t) = \frac{\frac{L/l^* + 4/3}{z_0/l^* + 2/3}\left\{\sinh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\right] + \frac{2}{3}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\cosh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\right]\right\}}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\right] + \frac{4}{3}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\cosh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle\Delta r^2(\tau)\right\rangle}\right]} \quad (2.5)$$

where l^* is the scattering mean free path, z_0 the penetration depth (assumed here to equal l^*), $k_0 = 2\pi/\lambda$, and L the sample thickness (4 mm here). l^* is obtained by performing an experiment on a water sample using 1% of latex beads, and fitting l^* using the accepted viscosity. Subsequently l^* for future samples is obtained by scaling the value obtained for water, based on the change in transmitted intensity when the sample is introduced, compared to the water experiment. It is known that for non-absorbing slabs of thickness *L*, the transmitted intensity is directly proportional to $(l^*/L)(1+4l^*/3L)$, so that by measuring the change in transmittance, the change in l^* can be calculated [27]. Once l^* is determined, the MSD can be obtained from the experimental correlation functions by inverting Eq (1) with a zero-crossing routine. That is; for each time point, the value of $\left\langle\Delta r^2(\tau)\right\rangle$ that is required in order for (2.5) to match the experimentally measured $g_1(t)$ is determined.

MPT Experimental Setup.

The Brownian motion of fluorescent polystyrene beads was monitored with an Olympus OH2 microscope, and using a 100× oil immersion lens. 45 frames s^{-1} were recorded with a UP800 CCD camera (UNIQVISION, USA), typically for 20 s, and digitalized with a PCDIG L frame grabber (Dalsa Coreco, CA). The motions of approximately 40 particles were determined using the tracking software in Image-Pro Plus (Media-Cybernetics, USA), in order to obtain the MSD as function of observation time. The one-point MSD was obtained by averaging over all the particles and all the initial times:

$$\left\langle\Delta r^2(\tau)\right\rangle_{OPMR} = \left\langle\left[r_\alpha(t+\tau) - r_\alpha(t)\right]^2\right\rangle_{t,\alpha} \quad (2.6)$$

where $r_\alpha(t)$ is the position of the particle α at time *t*, and τ, the lag time.

In the two-point correlation analysis, a correlated diffusion coefficient was calculated by averaging over all the particle combinations and all the initial times:

$$D_{rr}(R,\tau) = \left\langle\Delta r_R{}^i(t,\tau) \bullet \Delta r_R{}^j(t,\tau)\right\rangle_{t,i\neq j} \quad (2.7)$$

where $\Delta r_R^{\,i}(t,\tau)$ is the displacement during the observation time of particle *i* along the vector connecting the two particles *i* and *j*, and *R* is the distance between the two particles. For an incompressible fluid, with $R > a$, where *a* is the bead radius, it can be shown that [18]:

$$\hat{D}_{rr}(R,s) = \frac{k_B T}{2\pi R s \hat{G}(s)} \qquad (2.8)$$

with $\hat{D}_{rr}(R,s)$ and $\hat{G}(s)$ the Laplace transforms of $D_{rr}(R,s)$ and $G(s)$. Finally by drawing a parallel with the GSER a two-point MSD can be derived:

$$\left\langle \Delta r^2(\tau) \right\rangle_{TPMR} = \frac{2R}{a} D_{rr}(R,\tau) \qquad (2.9)$$

Bulk Rheology.

Rheological measurements were performed on a stress-controlled rheometer (Parr Physica UDS 300; Physica, Stuttgart, Germany) using the cone-plate geometry. The angle of the cone was 4° and its diameter 40mm. The sample was loaded into the rheometer and a solvent trap was fitted to avoid water evaporation. All the measurements were performed in duplicate at a constant temperature of 20° C.

3. Results and Discussion

Figure 1 shows the MSDs obtained from our microrheological investigations of a series of glycerol / water mixtures of 60, 80, 90 and 98% w/w, plotted as a function of time on logarithmic axles. In accordance with our expectations for purely viscous materials the data show linear dependences of the logarithm of the MSDs on logarithmic time, with slopes close to 1, over the whole frequency range examined. The size of the tracer particles used in the different experiments were different owing to the availability of labelled particles and the desire to observe significant displacements over short times in the DWS experiments. Therefore, in order to directly compare the datasets the measured MSDs have been scaled to a single particle size. The success of this procedure is evident in the figure and good agreement can be seen between the techniques as reported previously [9]. Although the DWS experiments proved difficult to perform at the highest concentrations, simply owing to the difficulty of including sufficient tracer particles to achieve reasonable l^* values, our particle tracking data are particularly robust and show no tendency towards anomalous sub-diffusive behaviour as has been reported previously

[1]. In addition, the predicted behaviour based on the accepted viscosity values of these solutions are shown on the figure and reasonable agreement can be seen.

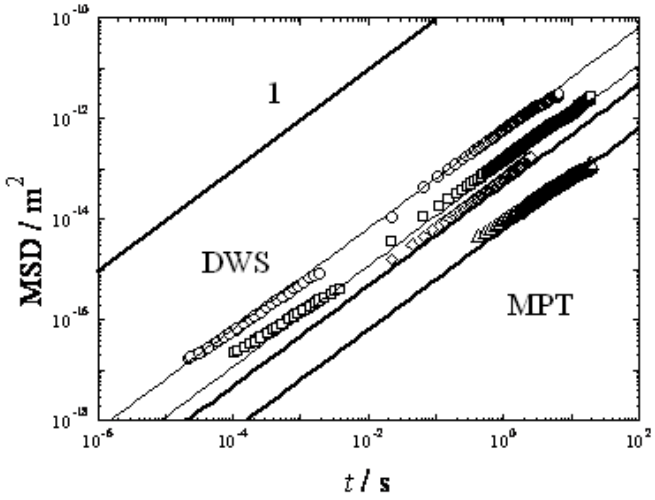

Figure 1: The MSDs obtained from our microrheological investigations of a series of glycerol / water mixtures of 60 (○), 80 (□), 90 (◇) and 98 (△)% w/w, plotted against time on logarithmic axes. The solid lines are predicted based on the accepted viscosity values of these solutions.

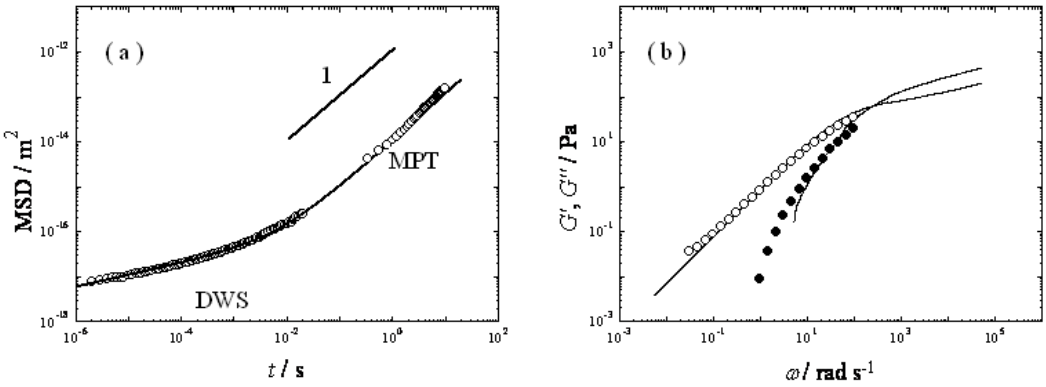

Figure 2: (a) The MSD obtained for a 4%w/w solution of PEO, prepared as described in the experimental section, plotted against time on logarithmic axes. The solid line shows the fit to a local power law. (b) The viscoelastic properties derived from the MSDs shown in figure 2(a) (Solid-line), compared with measurements made using a conventional rheometer, G' (●) and G'' (○).

Next attention was turned towards a viscoelastic solution of flexible polymers. Figure 2 (a) shows the MSD obtained for a 4% w/w solution of PEO, prepared as described in the experimental section. Once again the DWS and particle tracking data were scaled to account for differences in bead size, and found thereby to produce a consistent dataset, in good agreement with previous studies [9]. The MSD can be seen

to be well described by a power law behaviour as found previously [22, 28], with a long-time viscous behaviour, characterised by a linear region in the logarithm of the MSD versus logarithmic time plot with a gradient close to 1, and viscoelastic behaviour predominant at shorter times. Figure 2(b) shows the viscoelastic properties derived from the MSDs compared with measurements made using a conventional rheometer, and indeed within experimental uncertainties there is good agreement between the methodologies.

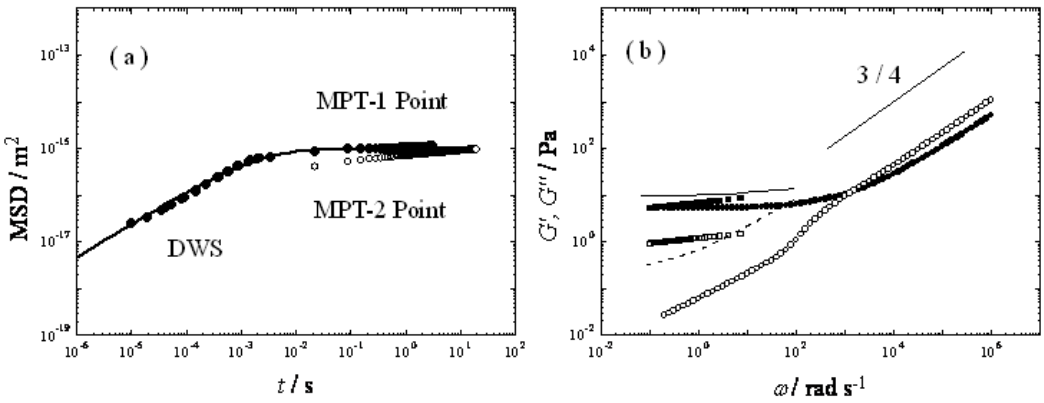

Figure 3: (a) The MSDs obtained from experiments using DWS, and multiple particle tracking, on an ionotropic gel of the polysaccharide pectin formed as described in the experimental section, plotted against time on logarithmic axes. The solid line is a fit to a theoretical model, described in the text. (b)The viscoelastic properties obtained from the transformation of this data. The solid guideline shows that the one-point microrheology results, G' (filled symbols) and G'' (open symbols), scale with $\omega^{3/4}$ at high frequencies. The filled and open squares show the two-point result for G' and G'' respectively and the solid and dashed lines the data obtained by conventional bulk rheometry.

Encouraged by the results from what might be considered as standard systems an ionotropic gel of the biologically important polysaccharide pectin was studied. The gel (R=0.4) was formed as described in the experimental section. The results obtained for the MSDs by DWS, and multiple particle tracking are shown in figure 3(a). There is a striking resemblance between the one-point results, that again are consistent with each other, and previous work reported on actin solutions [10, 29-31] and cross-linked actin networks [32, 33]. Motivated by this obvious similarity we endeavoured to fit our one-point MSD to a theoretical expression previously used in actin work [29]. The theoretical MSD for a small bead that couples to single filament eigenmodes that are themselves coupled to collective modes of an overdamped elastic background is predicted to have the form:

$$MSD(t) = A\{1-(\pi/t)^a\, erf(t^a)/2\} + B\{1-bt^b\Gamma[-b,t]\} \qquad (2.10)$$

where a=1/2, b=3/4, t is in units of τ_c, *erf* is the error function, Γ is the incomplete gamma function, and A and B are constants. The fit of the one point DWS and MPT data, to this functional form is shown in the figure 3a. Given the good agreement in the time domain it is unsurprising to find that the viscoelastic properties we obtain from the transformation of this data, also closely resemble that previously reported, in particular giving both elastic and viscous moduli (G' and G'') scaling with $\omega^{3/4}$ at high frequencies (Figure 3(b)). Furthermore, the switch to a frequency independent elastic modulus at low frequencies also looks extremely similar to data obtained on cross-linked actin filaments [32, 33].

These data provide strong evidence that, at least in this regime of cross-link density, the viscoelastic properties of the pectin gel can be modelled as a lightly cross-linked network of semi-flexible filaments, with scaling power of ¾ arising from the redistribution of bending modes of individual filaments induced on compression or stretching [34-36]. Although pectin gels are not traditionally considered to be particularly filamentous in character the individual polymers are themselves semi-flexible, with persistence lengths of around 15 sugar residues compared with a contour length of some 200-500 [37]. Furthermore, their polyelectrolytic nature and the postulated increased stiffness of calcium-crosslinked dimers, the elementary units of the network, makes them interesting model systems.

While the latest advances using fast cameras, laser traps and interferometers are able to measure two-point data over a large frequency range our frequency range is currently limited. Nevertheless, it is safe to say that the two-point data in this system shows some differences to the one point, that are indeed similar to that previously reported [31]. The two-point data, in magnitude at least, is slightly closer to the results of measurements carried out using conventional bulk rheology, also shown. However, in general all techniques agree reasonably within the restricted frequency range available, with variations in the elastic moduli similar to those found in other gelled systems [38] and typical of sample to sample variation. Future work will aim at controlling the equivalent filament length in our system, to investigate whether the observed enhanced viscoelastic relaxation at intermediate frequencies scales with the square of the filament length, as found in actin systems [31].

Additionally we sought to carry out preliminary investigations into the possibility of using these techniques for assessing the evolution of large scale heterogeneities in mechanical properties that may occur for example during gelation or breakdown processes. Firstly, experiments were carried out on macroscopically biphasic systems, generated as described in the experimental section. These results confirmed that, as

expected, the DWS data obtained when the beam spanned both phases was in fact representative of some average of the properties of the two individual phases. Using multiple particle tracking however, simply by focussing on two well separated areas either side of the interface, the mechanical properties of the two individual phases were easily distinguished and were, at least at reasonable distances from the interface, the nominal values obtained when the phases were measured independently.

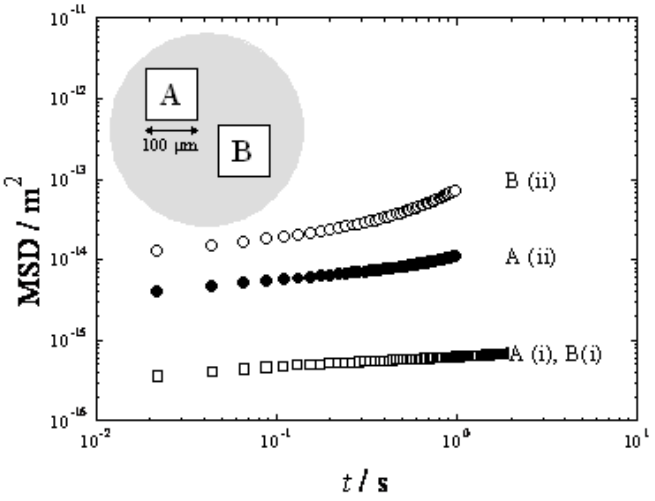

Figure 4: Spatially resolved MSDs, plotted against time on logarithmic axes, measured at different locations in a pectin gel (R-0.2), (i) prior to sonification and (ii) immediately after sonification of the gel.

Secondly, we took a pre-formed pectin gel and disrupted the network using sonification. Initially we estimated, simply by performing repeat measurements of the MSD as a function of position on the quiescent gel, that the spatial variation of the recorded MSD was on the order of 5%. However, immediately after sonification of the gel we clearly observed, simply from the bead movement, that some parts of the material were considerably less elastic than before the treatment. Furthermore, there was a significant spatial variation to the bead's movements now, far in excess of the 5% observed before the gel disruption. The MSDs shown in Figure 4 suffice to demonstrate this point. Finally, we observed that at a location in the gel where the MSD was significantly enhanced immediately following sonification, the mechanical properties evolved as a function of time, so that after one hour the properties more closely approximated that of the starting material (Figure 5). While these measurements are of a preliminary nature they reveal the potential to measure interesting re-healing dynamics of disrupted networks, including how the spatial heterogeneity of the network evolves.

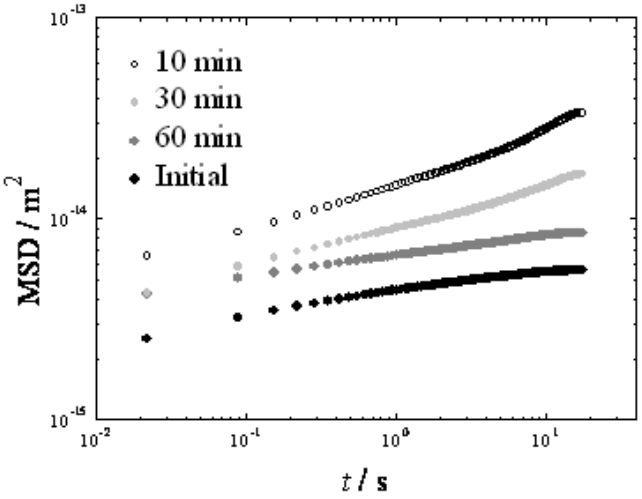

Figure 5: The time evolution of the MSD, plotted against time on logarithmic axles, measured on a pectin gel (R=0.2) following relaxation toward the initial state following sonification.

4. Conclusion

Results from glycerol / water mixtures and polyethylene oxide solutions were found to agree with expectations for purely viscous and flexible polymer solutions respectively, giving confidence in the techniques used. Moreover, the subsequent microrheological characterisation of a calcium-induced gel of the polysaccharide pectin, yields similar results to those obtained previously for actin, with a low frequency, frequency independent modulus ($G' > G''$) and a high frequency scaling of both G' and G'' with $\omega^{3/4}$. For this sample MPT results are analysed in order to yield both one- and two-point microrheological measurements and these are shown to compare favourably with those obtained by additional experiments carried out using diffusing wave spectroscopy (DWS) and those obtained using a conventional rheometer. Furthermore, preliminary results reported here indicate that these techniques are ideally suited to the study of the temporal evolution of the spatial distribution of mechanical properties in such polysaccharide samples, for example during gelation or digestion.

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Chapter III – Pectin gels as semi-flexible networks

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Abstract:

Microrheological measurements have been carried out on ionotropic gels made from an important cell wall polysaccharide, using diffusing wave spectroscopy (DWS) and multiple particle tracking (MPT). These gels were formed by the interaction of calcium ions with negatively charged groups on the polymer backbone; which is a co-polymer of charged and uncharged sugars, galacturonic acid and its methylesterified analogue respectively. The results suggest that semi-flexible networks are formed in these systems, with a low frequency, frequency independent storage modulus (G'>G'') and a high frequency scaling of both G' and G'' with $\omega^{3/4}$. The differences observed between gels obtained using polysaccharide samples with different amounts and patterns of the charged ion-binding groups could comfortably be accommodated within this theoretical framework, assuming that the elementary semi-flexible elements of the network are filaments consisting of two polymer chains bridged with calcium. In particular, a sample that was engineered to possess a blockwise intramolecular distribution of calcium chelating moieties clearly exhibited the high frequency scaling of both moduli with $\omega^{3/4}$ across some 3 orders of magnitude, and the concentration dependences of the elastic modulus, at both high and low frequency, were found to follow power laws with predicted exponents. Furthermore, quantitative agreement of the moduli with theory was found for realistic estimates of the molecular parameters, suggesting that the physics of semi-flexible networks is not only exploited by protein components of the cytoskeleton but also by polysaccharides in plant cell walls.

1. Introduction

It is well known that a plethora of biologically relevant soft materials exhibit heterogeneous hierarchical architectures that arise naturally from the propensity of constituent biopolymers to self-assemble and aggregate. These assembly processes

can yield specific supra-molecular structures, such as rods, helices, ribbons and tubules, and in soft biomaterials of which they form a part it is predominantly the nature and interaction of these structural elements that mediate the storage and dissipation of mechanical energy. Thus, in order to understand the mechanical properties of this important class of soft materials it must be appreciated that, in contrast to classical rubber-like networks of flexible chains connected by point-like cross-links, many biopolymer systems are assembled from semi-flexible filaments that can have a persistence length in excess of the distance between entanglements [1, 2]. This means, among other things, that the mechanical response of such systems may include a significant elastic contribution from bending modes and that networks easily strain-harden, a fact that presumably played no small part in the success of such evolved strategies in biomaterials.

Throughout the last decade, bolstered by progress in the development of microrheological techniques [3-12], a series of seminal works on F-actin, an important filament-forming protein of the cytoskeleton, have been carried out [13-29]. Indeed, F-actin has been the exemplary system studied by physicists working on network theories of semi-flexible polymers and excellent progress in both experimental and theoretical aspects have been made using this system.

More recently strain hardening has been demonstrated to be a common feature of many biopolymeric systems, originating from the semi-flexible nature of network strands [30], and an extensive set of rheological data for the biopolymer gelatin has been interpreted within the same framework, in which re-natured stiff triple helices form the elementary network elements [31]. While strain hardening has been reported experimentally in a number of polysaccharide gels [32] there has been no published interpretation of this behaviour and further, to our knowledge, there has been no convincing demonstration that the same underlying physics demonstrated to play such an important role in proteinaceous systems can govern the behaviour of soft polysaccharide based materials.

In this work the mechanical properties of ionotropic gels of the polysaccharide pectin are studied. Pectin is found in the cell walls of all land plants and although this is a complex biological matrix in which hemicelluloses, pectins, cellulose, proteins and lignin all play a role in determining structure and properties, it is known that the pectin component has considerable mechanical utility within the cell wall, in which the binding of calcium plays a significant role [33, 34]. Pectin is extracted commercially from lemon peel and apple pomace and consists mainly of a 1,4 linked α-D-galacturonate residues (typically ~90%). This dominant part of the pectin,

referred to as homogalacturonan, is a linear and relatively stiff anionic polymer [35], and is responsible for the manifest ion-binding. However, the sugar residue also naturally occurs in an uncharged, calcium-impotent, methylesterified form, as shown in figure 1(a). Pectins are usually characterised by this degree of methylesterification (DM) and their network formation capabilities are clearly related to this value, with DM<50% pectins forming ion-induced networks *in-vitro*. Interestingly this structural feature is known to be modified *in-vivo* as the plant engineers its constituent polymers, eliciting desired mechanical changes in order to facilitate varied physiological processes.

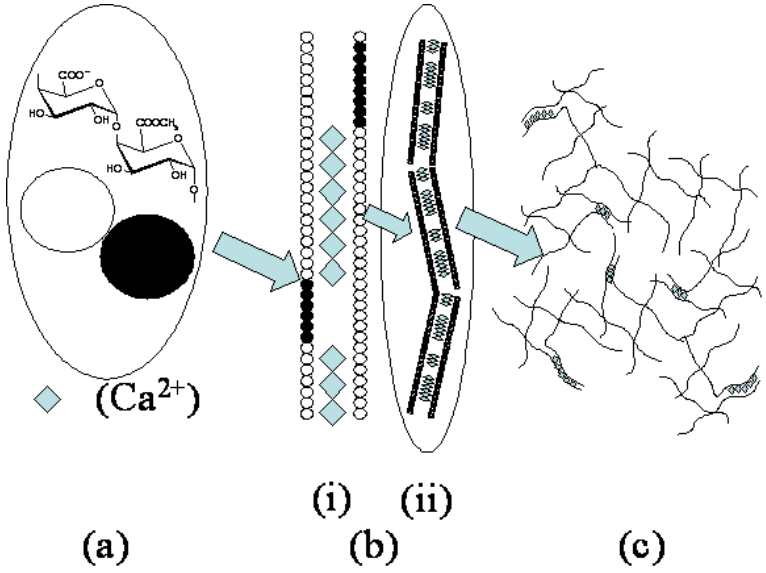

Figure 1: A schematic diagram of the proposed assembly process in ionotropic pectin gels; (a) charged galacturonic acid residues and uncharged methylesterified analogues are represented by open and filled circles respectively; (b) (i) galacturonic acid residues on neighbouring chains zip-up with calcium ions, represented by diamonds, (ii) forming stiff fibrils, (c) interfibrillar cross-links may also be formed with calcium depending on the amount of calcium available.

Broadly, calcium induced pectin networks are formed according to a so-called egg box model [36] in which gelation proceeds in two steps. The first step is a rapid formation of dimeric junction zones where calcium is chelated between the negatively charged carboxyl groups of two pectin chains, as shown schematically in figure 1 (b). Subsequent dimer-dimer aggregation, as depicted in figure 1(c) may also occur when there is sufficient calcium present. It is believed that the formation of a stable junction zone requires a block of approximately 12-16 consecutive free galacturonic acid (GalA) units along the polymer backbone [36, 37]. Hence, the *in-vitro* and *in-vivo* interaction with calcium ions, and the properties of the gels thereby formed, are expected to depend not only on the amount of ion-binding groups possessed by the polymer, but also on the distribution of such groups along the backbone.

Although pectin gels are not traditionally considered to be particularly filamentous in character the individual polymers are themselves semi-flexible, with persistence lengths of around 15 sugar residues compared with a contour length of some 200-500 [38]. Furthermore, their polyelectrolytic nature and the postulated increased stiffness of calcium-crosslinked dimers makes them interesting model systems. In addition, by modifying the concentration and fine structure of the polymer, changes to the phase volume, and entanglement and persistence lengths of constituent dimers can be made, and the consequences examined.

2. Experimental details

Materials

Pectins (M_w ~150 kDa), extracted from citrus peel with galacturonic contents of ~ 90%, and sample average degrees of methylesterification of 35 and 86% were kindly supplied by CP Kelco ApS, DK 4623 Lille Skensved, Denmark. The first sample was produced *in-house* by deesterification of a highly methylesterified pectin with a fungal enzyme, which, owing to its mode of action, produces a random arrangement of calcium binding groups along the polymer backbone. The second, highly esterified sample, was used to prepare i) a 48% DM sample, made using a base saponification in order to produce a random distribution of charged residues, and ii) a 50% DM sample, produced using a processive commercial pectin methyl esterase (PME) [EC 3.1.1.11] purchased from Sigma Aldrich (P5400) to remove the methylester groups and hence generate a highly blockwise charge distribution [39].

The sample average DMs of all the samples were determined using capillary zone electrophoresis (CE) as previously described [39-41]. In order to verify the differences in the intramolecular distribution of the calcium binding groups the polymers were incubated with a polygalacturonase [EC 3.2.1.15] that was kindly provided by Jacques Benen from the University of Wageningen. This is a pure endo-PG II isoform from *Aspergillus Niger* [42] and has an absolute requirement for the sugar residues in the active site to be unesterified in order for the chain to be severed, which yields the degradation pattern methylester-sequence dependent. Digest fragments were monitored using CE [43-45]. Large amounts of mono- di- and tri-galacturonic acid were liberated by the endo-PG II digest of the PME generated sample and in concert with the lack of detectable partially methylesterified low degree of polymerization fragments, these observations confirm its blockwise nature.

For microrheology experiments stock pectin solutions of 1-3% w/w were prepared by dissolving the pectin powder in water (minimum resistance 18.2 MΩ) and stirring overnight. The pH was adjusted to 5.6 with a solution of 0.1M NaOH, in order to ensure that the unmethylated galacturonic residues were fully charged (pK_a ~ 3.5).

$CaCO_3$ powder with a mean particle diameter of 1 μm was kindly provided by Provencale s.a., Avenue Frédéric Mistral, 83172 Brignoles Cedex, France.

Glucono-δ-lactone (GDL) was purchased from Fisher Scientific, Bishop Meadow Rd, Loughbourough, LE11 5RG, UK.

Latex particles of diameter 465 nm (2.62% w/v stock solutions) and fluorescent particles of diameter 541 nm (Fluoresbrite plain YG, 2.64% w/v stock solution) were purchased from Polyscience Inc. (Warrington, PA).

Sample Preparation.

Pectin Gels. Ionotropic pectin gels were obtained by slowly releasing calcium ions into pectin solutions [46] containing 1% w/v of latex beads (DWS) or 0.03% w/v of fluorescent beads (MPT). The appropriate amounts of pectin and bead stock solutions were mixed and stirred, and immediately prior to loading into appropriate test cells a salt solution was added, the final mixing of which achieves the final desired concentration of all components of the system. This aqueous salt solution was composed of $CaCO_3$ and GDL. The GDL hydrolyses with time, releasing protons that solubilise calcium ions from the $CaCO_3$. These components were introduced as powders into water, quickly mixed, and added to the pectin - bead solution as quickly as possible, in order to avoid significant calcium release before mixing with pectin. The quantity of $CaCO_3$ added determines the R value, R=2*[Ca2+]/[COO-] which can, by controlling the amount and extent of interchain association, be varied to tune the elasticity of the material. A stoichiometric ratio of GDL [GDL]=2*[Ca2+] is used in order to maintain the pH of the solution [46]. After the addition of the salts the final prepared solution was stirred for a few minutes, and the samples were loaded on the appropriate test cell and left overnight.

Microrheology

The aim of microrheology is to extract the rheological properties of soft materials from the motion of probe particles immersed in the material [47]. Passive microrheology, contrary to active microrheology, is the simple study of the particle's thermal motion. It is a non-destructive technique and recovers the linear response of the material, in contrast to active microrheology. For a viscoelastic fluid, the mean

square displacement (MSD) of a probe particle will vary as a local power law $\left\langle \Delta r^2(\tau) \right\rangle \sim \tau^{\alpha}$ with $0 \le \alpha \le 1$ depending on the nature of the medium, and τ is the observation time. In order to link the measured MSD to the viscoelastic properties, a generalized Stokes-Einstein relation (GSER) [10] can be used as described in the introduction, with the caveats that the fluid is incompressible and the boundary non-slip.

$$\hat{G}(s) = \frac{k_B T}{\pi a s \left\langle \hat{\Delta} r^2(s) \right\rangle}, \qquad (3.1)$$

with $\hat{G}(s)$ the shear modulus in the Laplace space, *s* the Laplace frequency and $\left\langle \hat{\Delta} r^2(s) \right\rangle$ the Laplace transform of the MSD. The complex shear modulus $G^*(\omega)$ is the Fourier transform of $G(t)$, with ω the Fourier frequency. A numerical method for determining the elastic (G') and viscous (G'') shear moduli from the MSD has been detailed by Mason [10]. The storage and loss moduli are given as a function of the frequency ω by:

$$G(\omega) = \frac{k_B T}{\pi a \left\langle \Delta r^2(1/\omega) \right\rangle \Gamma[1+\alpha(\omega)]} \qquad (3.2)$$

$$G'(\omega) = G(\omega)\cos[\pi a(\omega)/2] \qquad (3.3)$$

$$G''(\omega) = G(\omega)\sin[\pi a(\omega)/2] \qquad (3.4)$$

$\left\langle \Delta r^2(1/\omega) \right\rangle$ is the MSD at time $\tau = 1/\omega$, $\alpha(\omega) = \left| \partial \ln \left\langle \Delta r^2(\tau) \right\rangle / \partial \ln \tau \right|_{\tau=1/\omega}$ and Γ is the gamma function. We used this method and an extension in which second order derivatives are also taken in order to better characterise the local power laws [10, 48].

DWS Experimental Arrangement.

The DWS apparatus used in this study has been fully described previously [49]. The samples were contained in glass cells of width 10 mm, height 50mm, and path length *L* of 4 mm, and were illuminated with a 35 mW HeNe Melles-Griot laser operating at wavelength λ=633nm. The laser beam was expanded to approximately 8 mm on the surface of the cell. The transmitted scattered light was detected using a single-mode optical fiber (P1-3224-PC-5, Thorlabs Inc., Germany). The optical fiber was connected to a Hamamatsu HC120-08 PMT photomultiplier tube module, and the intensity autocorrelation functions of the scattered light were obtained using a Malvern 7132 correlator. Tests were run for 20 minutes to ensure low noise intensity autocorrelation functions.

For an expanded beam mode, the field autocorrelation function is $g_1(t)$ is obtained from the measured intensity autocorrelation function $g_2(t)$ using the Siegert equation $g_2(t) = 1 + \beta g_1^2(t)$, where β is a constant depending on the instrument. In the transmission geometry, the field autocorrelation function is given by [50]

$$g_1(t) = \frac{\frac{L/l^* + 4/3}{z_0/l^* + 2/3}\left\{\sinh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right] + \frac{2}{3}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\cosh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right]\right\}}{\left(1 + \frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right] + \frac{4}{3}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\cosh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right]} \tag{3.5}$$

where l^* is the scattering mean free path, z_0 the penetration depth (assumed here to equal l^*), $k_0 = 2\pi/\lambda$, and L the sample thickness (4 mm here). l^* is obtained by performing an experiment on a water sample using 1% of latex beads, and fitting l^* using the accepted viscosity. Subsequently l^* for future samples is obtained by scaling the value obtained for water, based on the change in transmitted intensity when the sample is introduced, compared to the water experiment. It is known that for non-absorbing slabs of thickness L, the transmitted intensity is directly proportional to $(l^*/L)(1 + 4l^*/3L)$, so that by measuring the change in transmittance, the change in l^* can be calculated [50]. Once l^* is determined, the MSD can be obtained from the experimental correlation functions by inverting Eq (1) with a zero-crossing routine.

MPT Experimental Setup.

The Brownian motion of fluorescent polystyrene beads was monitored with an Olympus OH2 microscope, and using a 100× oil immersion lens. 45 frames s^{-1} were recorded with a UP800 CCD camera (UNIQVISION, USA), typically for 20 s, and digitalized with a PCDIG L frame grabber (Dalsa Coreco, CA). The motions of approximately 40 particles were determined using the tracking software in Image-Pro Plus (Media-Cybernetics, USA), in order to obtain the MSD as a function of observation time. The one-point MSD was obtained by averaging over all the particles and all the initial times:

$$\left\langle \Delta r^2(\tau)\right\rangle_{OPMR} = \left\langle \left[r_\alpha(t+\tau) - r_\alpha(t)\right]^2\right\rangle_{t,\alpha} \tag{3.6}$$

where $r_\alpha(t)$ is the position of the particle α at time t, and τ, the lag time.

Our microrheological set-ups were thoroughly tested using glycerol-water mixtures of known viscosities and an archetypal flexible polymer, polyethylene oxide (PEO), and were shown to perform well in both cases [51].

3. Results and discussions

Figure 2 (a) shows the mean square displacement of tracer particles as a function of time, as measured by DWS and MPT, when embedded in a calcium induced gel made with a commercially available pectin sample. These initial experiments were carried out at a polymer concentration of 1% w/w using a sample with some 65% of the sugar residues charged and randomly distributed along the backbone; the gel being formed as described in the experimental section. The agreement between the techniques is good. In addition to supporting the DWS data, the MPT measurements also allow the spatial heterogeneity of the mechanical properties [11, 20, 52-54] to be assessed and it should be noted that Van Hove correlation functions for this system, and indeed all those described herein, were Gaussian and indicated that there was no significant difference in the local rheological properties experienced by the individual tracer particles, so that quantitative microrheology could safely be performed. Further work on this aspect of the gels in different concentration regimes will be reported elsewhere.

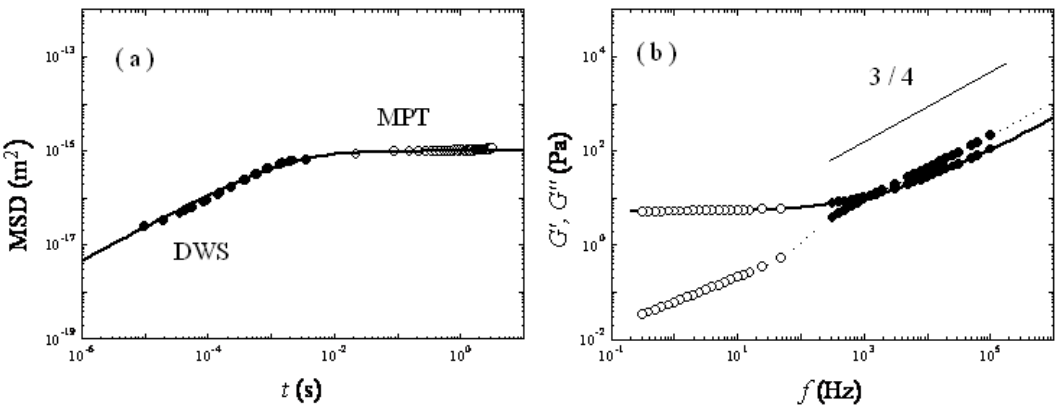

Figure 2: (a) The MSDs obtained from experiments using DWS (filled circles), and multiple particle tracking (open circles), on an ionotropic gel of the polysaccharide pectin formed as described in the experimental section. The solid line is a fit to a theoretical model, described in the text. (b)The viscoelastic properties obtained from the transformation of this data for the DWS data (filled circles) and the MPT data (open circles). The solid guideline shows G' (line) and G'' (dots) scaling with $\omega^{3/4}$ at high frequencies.

There is a striking resemblance between these one-point microrheological results and work reported on actin solutions [7, 17, 18, 29]. Motivated by this obvious similarity we endeavoured to fit our one-point MSD to the theoretical expression used in the actin work [29]. The theoretical MSD for a small bead that couples to single filament eigenmodes that are themselves coupled to collective modes of an overdamped elastic background is predicted to have the form:

$$\Delta x^2(t) = A\{1-(\pi/t)^a\, erf(t^a)/2\} + B\{1-bt^b\Gamma[-b,t]\} \qquad (3.7)$$

where a=1/2, b=3/4, t is in units of τ_c, *erf* is the error function and Γ is the incomplete gamma function. The fit of the one point DWS and MPT data, to this functional form is shown in the figure. Given the good agreement in the time domain it is unsurprising to find that the viscoelastic properties we obtain from the transformation of this data, also closely resemble that previously reported, in particular giving both elastic and viscous moduli (G' and G'') scaling with $\omega^{3/4}$ at high frequencies (Figure 2(b)). Although the adherence to the ¾ scaling within the data range of these preliminary experiments is clearer for G'' than G', the power law dependence of the elastic modulus is clearly revealed in the frequency response obtained by transforming the extrapolated time domain fitting function. Furthermore, the switch to a frequency independent elastic modulus at low frequencies also looks extremely similar to recent data obtained on lightly cross-linked actin filaments [28]. It is worth clarifying that experiments carried out on pectin solutions in the absence of calcium show that there is no tendency for the MSDs to exhibit a high frequency ¾ scaling of the moduli; crosslinks are not introduced between pre-existing filaments as in the actin sudy [28] but rather we will argue that semi-flexible filaments are generated by the addition of calcium (shown schematically in figure 1(b)), which can also lightly crosslink some filaments (figure 1(c)). To summarise, initial experiments carried out using a commercially available pectin sample with 65% of the backbone sugar residues charged and randomly distributed along the backbone, were suggestive that the calcium induced gels formed from such systems were lightly cross-linked entangled semi-flexible networks, with a low frequency, frequency independent elastic modulus ($G' > G''$) and a high frequency scaling of both G' and G'' with $\omega^{3/4}$.

In order to investigate this behaviour further, and in particular to gather additional evidence that the scaling power of ¾ arises from the redistribution of bending modes of individual filaments induced on compression or stretching, we examined systems in which existing molecular engineering skills were exploited in order to generate systems in a similar regime but where the persistence length of constituent fibrils was varied. Unlike actin systems where the relationships between the polymer concentration and filament density, and filament length and persistence length are reasonably straightforward, owing to the fact that all the polymer is incorporated into the semi-flexible filaments, in pectin systems the situation is not so clear cut. In these co-polymeric systems only the charged (unmethylesterified) groups have the potential to bind the calcium into junction zones, postulated to be the semi-flexible filaments of the network, as shown in figure 1(c). Furthermore, it has been proposed that a minimum length of sequential ion-binding groups is required to form stable calcium mediated bridges, which renders the pattern of methylesterification crucial to the ability of a particular region of the polysaccharide backbone to participate in a

calcium chelation zone or filament motif. Further still, the consequences of interspersing different lengths of “mismatched regions” containing methylesterified groups into otherwise calcium binding runs of charged sugars are not clear. While the bending modulus of the filament is likely to vary spatially along the chain if multiple lengths of calcium bound stiff dimeric regions are interrupted by methylester groups, defining whether or not such regions are “part of a filament” is not trivial. In addition, chain to chain variation in the degree of methylesterification also exists, and although measurements of this can be made [39, 40], it further complicates the matter in hand.

Despite the complexities of the exact relationship between filament properties and polymeric fine structure it is clear nevertheless that the amount and pattern of ion-binding groups on the backbone are likely to give rise to changes in both the filament density and average persistence length. Hypothesising that polymer dimers cross-linked with calcium do essentially form the fundamental filamentary elements of the network, experiments were subsequently carried out using polymer architectures that were engineered to possess 1) less calcium binding groups and 2) a more blockwise intramolecular distribution of ion-binding groups, when compared to the pectin used in the preliminary studies. While the former would be expected to possess smaller average persistence lengths of filaments, the later would be expected to form longer, stiffer dimers. Furthermore, having the ion-binding groups arranged blockwise ameliorates some of the difficulties described above thereby simplifying the estimation of the filament density. Such blockwise fine structures are also considerably closer to native structures found in plant cell walls.

Figure 3 shows the mean square displacement of the thermally activated movement of tracer particles, measured by DWS, in pectin gels made from polysaccharides engineered to have different amounts and patterns of ion-binding groups as described in the experimental section. While the polymer and calcium concentrations have also been controlled in order to satisfy a number of practical considerations regarding the gel formation, the significance of which will be addressed in due course, a number of points are nevertheless clear. For a random distribution of ion-binding groups the ¾ power law behaviour moves to higher frequencies as the number of ion-binding groups is reduced, while for a blockwise distribution where all the calcium chelating groups are able to participate in junction zone formation the semi-flexible network signature is clearly observable over nearly 3 orders of magnitude in time (from ~10^{-6} to ~10^{-3} s). This strongly suggests that indeed the elastic and viscous moduli (G' and G'') scaling with $\omega^{3/4}$ at high frequencies observed in pectinacious systems reflects the semi-flexible nature of network filaments that consist of calcium cross-linked polymer chains.

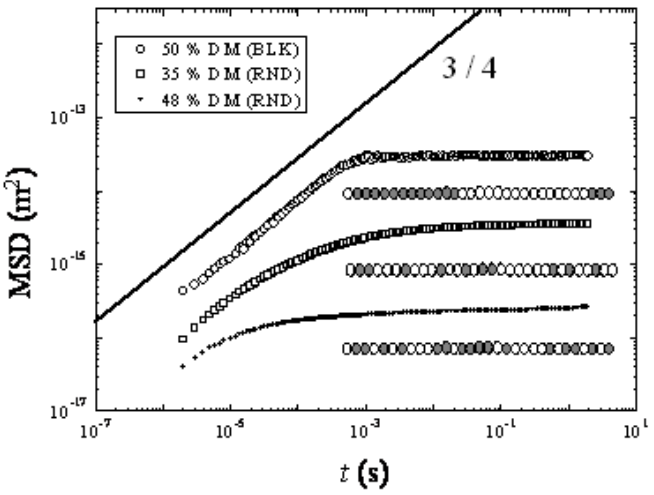

Figure 3: The MSDs obtained from experiments using DWS on ionotropic gels of pectin samples with different backbone architectures. Samples of 35 and 48 % DM having randomly distributed ion-binding groups (open circles in the schematic polymer structures inset), and a 50 % DM sample possessing a blockwise distribution of charged groups are shown. These were formed as described in the experimental section (●) 1 % w/w polymer, R=0.4; (□) 1% w/w polymer , R=0.15; (○) 0.2% polymer, R=0.15).

Not only does the sample possessing the blockwise charge distribution exhibit the clearest signatures of semi-flexible behaviour but, as described above, it is the easiest sample to model, where the assumption that all ion-binding groups have the potential to be part of a filament is likely to be a reasonable approximation. Bearing this in mind, as well as the intrinsic interest in its bio-mimetic connection, we carried out further studies on this sample. Figure 4 shows the measured MSDs of the tracer particles in calcium mediated gels of this blockwise sample at various polymer concentrations. The theoretical framework of the semi-flexible network model also provides predictions for the scaling exponent of the elastic moduli (or equivalently the MSD) with concentration at both high and low frequencies [2] and the data obtained in our experiments at 10^{-4} and 10^{-1} Hz are shown in figures 5 (a) and (b), where it can be seen that for concentrations below 0.8% w/w the agreement is excellent. This provides further evidence of the governing semi-flexible network physics in this regime. Above this concentration agreement with the concentration dependence predicted by this model is lost. It can be noted that concurrently it is at these concentrations in figure 4 that the measured data no longer show a clear asymptotic trend towards a scaling of ¾ at high frequency, in good agreement that at these concentrations we are entering a regime in which the network behaviour is dominated by another factor that we suggest to be the bundling of dimeric filaments. It is also worth noting that the comparison of these results reported in figure 4 with

those obtained using different polymeric fine structures (figure 3) confirms the hypothesis that differences in the latter cannot be explained solely by the differences in concentration of the samples that were necessitated by practical issues regarding the kinetics of gel formation, and reflect a significant contribution from variations in filament length.

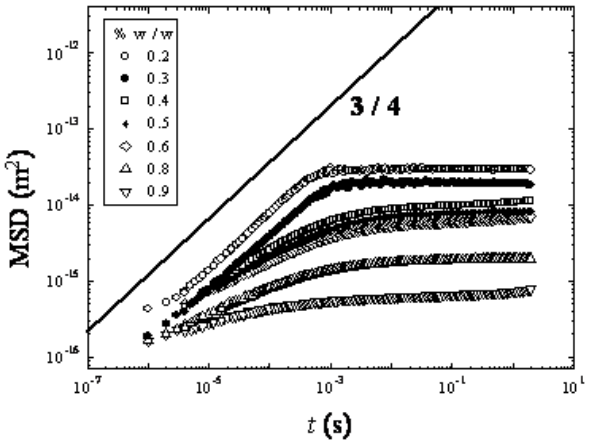

Figure 4: The MSDs obtained from experiments using DWS on ionotropic gels of pectin samples with 50% ion-binding groups arranged in a blockwise distribution, formed as described in the experimental section, as a function of concentration.

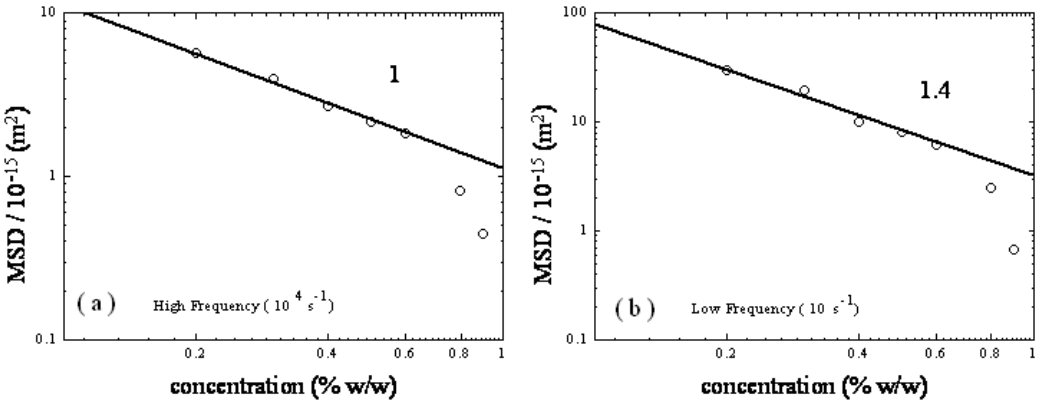

Figure 5: The concentration dependence of the mean square displacements of tracer particles in a 0.2% w/w gel of the 50% DM, PME generated, pectin. (a) at high frequency and (b) at low frequency.

Finally, still further evidence is presented in the form of a calculation of the moduli at high frequency based on the estimation of molecular parameters in the regime where we are claiming semi-flexible network physics is governing the mechanical properties. In order to compare the calculation of mechanical properties with the experimental data, the measured MSD for the 0.2% w/w blockwise sample was transformed to the frequency domain as described in the Experimental Details. It

should be noted that in order to perform the numerical derivatives the time domain data is routinely fitted to a smooth functional form, such double or reciprocal power laws [52, 55]. While the results shown in figure 2 suggest a fit to equation 3.8 would be useful in this regard we found that while the proposed form certainly captures the main features of the 0.2% data it struggled to reproduce the transition region in detail. The experimental points fell below the prediction at long times, as found previously in actin studies [29], but also were significantly above the theory in the transition region (not shown). The results of an MPT experiment were consistent with the measured long time displacement, and taken with the good agreement of this data with the concentration dependence of the MSDs shown in figure 5, there is reasonable experimental evidence supporting the sharpness of the measured low frequency cut-off. Therefore, in lieu of a physically significant functional form that captures the detail of the measured MSD across the whole measured time domain, a transform was carried out simply in the asymptotic regions. Figure 6 shows the experimental data transformed as described and the results of a theoretical calculation performed using [2]:

$$G(\omega) = \frac{1}{15}\rho\kappa l_p \left(-2i\varsigma/\kappa\right)^{3/4}\omega^{3/4} - i\omega\eta \qquad (3.8)$$

where ρ is the polymer concentration in length per unit volume, κ is the bending modulus, l_p is the persistence length and ζ is the lateral drag coefficient per unit length which we have approximated by $4\pi\eta$ / ln(ε/a), where η is the viscosity, ε is the mesh size and a is the radius of the filament.

By assuming the filament radius is of the order of 1 nm (which seems reasonable for a dimeric polysaccharide), and that the filament density can be calculated from the actual polymer concentration multiplied by the fraction of sugars that are charged (0.5) and by the fraction of those that have available calcium (0.15) then at 293 K and at a polymer concentration of 0.2% w/w the quantitative prediction is shown in figure 6, assuming a filament persistence length of some 300 nm [2, 28]. This filament persistence length is not unreasonable based on available microscopy of similar pectin systems [56], although it is the order of the chain contour length and is only a factor of 3 larger than the predicted mesh size. While this set of molecular parameters seems at least reasonable for an order of magnitude calculation, the lateral drag coefficient required in order to then produce the shown prediction is some two orders of magnitude less than that found for F-actin [28]. This in itself it perhaps not surprising, although calculating this value from the approximation given above seems to require a physically unrealistic viscosity of the medium exerting drag on the

filaments of 0.01 mPas. On the whole, considering the approximations made for this highly complex system, the calculation serves to show that it is not unreasonable that indeed these gels of structural anionic polysaccharides exhibit a regime in which their mechanical response is governed by the physics of semi-flexible networks. Although the plant cell wall is an extremely complex biopolymer matrix, as discussed, the positioning of this regime in terms of polymer and calcium concentration suggest that this may be operative *in-vivo* [33, 57].

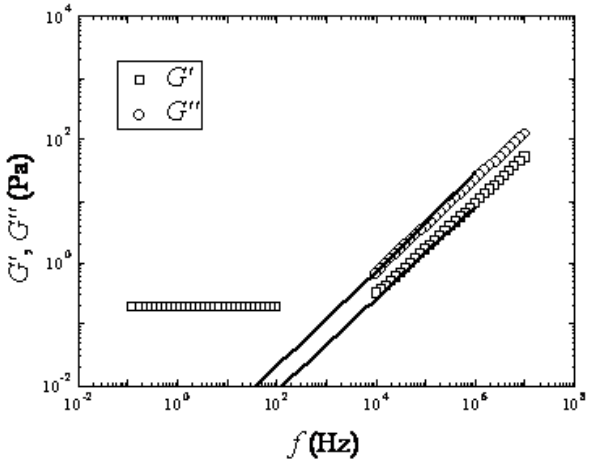

Figure 6: The frequency dependence of *G'* and *G''* measured on a 0.2% w/w gel of the 50% DM, PME generated, pectin. The solid line is a theoretical calculation performed as described in the text.

4. Conclusion

The anionic polysaccharide pectin is a crucial structural component in the cell walls of all land plants where it is, amongst other things, cross-linked with calcium ions. *In-vitro* microrheological characterisation of calcium-induced pectin gels revealed a regime that exhibits the signatures of lightly cross-linked, entangled semi-flexible networks, with a low frequency, frequency independent elastic modulus ($G' > G''$) and a high frequency scaling of both G' and G'' with $\omega^{3/4}$. Experiments subsequently carried out using polymer architectures in which the degree of calcium mediated association could be altered suggest that pectin chains cross-linked with calcium form the fundamental filamentary elements of the network. In particular, a pectin sample was modified by a plant enzyme in order to produce a more blockwise intramolecular distribution of ion-binding groups and when ionotropic gels were made using this polymer the predicted semi-flexible scaling regime was clearly observed over almost 3 orders of magnitude. Furthermore, the concentration dependence of the MSDs obtained from this sample, at both high and low frequency, followed power laws with

exponents predicted within the theoretical framework of semi-flexible networks; and quantitative agreement of the moduli with theory was found for realistic estimates of the molecular parameters.

While such a regime has been extensively investigated for other semi-flexible biopolymers, in particular F-actin and other intermediate filaments of the cytoskeleton, this is the first time that the same underlying physics has been experimentally demonstrated for a polysaccharide system. This work therefore demonstrates the utility of the semi-flexible network model beyond protein filament gels to polysaccharides with much smaller mesh sizes and persistence lengths. Furthermore, these findings suggest that it is possible that the same semi-flexible network physics that provides the mechanical framework of the cytoplasm, also has a role to play in understanding the cell wall. Interestingly, the polymer and calcium concentrations spanning this regime could well be representative of certain cell wall types under physiologically relevant conditions. Future work will include the investigation of the transition to bundling at higher concentrations, strain hardening phenomena, and the effects of the presence of other components such as cellulose micro-fibrils, in an attempt to move towards a mechanical understanding of more realistic cell wall mimics.

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Chapter IV – Pectin gels as chemically cross-linked flexible networks

As submitted:

R. R. Vincent, A. Cucheval, Y. Hemar, and M. A. K. Williams, *Bio-inspired network optimisation in soft-materials: Insights from the plant cell wall,* submitted to European Physical Journal E

Abstract.

The polysaccharide pectin is found in the cell walls of all land plants where it has considerable mechanical utility, predominantly mediated by the amount and pattern of calcium-binding sugar residues occurring on a largely co-polymeric backbone. This backbone consists of galacturonic acid and its methylesterifed counterpart and the amount and pattern of the former, which is responsible for the calcium cross-linking of the chains, is controlled *in-vivo* by the action of an enzyme, pectinmethylesterase (PME). The dynamic-mechanical response of ionotropic gels made from extracted pectins, whose primary structure was modified *in-vitro*, have recently been investigated by microrheological experiments and found to exhibit behaviour indicative of semi-flexible polymer networks. In this work we investigate the gelling behaviour of systems in which PME has been used to liberate calcium binding sites on initially highly methylesterified pectin in the presence of calcium-ions, rather than subsequently releasing calcium into solutions of pre-processed pectins. In stark contrast to the semi-flexible network paradigm of biological gels and the previous work on pectin, these gels exhibit the properties of chemically cross-linked networks of flexible polymers. The ability of plants to control local polymer, enzyme, and ion concentration, suggests that both semi-flexible and flexible networks may be relevant *in-vivo*, providing different functionalities.

1 Introduction

Soft biological materials are smart: a plethora of environmental signals can trigger dramatic changes in their structure and mechanical properties. Their constant optimisation, both spatially and temporally, ensures that different physiologically relevant functions can be performed. Such changes are facilitated through molecular remodelling and the control of intermolecular assembly. Understanding the structure - function relationships exploited in these systems will illuminate Nature’s design rules

and ensure that the abundant biopolymer resources of the planet can be used in an efficient, optimised way.

Many soft biological materials exhibit hierarchical architectures that arise naturally from the propensity of constituent biopolymers to self-assemble and aggregate. These assembly processes can generate specific supra-molecular structures, such as rods, helices and tubules, and typically yield semi-flexible filaments that can have a persistence length in excess of the distance between entanglements. F-actin, a filament-forming protein of the cytoskeleton has, for many years, been the exemplary system for the study of the mechanical behaviour of such systems and has motivated both the development of physical theories for semi-flexible networks [1-3], and experimental microrheology techniques (MR) [4, 5]. These experimental techniques, which give access to viscoelastic properties by studying the Brownian motion of micro-tracers, allowed the testing of semi-flexible network models by giving access to the mechanical properties at high frequency [6, 7] and subsequently they have become the paradigm for biological gels [8-10]. While F-actin filaments are extensive and can be easily visualised in microscopy, semi-flexible network signatures have also recently been found in systems in which the network filaments themselves occur on a somewhat smaller length-scale, in systems as diverse as polysaccharide gels [11] and worm-like micelle solutions [12].

In the present work, the dynamic-mechanical response of ionotropic gels made from the polysaccharide pectin is studied. While pectin is not the only important plant cell wall component, it is known to play a major mechanical role in many plant cell functionalities. Micro-indentation experiments carried out on pollen tubes showed that cell growth is strongly linked to the calcium binding abilities of its constituent cell wall pectins [13]. Furthermore, the calcium induced pectin network regulates cell-cell adhesion, whereas the other main cell wall components, cellulose and xyloglucan, do not appear to play a noticeable mechanical role in this regard [14].

The main sources of commercial pectin are orange peel and apple pomace, and although the detailed fine structure *in-vivo* is not known with certainty due to its complexity [15, 16] the extracted product is composed mostly of linear chains called homogalacturonans, with some 90% of the sugar residues being 1,4 linked α-D-galacturonic acid and its methyl-esterified counterpart, as seen in figure 1a. The proportion of the uncharged methyl-esterified residues, called the degree of methyl-esterification (DM), is closely linked to the ionic binding abilities of pectin. In addition, as there is data suggesting that between 8 and 15 consecutive charged residues are required to form a stable junction zone with calcium [17, 18], it is clear

that not only the quantity of charged residues but their distribution along the chain will greatly impact on the mechanical properties of ionotropic networks.

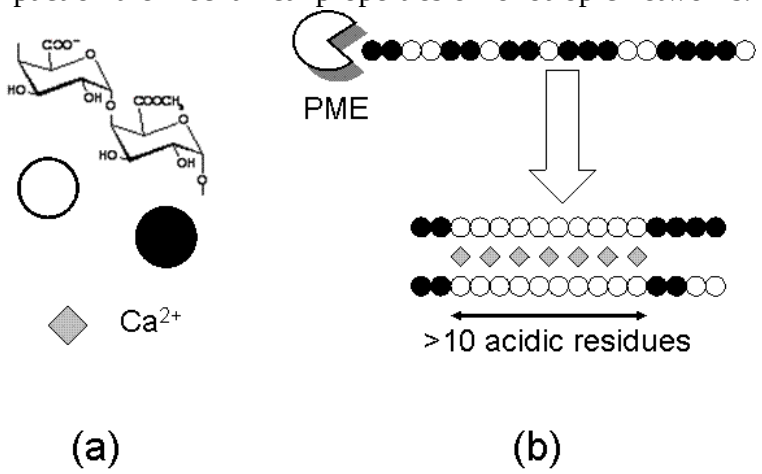

Figure 1: A schematic diagram of the pectin fine structure and its modification; (a) charged galacturonic acid residues and uncharged methylesterified analogues are represented by open and filled circles respectively; (b) (i) PME enzymes act on the polymer chains and remove the methyl-esterified residues (ii) allowing, when enough consecutive residues are charged, the formation of a stable calcium mediated junction zone.

In the plant cell, pectin is synthesized in the Golgi apparatus with a high DM and cannot therefore form cross-links with any present Ca^{2+} while being transported and deposited in the cell wall. In order to fulfil the desired and varied functionalities, the polymer chains are modified *in-muro* by pectinmethylesterase (PME) enzymes, which deesterifie the uncharged groups, and subsequently allow binding with Ca^{2+}. It is known from in-vitro studies that PMEs from plants (p-PME) do not simply deesterify the charged groups and that, although establishing the detailed resultant charge distribution and mechanism of processivity is still a work in progress [19], a multiple attack mechanism is a reasonable description of the deesterification process, where a limited number of consecutive residues are processed in each encounter [20]. Such a p-PME action carried out on a pectin of initially high DM will then generate a blocky distribution of formed acidic residues (figure 1b), and consequently improve the calcium binding abilities of the polymer greatly over a randomly deesterified substrate of the same DM.

Previous MR experiments on calcium induced pectin gels [11] showed that these systems can exhibit signatures of semi-flexible network under certain conditions; where, following the subsequent release of a substantial amount of Ca^{2+}, blockwise distributions of the acidic groups lead to the formation of relatively long and stiff dimeric zones. However, despite the fact that p-PME was used to modify the fine structure of the polymer, there are still substantial differences between the conditions

of the previously studied system and the *in-vivo* network. In particular the pectins in the cell wall are of a higher DM than those studied previously and, perhaps even more importantly, the naturally occurring deesterification process may take place in the presence of Ca^{2+}. Therefore, in this work we investigate the gelling behaviour of pectin systems that have been designed to be closer bio-mimetic models of the *in-vivo* pectin networks.

2 Experimental details

2.1 Materials

Pectin (M_w ~150 kDa), extracted from apple pomace with a galacturonic content of ~ 90%, was kindly supplied by CP Kelco ApS, DK 4623 Lille Skensved, Denmark as a starting material. The sample average DM of this sample and all others produced by modification were determined using capillary zone electrophoresis (CE) as previously described [21, 22].

Pectinmethylesterase (PME) from Orange peel [EC 3.1.1.11] was purchased from Sigma Aldrich (P5400) in order to remove methylester groups from the starting pectin substrate [24]. Stock solutions of the enzyme were prepared by dissolving 0.01g of dried PME in 20ml in Milli-Q water, and were stored at -20^{o}C in Eppendorf tubes. Aliquots were thawed prior to conducting experiments and used immediately. This PME has a peak of activity at pH 7.5, and this was maintained throughout our experiments with the use of a buffer. The specific activity of this enzyme is then 2.5 μkat/mg protein.

Polygalacturonase [EC 3.2.1.15] was used to digest the polymeric substrates in order to verify differences in the intramolecular distribution of the calcium binding groups. The enzyme was kindly provided by Jacques Benen from the University of Wageningen. It is a pure endo-PG II isoform from *Aspergillus Niger* [23] and has an absolute requirement for the sugar residues in the active site to be unesterified in order for the chain to be severed, which yields the degradation pattern methylester-sequence dependent. The pectin substrates were digested for 24 hours at 30°C. A 1 ml solution of substrate at a concentration of 0.5%w/w and pH 4.2 (acetate buffer 50 mM) was incubated with 20 μl of enzyme solution (0.094 mg/ml protein). At the end of the reaction, the enzyme was denatured at 90°C for 3 min. Under these conditions, the specific activity of endo-PGII is 36.5 μkat/mg protein. Digest fragments were monitored using CE [22].

$CaCO_3$ powder with a mean particle diameter of 1 μm was kindly provided by Provencale s.a., Avenue Frédéric Mistral, 83172 Brignoles Cedex, France.

Glucono-δ-lactone (GDL) was purchased from Fisher Scientific, Bishop Meadow Rd, Loughbourough, LE11 5RG, UK.

Latex particles of diameter 465 nm (2.62% w/v stock solutions, and fluorescent latex particles of diameter 541 nm (Fluoresbrite plain YG, 2.64% w/v stock solution) were purchased from Polyscience Inc. (Warrington, PA).

It should be noted that in preliminary experiments an effect of the presence of the micro-spheres on the rate of the enzyme induced gelation was observed. It appeared that in the presence of latex beads, samples gelled much more slowly, or in some cases not at all, which we hypothesised as being the result of some enzyme adsorbing to the latex, reducing the effective concentration available to interact with the pectin. Contrary to the pectin chains, PMEs are positively charged at pH 7.5 (isoelectric point pl > 9, [24]), and the slightly negatively charged latex beads, while being ideal for pectin microrheology may encourage the adsorption of enzymes. To solve this problem, a simple pragmatic solution was applied and a larger initial concentration of PME added until gelation was observed in a reasonable time.

2.2 Sample Preparation

Enzymatically induced pectin gels. For MR experiments, stock pectin solutions of 1.8% w/w were prepared by dissolving the apple pectin powder in a 0.01M HEPES buffer made with Milli-Q water, adjusting to pH7.5 with NaOH, and stirring for 1 hour. Subsequently, desired volumes of a $CaCl_2$ salt solution and of the stock bead solution were added in order to get a final concentration of 0.8% of spheres in solution. The quantity of $CaCl_2$ determines the R value, R=2*[Ca2+]/[COO-] which can, by controlling the amount and extent of interchain association, be varied to tune the elasticity of the material. Finally the sample is mixed with the desired volume of water, in order to reach a final polymer concentration of 1%w/w. As the starting pectin is of a high DM, it does not gel in the presence of the added Ca^{2+}. Just before loading the solution in the test chamber, 25μL of enzyme solution is added for 1.5mL of pectin solution, and mixed for 5minutes before starting the measurements.

Controlled calcium release gels. Ionotropic pectin gels were also obtained by slowly releasing calcium ions into solutions of pectin with fine structures determined by prior enzyme processing [25], containing 0.8% v/w of latex beads (DWS) or 0.03% v/w of fluorescent beads (MPT). The appropriate amounts of pectin and bead stock

solutions were mixed and stirred, and immediately prior to loading into appropriate test cells a salt solution was added, the final mixing of which achieves the final desired concentration of all components of the system. This aqueous salt solution was composed of $CaCO_3$ and GDL. The GDL hydrolyses with time, releasing protons that solubilise calcium ions from the $CaCO_3$. These components were introduced as powders into water, quickly mixed, and added to the pectin - bead solution as quickly as possible, in order to avoid significant calcium release before mixing with pectin. The quantity of $CaCO_3$ added determines the R value, as described above, and a stoichiometric ratio of GDL [GDL]=2*[Ca2+] is used in order to maintain the pH of the solution [25]. After the addition of the salts the final prepared solution was stirred for a few minutes, and the samples were loaded into the appropriate test cell and left overnight.

2.3 Microrheology

The aim of microrheology is to extract the rheological properties of soft materials from the motion of probe particles immersed in the material [26, 27]. Passive microrheology, contrary to active microrheology, is the simple study of the particle's thermal motion. It is a non-destructive technique and recovers the linear response of the material, in contrast to active microrheology. For a viscoelastic fluid, the mean square displacement (MSD) of a probe particle will vary as a local power law $\left\langle \Delta r^2(\tau) \right\rangle \sim \tau^{\alpha}$ with $0 \le \alpha \le 1$ depending on the nature of the medium, and τ is the observation time. In order to link the measured MSD to the viscoelastic properties, a generalized Stokes-Einstein relation (GSER) [28] can be used, with the caveats that the fluid is incompressible and the boundary non-slip.

$$\hat{G}(s) = \frac{k_B T}{\pi a s \left\langle \hat{\Delta} r^2(s) \right\rangle} \qquad (4.1)$$

with $\hat{G}(s)$ the shear modulus in the Laplace space, *s* the Laplace frequency and $\left\langle \hat{\Delta} r^2(s) \right\rangle$ the Laplace transform of the MSD. The complex shear modulus $G^*(\omega)$ is the Fourier transform of $G(t)$, with ω the Fourier frequency.

The DWS apparatus used in this study has been fully described previously [29]. The samples were contained in glass cells of width 10 mm, height 50mm, and path length *L* of 4 mm, and were illuminated with a 35 mW He-Ne Melles-Griot laser operating at wavelength λ=633nm. The laser beam was expanded to approximately 8 mm on the surface of the cell. The transmitted scattered light was detected using a single-mode optical fiber (P1-3224-PC-5, Thorlabs Inc., Germany). The optical fiber was connected to a Hamamatsu HC120-08 PMT photomultiplier tube module, and the intensity autocorrelation functions of the scattered light were obtained using a

Malvern 7132 correlator. Tests were run for 10 minutes to ensure low noise intensity autocorrelation functions.

For an expanded beam mode, the field autocorrelation function $g_1(t)$ is obtained from the measured intensity autocorrelation function $g_2(t)$ using the Siegert equation $g_2(t) = 1 + \beta g_1^2(t)$, where β is a constant depending on the instrument. In the transmission geometry, the field autocorrelation function is given by [30]. Once the light mean free path l^* is determined as in [31], the MSD can be obtained from the experimental correlation functions by inverting them with a zero-crossing routine.

3 Results and Discussion

3.1 Enzymatically induced pectin gels

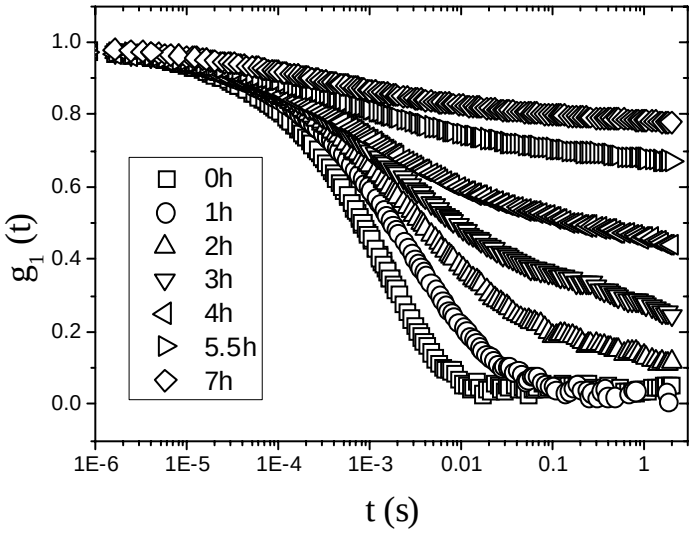

Figure 2: The temporal evolution of the transmission autocorrelation function measured using DWS, on an enzymatically formed pectin gel made as described in the text. The starting DM of the 1%w/w Apple pectin solution is 78%, and the added calcium is equivalent to an R value of 0.3 for an equivalent DM of 60%.

Figure 2 shows the light autocorrelation function $g_1(\tau)$ as a function of the time lag, as measured by DWS, for an initially high-DM pectin sample in the presence of Ca^{2+}, subsequently gelling as a consequence of PME action, as described in the experimental section. The sol-gel transition is clearly reflected in the dynamics of the probe particles. While for the first 2 hours after the addition of PME $g_1(\tau)$ is characteristic of a near Newtonian fluid, i.e. $g_1(\tau)$ goes to zero at long times, after 90 minutes, the correlation function does not decay to zero, showing that a visco-elastic gel is formed. After 5.5 hours, $g_1(\tau)$ tends to be quite flat and remains close to one even at longer time lags, corresponding to the presence of a quasi-elastic material.

MSDs obtained from $g_1(\tau)$ as described in the experimental section are shown in figure 3, for the first 4 hours of enzymatic action. In order to be confident that the extracted MSDs were not significantly influenced by heterogeneities the spatial variation in rheological properties from bead to bead was checked using Multiple Particle Tracking (MPT) [32, 33]. For the studied systems, the Van Hove correlation functions of the probe's Brownian motion were well described by Gaussian distributions, synonymous with spatial homogeneity of the beads motions at this length scale [34]. Corresponding to the indications in the autocorrelation function 90 minutes after the addition of PME, a plateau of MSD appears at low frequency, signaling the formation of a gel. It should be noted that instead of a real plateau, the MSD seems to have a small power law dependence at long times, indicating the material is not a completely elastic.

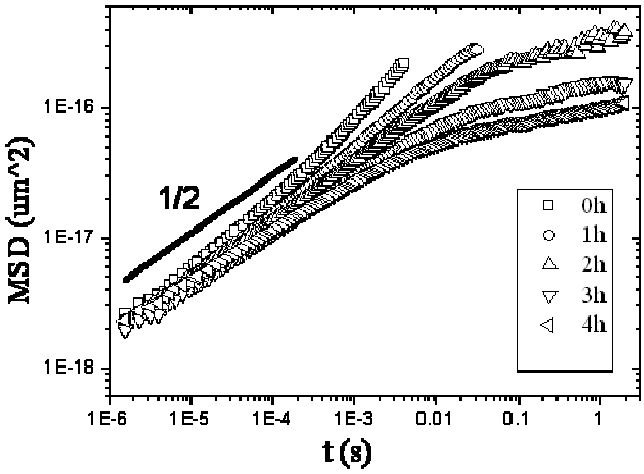

Figure 3: The temporal evolution of the MSD during enzymatically induced gelation, measured using DWS, obtained from processing the data shown in figure 2.

Having clearly been successful in observing gel formation by using PME to release ion-binding groups in the presence of Ca^{2+}; in contrast to the routinely exploited reverse situation where the ions are released into solutions containing pectins with pre-existing pPME-generated binding groups; our attention moved to the high frequency behaviour exhibited by the resultant network. Interestingly, at high frequencies, the MSD varies with τ with a power law with an exponent of ½, and so for all times after PME addition. This is dramatically different from the behaviour found in previously studied pectin gels where the ions themselves were released [11], [35], where the MSD at high frequency was observed to follow $\tau^{3/4}$, one of the main signatures of the presence of semi-flexible networks. In contrast, a slope of ½ in the double logarithmic plot at high frequencies is consistent with the Rouse model for

flexible polymers [36-38]. This exponent, resulting from the relaxation at high frequencies of flexible chains might be expected at the initial time, but the fact that it is conserved as the sample is gelling is a strong indication that the main building blocks in this enzymatically generated gel are flexible polymers, and not stiffer cross-linked dimers of large persistence length whose bending modes would play an important role in determining the materials properties. After 4 hours, there is some indication that this signature of ½ moves to slightly higher frequencies, as the Rouse time decreases for the gelling sample.

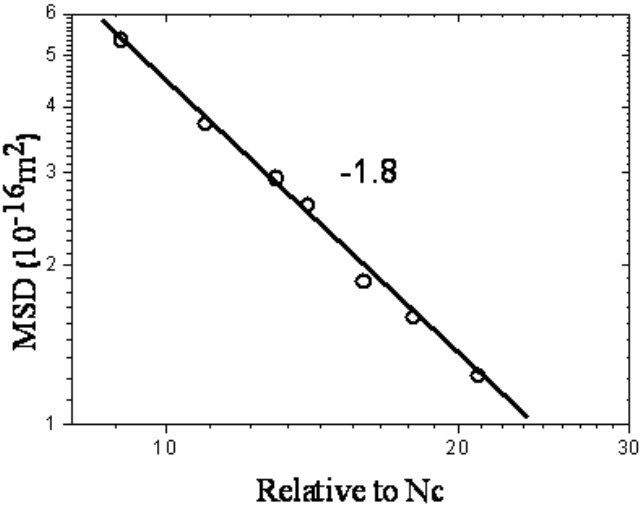

Figure 4: The cross-link density dependence of the long times plateau values of the MSD obtained from the data shown in figure 3, assuming that the cross-links density is proportional to the enzyme action time.

As the high frequency behavior corresponds to small displacements, of the order of 1-10 nm, it predominantly probes the nature of the network's constituent filaments. In order to get some information about the nature and the density of the cross-links, the study of the long time behavior is essential, giving access to the state where large bead displacements can significantly strain the network. In figure 4, the plateau values of the evolving MSDs shown in figure 3 (inversely proportional to the elastic modulus) are plotted against the relative cross-links density N_C, assuming that the number of cross-links is proportional to the time passed since the initiation of enzymatic action [39]. This assumption is reasonable assuming a simple Michaelis-Menton model [40, 41] for the enzymes action and is supported by preliminary results obtained using NMR to measure the rate of methanol liberation [42]. The data is well described by a power law with an exponent of 1.8. While again this is not consistent with the semi-flexible behaviour found in previous studies, it does agree well with previous experiments carried out by DWS micro-rheology on chemically cross-

linked gels made of poly (vinyl alcohol) [43]. While the comparison of the two systems is insightful and a lightly cross-linked semidilute solution of branched flexible polymers seems an appropriate description of our system, as far as we know, that there are no theories concerning the origin of this exponent.

In order to explain the contrasting behaviour observed in the MR experiments depending on the order of Ca^{2+} release and p-PME action we hypothesize the following model: when pectin is pre-processed by pPME with no Ca^{2+} present, the enzyme is free both to continue to create longer charged regions in a single chain contact and also create longer acidic blocks by re-binding the chain in the vicinity of a previously deesterified block. Thus longer charged-residue blocks can be formed, which subsequently, on the introduction of calcium, favour zipping into long stiff dimeric filaments which entangle and lightly crosslink with excess calcium; explaining the semi-flexible nature of these gels ([11]).

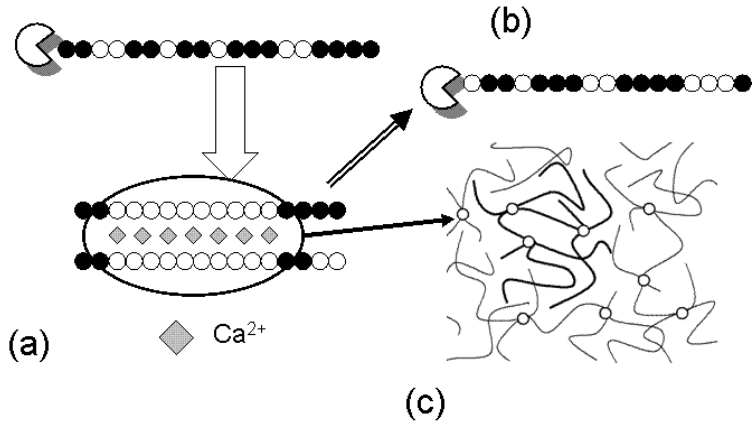

Figure 5: Schematic of the proposed model for the network formation of enzymatically made pectin gels; (a) when ~10 consecutive residues are deesterified by a PME enzyme, a stable calcium-mediated cross-link is formed, (b) protecting this area of the chain from further PME catalysed processing, which continues on another pectin chain, where a new cross-link is formed. (c) Consequently, a randomly branched polymer network is formed.

In contrast, with Ca^{2+} omnipresent as depicted in figure 5, as soon as the enzyme action has created a run of charged residues sufficiently long in order to bind to another similar region on a different chain it will do so. Subsequently, owing both to steric crowding and to conformational changes in the chain concomitant with ion binding and described by the egg-box model [17], the PME is unable to act further around the binding region (figure 5b). Furthermore, additional enzymatic processing in the vicinity of the established crosslink is severely disadvantaged and the PME moves to another pectin chain (figure 5c), where it will create another new calcium binding site by locally charging the polygalacturonic residues. Thus, short dimeric

connections of the minimum length required to bind calcium are distributed through the system acting as punctual chemical cross-links, with single pectin chains forming the network connections between nodes; explaining the crosslink density dependence and the flexible nature of the network elements.

This model agrees well with the experimental results. For a solution of randomly branched flexible polymers, the classic Rouse model [36, 37] predicts the slope of ½ at high frequencies. For times higher than the Rouse time τ_R, the applied constraints are relaxed by reptation of the polymers, which explains the slight diffusion of the probes particles at low frequencies, or equivalently the time dependence of the MSD at long times. τ_R should decrease with an increase of M_W, which is found experimentally, and leads to an increase of the stiffness of the gel. This stiffness scales with N_C with a power law of 1.8, which has been found previously by other groups, but is not predicted by a theory yet. Indeed, there is a lack of theories for the case of semi-dilute solutions of branched flexible polymers, where the De Gennes scaling principles are not applicable [44, 45].

3.2 Fine structure of the resulting polymer

In order to test the proposed model (figure 5) which would imply different polymeric fine structures, and in particular different blocklengths of ion-binding residues when pPME action is accompanied by the presence of Calcium, the polymer was recovered from a gel and its structure analysed. The results obtained from the analysis of this pectin sample were compared to a second sample, modified by pPME but not in the presence of calcium. While this control, pre-processed pectin could easily be recovered for analysis by dialysis against water and freeze drying, the sample modified in the presence of Ca^{2+} results in the formation of a gel, so that these ions need to be removed from the system as part of the extraction procedure. Although a reasonably successful methodology for performing this task was developed, as described below, this requires a moderately deesterified sample in order to keep the gel reasonably weak, and thus facilitate the extraction process. In preliminary experiments, the reaction was quenched shortly after the gelation of a calcium containing deesterified sample was observed visually, and subsequently the resulting polymer extracted. The final DM of the extracted polymer was then determined experimentally by CE and served to determine the rate of DM decrease by PME deesterification, assuming that the DM decrease was linear with the time. The same was done on pectin processed in the absence of calcium. Knowing these two decrease rates, it was possible to reach a target DM by simply adjusting the time of enzymatic action. Interestingly, under the same temperature and buffer conditions, the reactions

in presence of calcium were faster (-5.8%DM/hour) than without (-4%DM/hour). This agrees well with previous work that demonstrated that the action of pPME is boosted by the presence of calcium [46].

To remove the Ca^{2+} from the gel in order to recover and analyse the fine structure of the polymer that had been generated and assembled, two methods were tested. Firstly the gel was added to a solution of the well known chelating agent EDTA. However, after several attempts the resulting solution was still found to exhibit a high viscosity and proved difficult to analyse. This motivated the trial of an alternative method to remove the calcium, by using acidic conditions to protonate the charges on the pectin chains, and thus free the Ca^{2+}. Here the sample was dialysed many times against a pH 2 HCl solution, and ultimately against Milli-Q water. Control experiments confirmed that the pectin chains themselves were not substantially degraded under these conditions. Atomic absorption measurements were used in order to check the quantity of the remaining calcium, and confirmed that only traces of calcium remained in solutions of the extracted material following the acidification protocol, while the samples treated with EDTA contained a substantially higher concentration. Further experiments were therefore carried out with the material extracted by acidification.

In this way, two daughter pectin samples were generated from a single mother material with an initial DM of 78%. Both were deesterified by pPME to a DM value of 62%, as measured experimentally on recovered samples post-processing, but in one case Ca^{2+} had been present during the deesterification process. In order to test the hypothesis advanced to explain the microrheological data; that is, that the presence of Ca^{2+} during pPME action limits the length of charged blocks, the intramolecular distribution of methyl esterification in these two samples was probed. In order to do this, a fragmentation approach was utilised in which the polymer chains are disassembled and attempts are made, based on derived digests, to infer something of the properties of the pre-digested substrate.

Figure 6 (a) and (b) show the CE results of incubating the engineered pectic polymers with a degrading enzyme, a polygalacturonase from the fungus Aspergillus niger, endo-PG II, as described in the experimental section. It has previously been shown that the separation of the resultant oligomeric digest fragments can be performed by CE [22], as used here, and the peaks are labelled according to the assignments made in previous work. (The notation n^m is used throughout in order to indicate an n-mer of galacturonic acid with m groups methylesterified.) The use of such digests in inferring the intramolecular methylesterification properties of the pre-digested substrate has been the subject of much recent interest [47, 48]. It is not the purpose in

this study to discuss, or to model in detail, the enzymatic degradation process, rather to use established wisdom to demonstrate that the pectin substrate generated in the absence of Ca^{2+} has a longer average blocklength of unesterified residues compared with its sister sample.

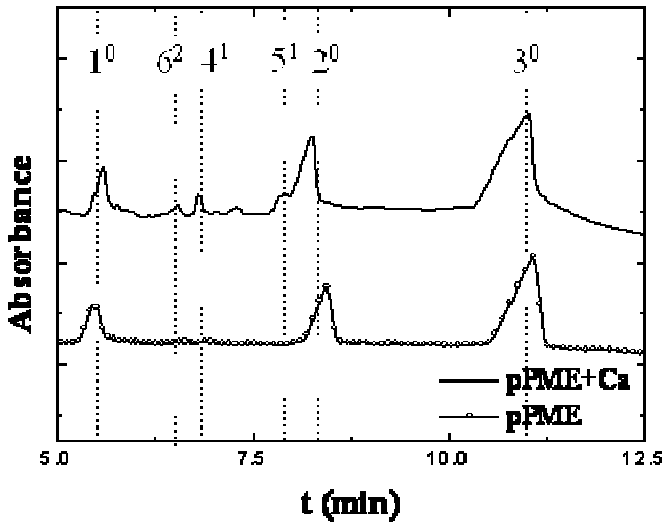

Figure 6: Electropherograms showing the oligomeric fractions resulting from incubating the engineered pectic polymers with endo-PG II, as described in the experimental section. The fine structures have been obtained by deesterification with pPME in the presence and absence of Ca^{2+}. Tentative assignments of the partially methylesterifed fragments have been made based in previous work

It is generally accepted that more blocky pectic polymers release more un-methylated fragments as digest products when compared to less blocky substrates of the same DM (endo-PG II has a strict requirement for unesterified residues in its active site and a preference for unesterifed substrates). Indeed, a blockiness scale has been proposed based on the amount of galacturonic acid released in the form of un-methylated mono-, di- and trimers [48]. In addition the ratios of liberated monomeric galacturonic acid to the dimer and trimer are dependent on blocklength to some degree. However, it can be shown, by considering the results obtained from the digestion of unesterified galacturonic acid oligomers of varying degrees of polymerisation [49] that the ratio of monomer / dimer / trimer released from unesterified blocks whose length is only in excess of around 10 would already be indistinguishable experimentally. Lastly, it seems reasonable that differences in the amount of small partially methylesterifed fragments liberated would also be indicative of differences in the intramolecular pattern of methylesterification; with larger amounts of these arising from less blocky, more punctuated structures.

Considering the data shown in figure 6 we can ascertain i) that large amounts of mono- di- and tri-galacturonic acid have been liberated by the endo-PG II digest of both samples compared with what would be expected for randomly deesterified substrates [22] indicating some kind of blocky sequence in both substrates ii) that as the ratios of the monomer to dimer and trimer are similar the blocks in both cases should be larger than approximately 10 residues and iii) it is particularly clear that there is a significantly greater presence of intermediate peaks corresponding to partially methylesterified fragments for the pectin made in presence of Ca^{2+}, giving a strong indication that indeed this intramolecular distribution does contain shorter blocks.

3.3 Controlled release gels of pectins whose architecture was laid down by pPME in the presence of calcium

Having recovered pectic polymers from the enzymatically created bio-mimetic gels described herein, enabling structural evidence that a distinct fine structure is evolved under these conditions to be gathered, we sought to demonstrate further that this was indeed the dominant effect in determining the flexible nature of the networks by re-gelling these polymers using a conventional methodology. A calcium induced gel of the pectin retrieved from the enzymatically triggered gel was made using a slow release method as described in the experimental section [25]. Specifically, a gel was made with 1% w/w of this pectin, with an R value of 0.05 and was studied via DWS. In addition the experiment was repeated using the sister 62% DM sample that was deesterified in the absence of calcium.

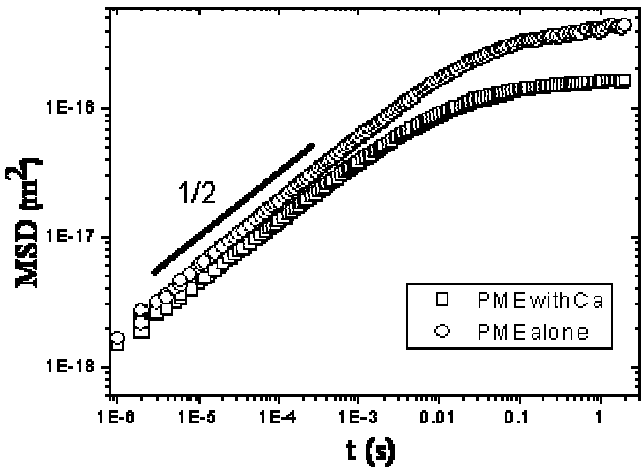

Figure 7: The resulting MSDs obtained from probe latex particles embedded in gels formed by the slow release of calcium (as described in the experimental section) for two samples of DM 62 which have been obtained by deesterification with pPME in the presence and absence of Ca^{2+} respectively.

Figure 7 shows the resulting MSDs recorded for the two DM 62 samples. Firstly, it is clear that the microrheological data obtained from the gel formed from pectin that had previously formed the enzymatically created gel still shows the $\tau^{1/2}$ power law at high frequencies. It is also worth noting that this is despite of the fact that the slow calcium release methodolgy yields quite different assembly kinetics. Again this supports the contention that by having Ca^{2+} present during deesterification a flexible network is formed owing to the modified fine structure created, and that indeed if this fine structure can be generated in some initial processing even gels subsequently formed from it by conventionally introducing ions will exhibit the flexible nature.

Secondly, it is interesting to note that the sample deesterified in the absence of calcium also exhibits the same high frequency behaviour. This would seem to indicate that simply by limiting the amount of pPME deesterification (78 – 62% DM here) a fine structure consisting of small blocks of unesterified galacturonic acid can be formed that, while of a length in excess of that required for the co-operativity of Ca^{2+} binding to ensure the formation of stable junction zones, might not be considerably longer than those formed with Ca^{2+} present. This supports the idea that has been emerging from other studies [19, 20] that pPMEs may, in any case, have limited processivity following a single binding event. Extremely blocky structures found after more extensive removal of methylesters [20] would then be largely a result of multiple attacks in the same region of the chain rather than been formed in one contiguous run.

While for both samples the MSD follows the $\tau^{1/2}$ power law at high frequencies, the long time plateau is lower for the pectin deesterified in presence of Ca^{2+} by a factor of approximately 2, indicating a more elastic gel. A simple interpretation of this behaviour is that the cross-link density is higher for the fine structure generated in the presence of Ca^{2+}. This is entirely consistent with evidence from the structural analysis which suggests that the average block length is shorter for this sample, so that by implication there are more blocks (and a higher potential cross-link density) for a fixed DM. This strongly suggests that in situations where the required function of pectin is to assemble with calcium into a network which is the most elastic possible then such a specific pattern of short blocks is the optimal solution. Indeed, by using calcium binding itself to restrict the further growth of runs of charged sugars in the generation of the fine structure, the length of the unesterified blocks is set at the minimal number of residues required to form a stable calcium bond and thus, the cross-link density in subsequently formed gels is maximized. It is also interesting to note that although the presence of calcium clearly does contribute to the optimisation, and would be expected to so even more significantly at lower DM values, it looks

increasingly likely that the processivity of pPME has evolved to be in at least the same ballpark as that of the minimum blocklength required to form a stable intermolecular association. If this is the case then, at the relatively high DMs studied here, which noteably are close to those actually found in the cell wall, simple pPME action might also select for elastic flexible networks.

In order to further demonstrate these ideas a relatively minor deesterification (8% DM) of a relatively high starting DM material (78% DM) was performed with pPME in the presence and absence of calcium; essentially as described previously, but this time creating samples of 70% DM, instead of 62%. We reasoned that in both cases we had inserted short, isolated, ion-binding blocks into the polymeric backbone, optimised for calcium binding where the ion was included in the deesterification process and slightly longer on average in the case without calcium, depending on the exact processivity of the pPME. Furthermore, we considered that the only difference between these samples and the 62% DM samples was the number of these calcium-connectable blocks. In order to test this idea we carried out microrheological experiments on these systems using a controlled calcium release methodology, and introduced the identical concentration of Ca^{2+} ions into the pPME generated samples of 62 and 70% DM; and into the samples generated by pPME in the presence of calcium of 62 and 70% DM. By ensuring that this calcium concentration used was significantly lower than that required to saturate even the available 70% DM sites we hypothesised that if indeed the total number of available calcium-connectable blocks was the only difference, the samples should exhibit equivalent rheological responses.

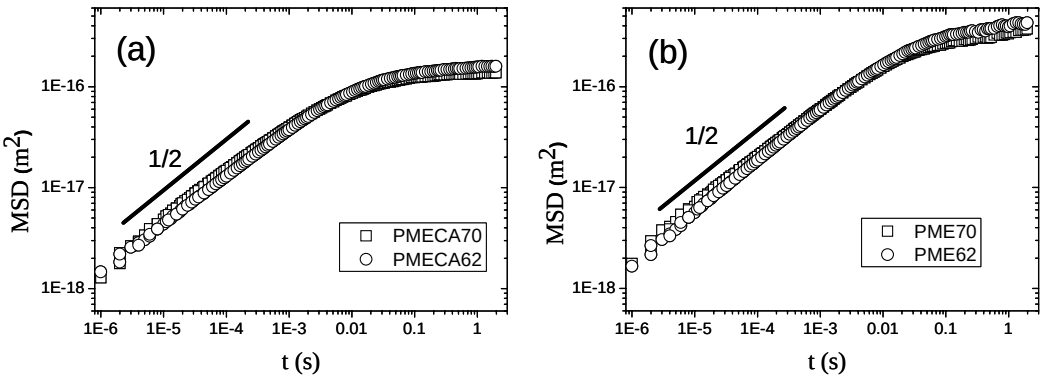

Figure 8: The resulting MSDs obtained from probe latex particles embedded in gels formed by the slow release of calcium (as described in the experimental section) for the pectin samples of DM 62 and 70, that had been modified with pPME in the (a) presence and (b) absence of calcium; gelled with the same low concentration of calcium as described in the text.

Figure 8 (a) and (b) shows the results from these experiments for the pectin samples that had been modified with pPME in the presence and absence of calcium

respectively. It is indeed clear that, with the number of calcium-mediated cross-links limited by the available calcium, the response from the DM 70 and DM 62% samples is indistinguishable within experimental uncertainty. It is remarkable to see how elastic this calcium induced gel of a 70% DM pectin is; randomly distributed fine structures would be expected to have more than 50% of the methyl groups removed before any sort of gel could be formed. A preliminary experiment carried out in a conventional rheometer showed that these enzymatically made gels can reach an elastic modulus of a few thousand Pascal, which is very large for pectin gels of this polymer concentration of 1%w/w.

4 Conclusion

The ionotropic gelling behaviour of the ubiquitous cell wall polysaccharide pectin has been investigated with DWS using polymeric architectures that have been designed to be realistic bio-mimetic models of *in-vivo* fine structures. These have been engineered by 1) releasing a limited number of calcium binding groups using p-PME and 2) by using pPME to liberate such binding sites *in the presence of calcium-ions*, rather than subsequently releasing calcium into solutions of pre-processed pectins. In stark contrast to the semi-flexible network paradigm of biological gels and previous work on pectin, it has been shown that when the length of ion-binding regions is restricted, gels are formed that exhibit the properties of chemically cross-linked networks of flexible polymers (the high frequency moduli scale with $\omega^{1/2}$, and with a cross-link density dependence of the elastic plateau follows $N_C^{1.8}$).

The ability of plants to control local polymer, ion and enzyme concentration, suggests therefore that both semi-flexible *and* flexible networks may be relevant *in-vivo*, and could provide different functionalities. For example, while the semi-flexible networks previously observed are more likely to exhibit stain hardening, the flexible networks described herein optimise the elasticity of calcium-bonded networks by utilising a specific pattern of short ion-binding blocks. It is also interesting to consider that these network types will have vastly different transport properties and the ability to select between these may well play a role in controlling the porosity of the cell wall.

The work clearly suggests which polymeric fine structure maximises the elastic properties of calcium-induced networks, and shows how Nature might be tailoring such architectures by using PMEs of optimised processivity or controlling the local calcium concentration. Further, it is interesting to see that studying the physics of resultant networks can yield conclusions regarding the action patterns of important remodelling enzymes.

Acknowledgments

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Chapter V – General discussion – A unifying framework for the different networks

As submitted in:

R. R. Vincent, M. A. K. Williams, *Microrheological investigations give insights into the microstructure and functionality of pectin gels*, submitted to a special issue of Carbohydrate Research.

Abstract

Many of the functional attributes of pectin, whether in the plant cell wall or in engineered food materials, are linked to its gelling properties and in particular its ability to assemble in the presence of calcium. Pectin's fine structure and local concentration relative to that of its cross-linking ion play a major role in determining resultant gel micro-structures, and consequently the mechanical and transport properties of pectin matrices. Recent studies have sought to probe the basic properties of such calcium induced matrices, using a light scattering technique called diffusing wave spectroscopy (DWS). Microrheological measurements carried out with DWS are able to determine the high frequency mechanical behavior, which is closely linked to the response of the basic strands of the network; in addition to the low frequency behavior, which provides information about the nature and density of cross-links. By using these microrheological measurements, two distinct regimes have been identified into which pectin gels fall: one corresponding to the presence of semi-flexible networks, a generally accepted paradigm in biological gels, and another where flexible networks dominate. In order to explain the origin of these dramatically different networks, distinct assembly pathways have been proposed in which the relative importance of the free energy gained by association and the frictional barrier to polymeric re-arrangement during network formation can differ significantly. By manipulating the local environment in the plant cell wall it is possible that Nature makes full use of both of these network types for fulfilling different tasks; such as providing strain-hardening, maximizing local elastic properties or controlling macromolecular transport.

Keywords: pectin gels, calcium association, microrheology, networks, mechanical properties

1 Introduction

The gelling abilities of pectin have been exploited by humans for a long time. The Romans learned from the Greeks that quinces, a pectin rich fruit, slowly cooked with honey would set when cooled, giving the recipe for Roman marmalade [1]. Since then, pectin has gained substantial interest from a plethora of fields, as predicted by Braconnot [2], who wrote in 1825 that pectin plays an important role in the plant functions, and will have many applications in the art of the confiseur. Indeed, research from plant biologists and food scientists on pectin has more than validated these two predictions, and currently pectin is of interest in a myriad of research areas including its possible medicinal benefits for the treatment of many diseases [3-5]. These varied mechanical and chemical functionalities are possible only due to pectin's large fine structure variability and rich interaction possibilities.

Pectin is a ubiquitous polysaccharide of the plant cell wall, where it is known to play various mechanical roles; well-studied examples including controlling the mechanical properties of the pollen tube during growth [6] and mediating cell-cell adhesion [7]. Although the complete *in-vivo* fine structure of this complex polysaccharide is still a matter of debate [8, 9], it is known that the homogalacturonan sections play the major role in determining its gelling abilities [10]. Homogalacturonan, which comprises the major part of commercially available pectins (typically > 85%), is a linear co-polymer consisting of galacturonic acid residues and its methyl-esterified counterpart. For both the Golgi-synthesized biological substrate and the typical commercially extracted pectins, the relative quantity of the methyl-esterified residues (Degree of Methyl-esterification, DM) is high, and these polymers do not gel in presence of the main biological cross-linking agent, calcium cations.

In the plant cell wall, when calcium cross-linking is physiologically required, pectin is deesterified *in-muro* by pectinmethylesterase (p-PME) enzymes. This deesterification takes place in the presence of a locally controlled concentration of its main binding ion, calcium. In contrast, for industrial applications, pectins extracted from apple pommace or citrus peels are typically deesterified enzymatically or chemically before being sold, in order to increase calcium sensitivity and, when used in applications, are gelled with the subsequent introduction of Ca^{2+}. It is well known that the use of different chemical or enzymatic methods of deesterification leads to different patterns in the intramolecular distributions of the liberated charged residues: pectinmethylesterase enzymes from land plants (p-PME) are known to produce a somewhat blocky pattern, while PMEs from fungus or a deesterification carried out under alkali conditions will result in a random distribution of the exposed

polygalacturonic acid residues. This large variability in the resultant polymeric fine structure gives ample opportunity to control the physical properties of resulting calcium induced gels.

Indeed, a specific number of consecutive unesterified galacturonic acid residues are required to form a stable egg box junction zone with calcium with estimates for this number varying between 8 and 15 [11, 13]. It is unsurprising then that, in general, a pectin sample with a blocky intramolecular DM distribution will tend to gel more easily, at an equivalent DM, when compared to a randomly patterned substrate. In order to characterize how "blocky" a particular pectin is, two different degrees of blockiness (DB [14], and Db_{abs} [15]) have been defined, based on the quantity of short galacturonic acid oligomers which are liberated from the substrate by pectin degrading enzymes.

While the precise detail of the binding of calcium with pectin is still an active area of research, the egg-box model [11] provides an excellent framework for its investigation; motivated by the success of this model in describing the association of calcium with alginate, another anionic polysaccharide molecule with several similarities to pectin. More recently the interaction with calcium has been investigated by sophisticated molecular modeling techniques [12]; which, for both polymers, corroborated the idea of a two-stage process in the mechanism of calcium assembly, where the formation of strongly linked dimers is followed by the formation of weak inter-dimer associations. Such a multi-step mechanism has been verified experimentally for alginate by SAXS [16], and in addition a third 'early' mono-complexation stage in the binding process has been suggested by calorimetric experiments [17], corresponding to the interaction of Ca^{2+} with sugar residues prior to egg-box formation. Although it is tempting to assume the same behaviour will be found for pectin systems, such direct comparisons currently lack relevant experimental evidence. At present, no SAXS data exists for pectin systems and while the calcium association processes in alginate and amidated pectin has been compared using isothermal titration calorimetry (ITC) [18], which is undoubtedly of great interest in its own right, the presence of the positively charged residues on the polymer backbone is expected to have a significant effect, rendering the prediction of behaviour for unamidated pectin difficult.

While the ionic associations have been previously studied in dilute solution, in particular by electrochemical methods [19, 20], little is known about how the Ca^{2+} association in more concentrated solutions orchestrates the network assembly and how relevant dynamics might by harnessed in order to generate distinctly different

functional networks. While state-of-the-art microscopy can generate high resolution images, it is still difficult to imagine the details of the self-assembly mechanism and the constitution of the network strands from the observation of the final pre-formed gel [21-23]. Furthermore, such high resolution images of networks require complex sample preparation that, while skillfully mastered by a few groups, yields direct imaging of network strand dynamics challenging.

An alternative way of obtaining information on the dynamics of the stress bearing filaments of the network is to study the rheological behavior of the induced materials. Bulk rheometers are routinely used to characterize visco-elastic materials and indeed the linear mechanical properties of calcium induced pectin gels have been investigated previously using such techniques [24-26]. However, classic rheometry offers limited information about the micro-structure of the studied material owing to the relatively narrow frequency range accessible. It is typically limited to obtain information at low frequencies, where studies potentially measure an elastic plateau and provide data primarily related to the cross-link density. In order to learn about the basic constitutive strands of the network, the study of the high frequency behavior is essential. While recent advances have been made in the area of the development of high frequency rheometers [27, 28], microrheological techniques (MR) [29, 30] and in particular a well established multiple-light-scattering technique called diffusing wave spectroscopy (DWS) still surpass in this area.

The first reported MR studies carried out on calcium induced pectin gels have shown that these systems can exhibit the signatures of semi-flexible networks [31, 32], a paradigm for biological gels [33]; while more recent results have demonstrated that they can also behave as punctually cross-linked flexible networks [34]. Here we discuss a unifying framework encompassing of the origin of both behaviours; show that the same pectin can be assembled so as to exhibit either kind of network, and tentatively propose a state diagram for the different modes of association of pectin chains.

2 Materials and methods

2.1 Materials

Pectin (M_w ~30-100 kDa), extracted from apple pomace, was purchased from Fluka Biochemika (Sigma Aldrich, Switzerland) as a starting material. The sample average DM of this sample and all others produced by modification were determined using capillary zone electrophoresis (CE) as previously described [35, 36].

Pectinmethylesterase (PME) [EC 3.1.1.11] was purchased from Sigma Aldrich (P5400) in order to remove methylester groups from the starting pectin substrate [37]. Stock solutions of the enzyme were prepared by dissolving 0.01 g of dried PME in 20 ml in Milli-Q water, and were stored at -20 ^{o}C in Eppendorf tubes. Aliquots were thawed prior conducting experiments and used immediately. This PME has a peak of activity at pH 7.5, and this was maintained throughout our experiments with the use of a buffer, as described below.

Polygalacturonase [EC 3.2.1.15] was used to digest the polymeric substrates in order to verify differences in the intramolecular distribution of the calcium binding groups. The enzyme was kindly provided by Jacques Benen from the University of Wageningen. It is a pure endo-PG II isoform from *Aspergillus Niger* [38] and has an absolute requirement for the sugar residues in the active site to be unesterified in order for the chain to be severed, which yields the degradation pattern methylester-sequence dependent. The pectin substrates were digested for 24 hours at 30 °C. A 1 ml solution of substrate at a concentration of 0.5%w/w and pH 4.2 (acetate buffer 50 mM) was incubated with 20 µl of enzyme solution (0.094 mg/ml protein). At the end of the reaction, the enzyme was denatured at 90 °C for 3 min.

$CaCO_3$ powder with a mean particle diameter of 1 µm was kindly provided by Provencale s.a., Avenue Frédéric Mistral, 83172 Brignoles Cedex, France.

Glucono-δ-lactone (GDL) was purchased from Fisher Scientific, Bishop Meadow Rd, Loughbourough, LE11 5RG, UK.

Latex particles of diameter 465 nm (2.62% w/v stock solutions) were purchased from Polyscience Inc. (Warrington, PA).

2.2 Pectin fine structure engineering

Pectin solutions of 1%w/w were made by dissolving the pectin powder in a 50mM HEPES buffer, adjusted to pH 7.5 with NaOH. A $CaCl_2$ salt was then added for the p-PME deesterification in the presence of Ca^{2+}, or the equivalent quantity of Milli-Q water when the deesterification was performed without Ca^{2+}. The quantity of added $CaCl_2$ was in slight excess over the final quantity of deesterified residues, in order to ensure that every created block capable of binding calcium into a stable junction did so at any stage of the reaction. 0.5 mL of enzyme solution was then mixed into 30mL of solution, and the solutions were left at 20 ^{o}C for a chosen time, depending of the final DM decrease required and of the rate of the deesterification processes. Assuming the enzymes followed a simple Michaelis-Menton model [46] and were in

the linear section of the product production curve (which was monitored independently using NMR to follow the liberation of methanol), preliminary experiments were used to determine the rate of DM decrease as -4%/hour for the p-PME deesterification without Ca^{2+}, and -5.8%/hour in presence of Ca^{2+}.

Thus, the PME action could be stopped after a pre-requisite time in order to achieve a desired final DM. This was carried out by decreasing the pH and subsequently heating the solution at 80 ^{o}C for 5 mins to denature the enzyme. For a final DM of 62%, this was performed easily when no calcium was present, although a strong mixing was necessary to break the gel formed in presence of calcium [34]. Subsequently, the modified polymers were extracted by dialysing the sample twice under acidic conditions against a 10^{-2} M solution of HCl, and finally twice against Milli-Q water. The dialyzed solutions were freeze dried and the engineered polymers recovered and stored dry. The recovered pectin samples had their fine structures analysed by capillary electrophoresis (CE [37]), characterising the sample average degree of methyl-esterification of the pectin, and providing qualitative information about the distribution of the acidic residues by analysing the endo-PGII-digest products [39,40].

2.3 Enzymatically induced pectin gels

For MR experiments, stock pectin solutions of 1.8%w/w were prepared by dissolving the pectin powder in a 50 mM HEPES buffer made with Milli-Q water, adjusting to pH 7.5 with NaOH, and stirring for 1 hour. Subsequently, desired volumes of a $CaCl_2$ salt solution and of the stock bead solution were added in order to achieve a final concentration of 0.8% of microspheres in solution. As described in section 4.2, the quantity of $CaCl_2$ was again in excess when compared to the final quantity of deesterified residues. Finally the sample was mixed with the desired volume of water, in order to reach a final polymer concentration of 1%w/w. As the starting pectin is of a high DM, it does not gel in the presence of the added Ca^{2+}. Just before loading the solution in the test chamber, 25 µL of enzyme solution is added for 1.5 mL of pectin solution, and mixed for 5 minutes before starting the measurements.

2.4 Calcium induced gels

Ionotropic pectin gels were also obtained by slowly releasing calcium ions into buffered (HEPES 50 mM) solutions of pectin with fine structures determined by prior enzyme processing [41], containing 0.8% of 465 nm latex beads. The appropriate amounts of pectin and bead stock solutions were mixed and stirred, and immediately

prior to loading into appropriate test cells a salt solution was added, the final mixing of which achieves the final desired concentration of all components of the system. This aqueous salt solution was composed of $CaCO_3$ and GDL. The GDL hydrolyses with time, releasing protons that solubilise calcium ions from the $CaCO_3$. These components were introduced as powders into water, quickly mixed, and added to the pectin - bead solution as quickly as possible, in order to avoid significant calcium release before mixing with pectin. The quantity of $CaCO_3$ added determines the R_{eff} value, described in the text, which can, by controlling the amount and extent of interchain association, be varied to tune the elasticity of the material. R_{eff} is related to the R value R = $[Ca^{2+}]$ / $[COO^-]$, which is commonly used to describe the calcium binding to pectin and alginate, as described in the main text. A stoichiometric ratio of GDL [GDL] = 2 * $[Ca^{2+}]$ is used. After the addition of the salts the final prepared solution was stirred for a few minutes, and the samples were loaded into the appropriate test cell and left overnight.

2.5 Microrheology

The aim of microrheology is to extract the rheological properties of soft materials from the motion of probe particles immersed in the material [29, 30]. Passive microrheology, contrary to active microrheology, is the simple study of the particle's thermal motion. It is a non-destructive technique and recovers the linear response of the material. For a viscoelastic fluid, the mean square displacement (MSD) of a probe particle will vary as a local power law $\left\langle \Delta r^2(\tau) \right\rangle \sim \tau^{\alpha}$ with $0 \le \alpha \le 1$ depending on the nature of the medium, and τ the observation time. The measured MSD can be linked to the viscoelastic properties, through a generalized Stokes-Einstein relation (GSER) [42], with the caveats that the fluid is incompressible and the boundary non-slip, such that:

$$\hat{G}(s) = \frac{k_B T}{\pi a s \left\langle \hat{\Delta} r^2(s) \right\rangle} \qquad (5.3)$$

with $\hat{G}(s)$ the shear modulus in the Laplace space, *s* the Laplace frequency and $\left\langle \hat{\Delta} r^2(s) \right\rangle$ the Laplace transform of the MSD. However, valuable physical insight can be gained purely from the behaviour of the MSD, with various well-established theories making distinct predictions based on different microstructural models.

The DWS apparatus used in this study has been fully described previously [43]. The samples were contained in glass cells of width 10 mm, height 50mm, and path length *L* of 4 mm, and were illuminated with a 35 mW He-Ne Melles-Griot laser operating at wavelength λ=633nm. The laser beam was expanded to approximately 8 mm on

the surface of the cell. The transmitted scattered light was detected using a single-mode optical fibre (P1-3224-PC-5, Thorlabs Inc., Germany). The optical fibre was connected to a Hamamatsu HC120-08 PMT photomultiplier tube module, and the intensity autocorrelation functions of the scattered light were obtained using a Malvern 7132 correlator. Tests were run for 10 minutes to ensure low noise intensity autocorrelation functions.

For an expanded beam mode, the field autocorrelation function is $g_1(t)$ is obtained from the measured intensity autocorrelation function $g_2(t)$ using the Siegert equation $g_2(t) = 1 + \beta g_1^2(t)$, where β is a constant depending on the instrument. In the transmission geometry, the field autocorrelation function is given by [44]:

$$g_1(t) = \frac{\frac{L/l^* + 4/3}{z_0/l^* + 2/3}\left\{\sinh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right] + \frac{2}{3}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\cosh\left[\frac{z_0}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right]\right\}}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right] + \frac{4}{3}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\cosh\left[\frac{L}{l^*}\sqrt{k_0^2\left\langle \Delta r^2(\tau)\right\rangle}\right]} \quad (5.4)$$

where l^* is the scattering mean free path, z_0 the penetration depth (assumed here to equal l^*), $k_0 = 2\pi / \lambda$, and L the sample thickness (4 mm here). l^* is obtained by performing an experiment on a water sample using 0.8% of latex beads, and fitting l^* using the accepted viscosity. Subsequently l^* for future samples is obtained by scaling the value obtained for water, based on the change in transmitted intensity when the sample is introduced, compared to the water experiment. It is known that for non-absorbing slabs of thickness L, the transmitted intensity is directly proportional to $(l^*/L)(1+4l^*/3L)$, so that by measuring the change in transmittance, the change in l^* can be calculated [52, 53]. Once l^* is determined, the MSD can be obtained from the experimental correlation functions by inverting Eq (4) with a zero-crossing routine. That is; for each time point, the value of $\left\langle \Delta r^2(\tau)\right\rangle$ that is required in order for Eq. 4 to match the experimentally measured $g_1(t)$ is determined.

3 Results and discussion

3.1 Characterization of the different regimes

Figure 1 shows the evolution of the mean square displacement (MSD) of embedded tracer particles versus lag time, measured during the gelation of an enzymatically induced pectin gel, made as described in detail in the experimental section 4.3. In this system, the initially high DM pectin is impotent to bind the calcium ions (which are

already present) into stable junction zones, until gelling is initiated due to a release of calcium binding sites by the addition of p-PME. This kind of gelation mechanism, where pectin is deesterified in the presence of calcium, might present a reasonable mimic of gel formation *in-vivo*. Remarkably, throughout the gel formation, the MSD at short times (high frequency) scales with the time lag, τ, according to a power law with an exponent close to one half. This is significant as it is consistent with the predictions of Rouse theory [45], i.e. with the presence of a network made of *flexible* polymers.

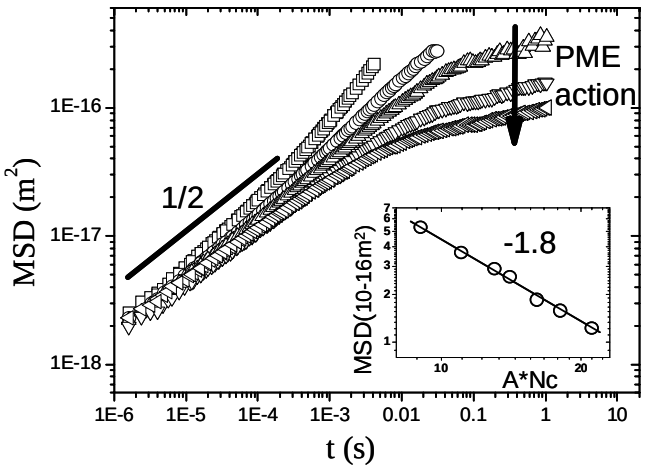


Figure 1: The temporal evolution of tracer particle MSDs versus lag time, measured using DWS, during the formation of an enzymatically triggered pectin gel made as described in the text. The starting DM of the 1%w/w pectin solution is 78%, and the added calcium is equivalent to an R value of 0.3 for an equivalent DM of 60%. Inset: The plateau value of the MSD (10^{-16} m^2), taken at 0.1 s, plotted against the time after introducing the enzyme, assumed to be proportional to the cross-link density N_C.

To further test this idea, the long time behavior, which is linked to cross-links present in the network, was also studied. Assuming that the de-esterfication process follows Michaelis-Menton kinetics [46], and that each substrate encounter yields a stable calcium binding site of the same galacturonic acid block length, then the number of cross-links, N_C, will increase linearly with time after enzyme addition. Thereby, a plot of the MSD against time after enzyme addition is essentially equivalent to investigating how the network modulus (proportional to the reciprocal of the MSD) scales with the cross-linking density (proportional to the enzyme action time). The inset in figure 1 shows such a plot of the low frequency MSD, taken at 0.1 Hz, versus time after enzyme addition, on double logarithmic axes, where a power law scaling with an exponent of -1.8 is evident. This is indeed consistent with previous work carried out on chemically cross-linked networks of *flexible* polymers [47]. To

summarize: both the measured high frequency scaling of the MSD and the cross-link density dependence of the low frequency data are consistent with the presence of a network of randomly branched nature, in which the cross-links are short and punctual, and are bridged by flexible strands. It has been previously hypothesized [34] that these punctual cross-links are a result of the presence of short blocks of charged residues of the minimal length possible in order to form a stable calcium-mediated junction zone, which arise from p-PME action being curtailed by the presence of calcium facilitating intermolecular interactions that prevent the enzyme from acting again around the same position.

In order to characterize the pattern of ion-binding groups generated in such an experiment, and to provide further evidence that indeed the intra-molecular charge distribution might be modified by performing deesterification *in the presence of calcium*, two pectins were engineered from the same starting material. One was deesterified by p-PME in presence of Ca^{2+} and one without Ca^{2+}, as detailed in the experimental section; where the pectin engineered in the presence of its binding ion is hypothesized to contain shorter blocks of acidic residues. While capillary electrophoresis (CE) gave a sample average DM of 62% for both pectins, the analysis of the products liberated when the samples were digested by the polygalacturonase, endo-PGII, revealed a notable difference in the average block length, as the digestion of the polymer made with PME in presence of calcium (PME62Ca) exhibited intermediate peaks in the electrophoregram corresponding to partially methylesterified oligomers, not present for the other pectin (PME62) [34]. This is a strong sign that indeed PME deesterification in presence of Ca^{2+} does produce shorter galacturonic acid blocks when compared with the outcome of the PME action without Ca^{2+} present. These short blocks facilitate the formation of punctual cross-links and flexible networks.

Figure 2 shows the MSDs of embedded tracer particles, measured by DWS, for gels made by a slow release of calcium into solutions of the two polymer substrates engineered as described above, and detailed in the experimental section. The chosen conditions were a relatively high polymer concentration, 1%w/w, and an R value equal to 0.05, i.e. a low calcium concentration. A low R value would be expected to result primarily in the formation of egg-box dimeric associations as suggested by SAXS [16] and calorimetric experiments [17] on the interaction between alginate and calcium. Typically alginate has a blocky distribution of its guluronic acid residues (unless specifically engineered to be alternating [48]), so it might be expected that blocky pectins would exhibit similar interactions with calcium. It is clear that the slope of the MSD at high frequencies (short times) on the double logarithmic plot

again follows the Rouse model [45, 49], as shown in figure 1, scaling with time with a power law with an exponent of ½ for both kinds of pectin. This is again consistent with the formation of short dimeric cross-links acting as punctual chemical cross-links in a solution of flexible polymers. While this was expected for the PME62Ca sample containing short charged blocks capable of forming dimeric egg box zones, these results also suggest that PME62 pattern is also made of relatively short blocks, despite the fact that the action of p-PME enzymes is well-known (at least for substrates modified to low DM) to produce very blocky charge distributions. This is further evidence that the degree of processivity of PMEs may in fact be relatively small [50, 51] yielding patterns of unesterified galacturonic acid residues being described by numerous relatively short blocks at high DM, with longer blocks emerging at higher degrees of deesterification.

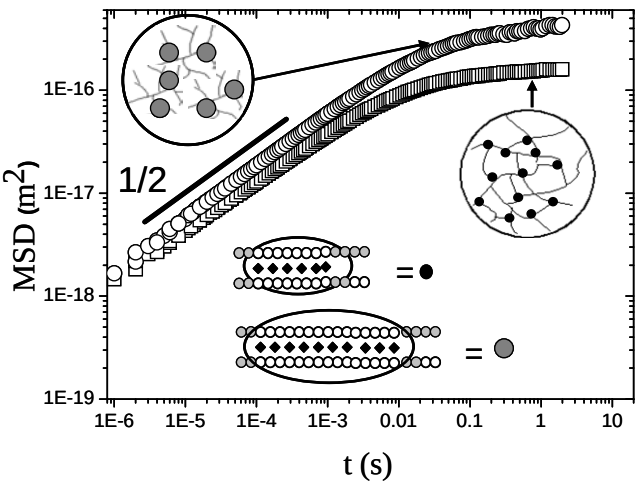


Figure 2: The resulting MSDs obtained from probe latex particles embedded in gels formed by the slow release of calcium (as described in the experimental section) for two pectin samples of DM 62% which have been obtained by deesterification with pPME in the presence (squares) and absence of Ca^{2+} (circles) respectively. The polymer concentration is 1%w/w, and the calcium concentration is equivalent to an R value of 0.05. The PME62 sample (containing longer but less numerous calcium junction-zone forming runs) is more characteristic of a branched polymer solution while the PME62Ca sample (containing shorter but more numerous charged calcium junction-zone forming runs) has a higher cross-link density and can be viewed more as a rubber-like network.

The analysis of the plateaus of the MSDs seen at longer times in figure 2 is also consistent with the presence of shorter blocks for the pectin deesterified in the presence of calcium, PME62Ca, as the MSD plateau is lower than that obtained from the gel made with the PME62 sample, a sign of a higher cross-link density. As the (limiting) Ca^{2+} concentration is the same in both gels, this implies that the length of

the calcium junction zones is smaller for the gel made with PME62Ca. Indeed, this result has much to say about the strategy for maximizing the elastic properties of gels made from pectins of a fixed DM: by generating a fine structure in which each calcium-binding junction zone is only the minimum length possible for ensuring stability at a prerequisite temperature it is possible to maximize the number of such connections. For flexible networks of this type, any further increase in the length of these junctions simply wastes charged residues that could be utilized elsewhere. While locally terminating p-PME action by the presence of calcium ions is an effective way of generating such fine structures containing the minimum calcium-interacting lengths of galacturonic acid residues, p-PME itself also seems programmed, at least to some degree, to create short (but long enough) blocks.

The gradient of the long-time "plateau" is another interesting aspect of the data shown in figure 2: the MSD of the tracer particles in the PME62Ca gel can be seen to be approximately flat at long times, which is clearly not the case for the PME62 material. While the relative constancy of the MSD in the former can be seen as an indication of a more rubber-like truly elastic network, the latter evidently does not reach a state that is totally percolated by long-lived cross-links, and is better described as a solution of highly branched high molecular weight flexible polymers, with the slow reptation of these high molecular weight assemblies explaining the slight relaxation at longer times. It would be reasonable then to estimate that the cross-link density required for percolation, N_{Cp}, lies between that exhibited by these two systems. If we consider that each PME62Ca chain has a minimum of two calcium chelating cross-links, thereby explaining the appearance of a percolated network, then this implies that around 8 of these regions (allowing for the limiting calcium concentration) were introduced into each chain by the deesterification process that reduced the DM by some 16% (78% →62%). Ignoring the original randomly distributed unesterified regions in the starting high DM pectin owing to the low probability of sufficiently long bare galacturonic acid runs to be able to form stable cross-links existing, and using an average degree of polymerization of some 350 residues, a rough estimate of the length of a junction zone of about 7 residues is found. Interestingly, this is indeed close to other estimates of the minimum number of consecutive acidic residues necessary to form a stable junction zone. As noted, the PME62 sample has a larger long-time displacement and hence smaller modulus by a factor of around two, and assuming now that this system is close to percolation, this roughly reflects the presence of half the junction zones; or equivalently a doubling of the estimated junction zone length to approximately 14 residues, again reasonably close to emerging ideas of p-PME processivity.

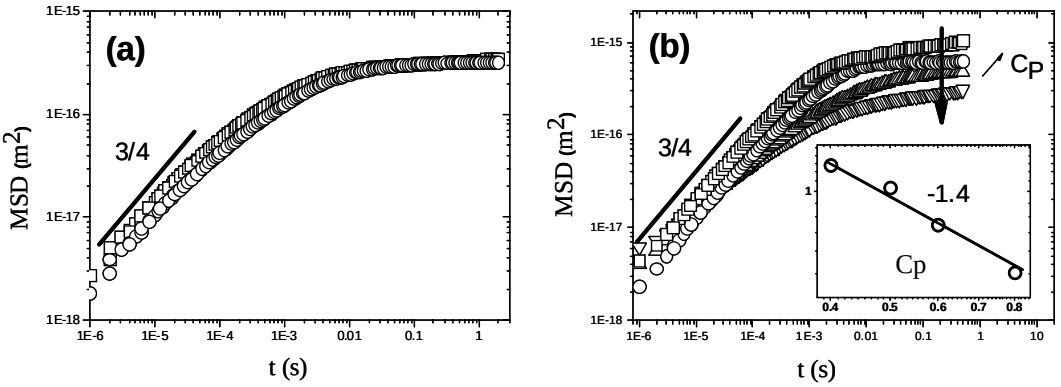


Figure 3: (a) The resulting tracer particle MSDs obtained by DWS from gels created by the slow release of calcium (as described in the experimental section) from PME62 (squares) and with PME62Ca (circles), with polymer concentrations of 0.5%w/w and R = 0.25 (R_{eff} = 0.6). (b) The resulting MSDs obtained from DWS for the PME62 for R = 0.25 (R_{eff} = 0.6), as in (a), but here for concentrations ranging from 0.4 to 0.8%w/w. Inset: The plateau value of the MSD ($/10^{-15}$ m^2), taken at 0.1 s, plotted against the pectin concentration (%w/w).

Interestingly, all the systems studied in this "flexible regime" exhibit behaviour in stark contrast to that found in previous work [31], in which microrheological studies were carried out on pectin gels which clearly showed the signatures of semi-flexible networks theories [52, 53]. The main predictions of these theories are a power law scaling of the high frequency MSD with an exponent of ¾ with the lag time, and a scaling of the plateau MSD at long time with an exponent of 1.4 with the polymer concentration. The extensively studied pectin sample used in these previous studies [31] was of a lower DM (50%), was considered to have a very blocky intramolecular pattern of unesterified residues owing to the fact it was deesterified by p-PME, and was subsequently gelled after deesterification, using a controlled release of calcium. At first glance the different behaviour of that system compared to that shown here in figures 1 and 2 might be simply assigned to fine-structure differences, i.e. that the semi-flexible regime might only be manifest by pectins containing unesterified blocks of significant length, where long calcium bound dimers form the actual network strands themselves, rather than simply connecting together more flexible filaments. To attempt to test such a hypothesis and better understand the origins of the different regimes, further pectin gels were assembled from the two polymers described above, PME62 and PME62Ca, but now at lower polymer, and higher calcium, concentrations, in order to match the conditions found in the previous semi-flexible network study. (These conditions had evolved largely as a practical response to restricting the modulus and gelation rate of systems of very blocky pectins while still creating percolated networks.) The results are shown in figure 3(a), and perhaps surprisingly a power law scaling of the MSD with lag time with an exponent of ¾

was observed at short times, for both pectins. Furthermore, the MSD of both gels is very similar over the whole frequency range, a sign that the density of the semi-flexible filaments, as well as the cross-link density is about the same for both gels.

To further corroborate the existence of a *semi-flexible* regime, even for the polymers possessing a fine structure that so convincingly illustrated *flexible* network behaviour in figure 2, DWS experiments were carried out on the PME62 gels made with a slow release of calcium, for different polymer concentrations. As depicted in figure 3(b), the familiar ¾ high frequency scaling of the tracer MSD with lag time is found for concentrations ranging from 0.4 to 0.8%w/w, and indeed in this region the MSD plateau value scales reasonably well with the polymer concentration with a power law of -1.4 (Figure 3(b) Inset). These results agree well with predictions based on the presence of a semi-flexible network, although the pectins used are made of relatively short charged blocks. It seems unlikely then that the actual semi-flexible filaments could be only dimeric junction zones as initially postulated [31], suggesting the possible assembly of stiff network strands with a certain chain multiplicity.

While relating the persistence length of formed semi-flexible filaments to their chain multiplicity is not trivial it is possible that individual dimers are not in fact sufficiently stiff so that there bending energy contributes significantly to their resistance to deformation. Alternatively, there is a precedent for the existence of higher order multiple chain associations at high concentrations of calcium. In alginate systems, which we have argued are a reasonable analogue of blocky pectin systems, previous studies [16, 17] observed that at *high calcium concentrations*, there was indeed a lateral association of egg-box dimers, as also suggested by modeling [12]. While these studies were carried out at relatively low alginate concentration where such stiff filaments would fail to percolate, in our semi-dilute systems these filaments form a topologically entangled semi-flexible network, yielding the observed concentration dependence, with light cross-linking by longer lived associations providing the long-time dependence.

However, in conditions of higher concentrations of polymer and low concentrations of calcium, such as the systems studied in figure 2, the same polymeric fine structure yields flexible networks. There is some evidence [16, 17] that in dilute systems multiple chain associations simply do not form below a certain concentration of calcium, presumably because they are simply out-competed by the formation of egg-box type dimers. Additionally, even at higher concentrations of calcium, it seems reasonable to argue that in semi-dilute and concentrated systems extensive lateral chain associations that would otherwise yield semi-flexible filaments, could be

prohibited because the high degree of mobility required for the polymers to rearrange into such configurations is curtailed. The slower reptative dynamics characterizing the systems ability to rearrange at higher concentrations means that as small amounts of calcium are introduced and mimimum-length calcium-binding junction zones are formed, “spot-welds” are introduced which dynamically trap the entangled polymer solution in a state where the stress bearing elements are flexible polymers linked by short, punctual, dimeric cross-links.

3.2 State diagram

How different conditions ultimately yield network regimes; characterized by the way the calcium induced associations either (i) pin flexible entangled systems with punctual cross-links or (ii) drive rearrangement and bundling of multiple polymers into semi-flexible filaments, can be summarized in a state diagram. Clearly such a diagram will contain regions corresponding to basic dimeric association and lateral association of the initial dimers.

An initial somewhat pragmatic approach to such a diagram would be to plot the described regions as a function of polymer concentration and R value. However, the traditionally calculated R value appears of questionable use as a unifying parameter able to cope with fine structures differences, counting, as it does, the quantity of calcium relative to the *total* number of charged galacturonic residues, despite the fact that it is often clear that a substantial portion of these residues can never form stable egg-box junction zones, owing to the nature of the intramolecular charge distribution. When searching for universal behaviour resulting from calcium binding it is possible that another parameter, R effective or R_{eff}, might prove to be more useful; defined as a ratio in the spirit of the well-known R value:

$$R_{eff} = \frac{\left[Ca^{2+}\right]}{2 \cdot \left[COO^{-}\right]_{eff}} \qquad (5.1)$$

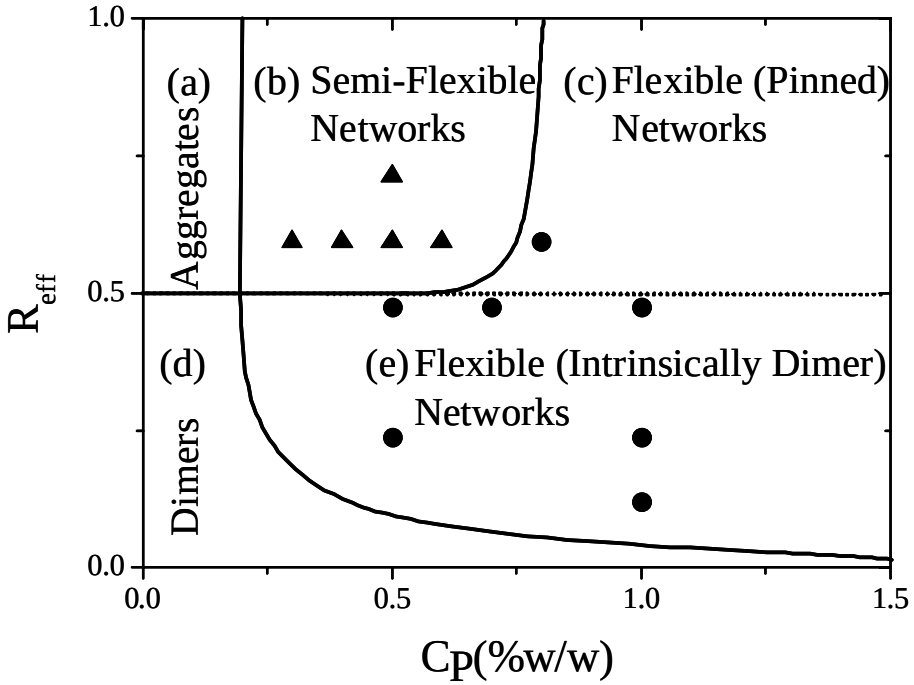
but where $\left[COO^{-}\right]_{eff}$, rather than counting all galactuonic acid, is the quantity contained in blocks of consecutive unesterified residues longer than a defined amount, specifically that amount required to bind calcium into a stable junction zone. This definition of R_{eff} could readily be used for pectins possessing blocky or indeed random intramolecular distributions of methylesterification, but strictly its calculation requires knowledge of a precise minimum number of consecutive residues required in order to form a stable junction zone at the temperature of interest. At present experimental estimates of this number range between 8 and 15 [11, 13], consistent with our estimates from this work.

However, for the pectins used in this study this lack of detailed knowledge can be circumvented by making two assumptions (i) that the initial 78% DM pectin (that was subsequently deesterified with p-PME to produce the investigated samples) does not contribute any residues to this ratio i.e. the probability of having even 8 consecutive unesterified resides in the starting material is negligible and (ii) that owing to the raison d'être of p-PME all the residues that were introduced by its deesterification of the starting substrate do contribute to the ratio. Thus, for the systems investigated here we have:

$$R_{eff.PME} = \frac{100 \cdot [Ca^{2+}]}{2 \cdot \Delta DM \cdot [Gal]} \qquad (5.2)$$

where *ΔDM* (%) is the decrease of DM generated by p-PME deesterification, and *[Gal]* the quantity of the galacturonic residues.

Figure 4 shows a proposed state diagram that has been experimentally mapped out, using this definition of R_{eff}, for gels made from the PME62 pectin, with boundaries estimated at the demarcation of the different regimes. Several key features of the arguments elaborated in the text are evident: For example, for relatively high calcium concentrations and low enough polymer concentration, lateral association of the primary dimers, permitted due to high system mobility, result in multiple chains assembling into semi-flexible filaments. In dilute solution, such filaments have previously been reported as multiply associated chains, as described for alginate in ITC experiments at high R values [17] (Region(a)). At higher polymer concentrations percolated semi-flexible networks based on these filaments are formed, as observed in microrheological studies described previously and herein (Region (b)). As the polymer concentration is increased still further, the system mobility is reduced significantly, and extensive lateral association of the dimers is dynamically forbidden even at high calcium concentrations, resulting in flexible networks in which the polymers are dynamically pinned (Region (c)). For low Ca^{2+} concentrations the scheme is simpler with both the dilute and semi-dilute regimes containing predominantly dimeric egg-box junctions that are manifest either as analogous chain associations to those detected in ITC experiments of alginate (Region (d)) or yield cross-linked flexible networks respectively (Region (e)).



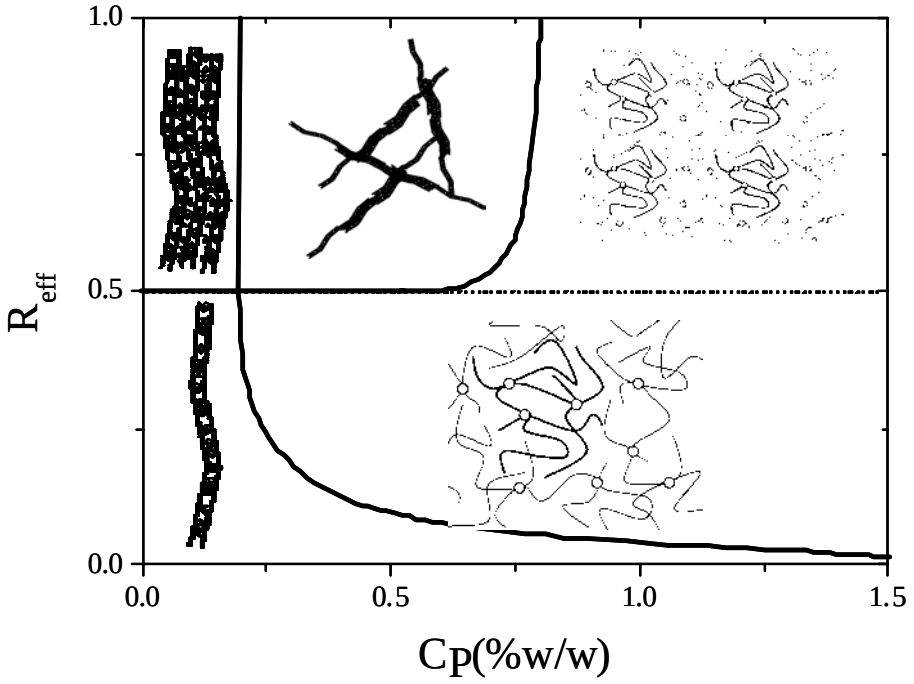


Figure 4: A proposed state diagram for PME62. (a) The points shown correspond to the results of the DWS experiments presented in Figure 2 and Figure 3, plus additionally measured points (raw data not shown). Triangles are plotted where, at that polymer concentration and R_{eff} value, semi-flexible signatures were found; and similarly circles denote systems where the experimentally determined signatures of flexible networks were observed. The different sket ched regions (a)-(e) are described section 2.2. (b) Schematic

According to our microrheology experiments the calcium concentration below which we cannot observe semi-flexible network behaviour is approximately at $R_{eff} = 0.5$ and it is indeed interesting to note that it has been proposed that this is the limit when all the possible dimers are formed in alginate [16], with the lateral association of these dimers occurring at higher R_{eff} (when not dynamically constrained). For alginate, which typically has a blocky pattern of ion-binding groups, R would be equivalent to R_{eff}. Other work also found that for alginates that inter-dimer associations occurred for R values higher than 0.55 [17], close to the boundary proposed here. By using R_{eff} as the parameter embodying this relative calcium concentration this boundary should be more robust to changes of pectin fine structure, although investigations using randomly distributed patterns of methylesterification are worthy of investigation and form part of ongoing work. The other significant boundary is found upon increasing the polymer concentration, signaling the point at which the polymeric mobility in the embryonic networks is reduced (owing to extensive entanglement and also pinning by the initial bound calcium) to an extent where further network rearrangements and lateral aggregation are not possible. This point will be linked primarily to a specific entanglement or cross-link density capable of modifying the system dynamics sufficiently in order to prevent substantial rearrangements. As such, the average block length of the calcium binding sugars would be expected to modify this limit, where increasing the block length should move it towards higher polymer concentrations, at a chosen R_{eff} value. The proposed influence of pectin fine structure on such a state diagram is proposed in figure 5.

It is expected that ultimately, as more information becomes available on the energetics of calcium binding and more experimental techniques are brought to bear, in an effort for example to directly measure the local polymer mobility during gelation, that such fine structure dependent state diagrams might be collapsed onto a universal plot in which the physics is embodied in the relative importance of the free energy gained by associations of various types and the frictional barriers to polymeric re-arrangement.

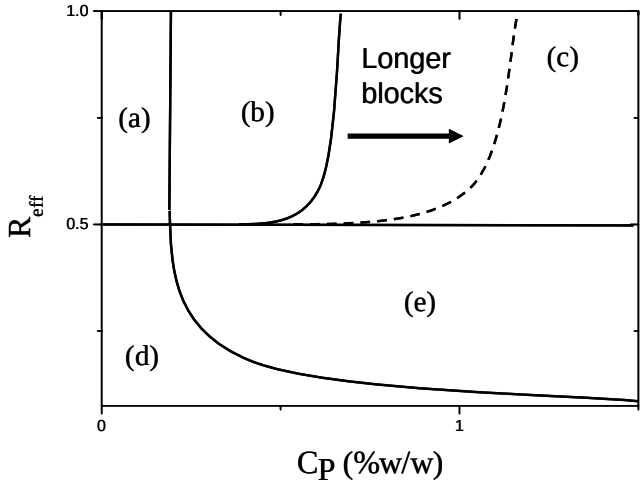


Figure 5: Proposed evolution of the suggested state diagram depending of the pectin fine structure: charged residues distributed in short blocks (bold), long blocks (dash). The regions (a)-(e) are the same as in figure 4.

4 Conclusion

Recent microrhelogical studies have been offering insights into the fundamental nature of pectin gels by giving unprecedented access to the high frequency mechanical properties, which reflect the properties of the network strands; in addition to the low frequency data which acts as a valuable indicator of more global network properties such as the cross-link density. While initial studies of the dynamic-mechanical response of ionotropic gels made from extracted pectins, whose primary structure was modified in vitro, exhibited behaviour indicative of semi-flexible polymer networks, (the high frequency moduli scale with $\omega^{3/4}$, and with a cross-link density dependence of the elastic plateau following $N_C^{1.4}$); subsequently studied systems in which PME was used to liberate calcium binding sites on initially highly methylesterified pectin in the presence of calcium-ions clearly showed the signatures of chemically cross-linked networks of flexible polymers (the high frequency moduli scale with $\omega^{1/2}$, and with a cross-link density dependence of the elastic plateau following $N_C^{1.8}$).

In this work microrheological studies have been performed on calcium induced gels made from two different high DM (62%) blocky pectins with controlled fine structures, one engineered to contain the shortest possible blocks capable of binding calcium into a stable junction zone, and one with longer (though still short) blocks. It was found that both of these fine structures were able to produce gels exhibiting behaviour characteristic of *either* the semi-flexible or flexible regime, depending on

the polymer concentration and the relative calcium concentration. Thereby, using polymer concentration and a newly defined parameter, the effective R value, R_{eff}, (which quantifies the calcium concentration relative to only the number of charged galacturonic acid residues which can potentially bind Ca^{2+} *into a stable cross-link*) a state diagram has been mapped out describing regions of the parameter space where each behaviour might be expected. Futhermore, the origin of these dramatically different networks has been rationalized with recourse to basic physical principles into a common framework in which the relative importance of the free energy gained by association and the frictional barrier to polymeric re-arrangement during network formation play significant competing roles.

The ability of plants to control local polymer and ion concentration, suggests that both semi-flexible *and* flexible networks may be relevant *in-vivo*, and could provide different functionalities. For example, while the semi-flexible networks previously observed are more likely to exhibit strain hardening, the flexible networks described herein optimise the elasticity of calcium-bonded networks by utilising a specific pattern of short ion-binding blocks. It is also interesting to consider that these network types will have vastly different transport properties and the ability to select between these may well play a role in controlling the porosity of the cell wall.

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Chapter VI – Conclusion

Scope:

The general aim of this study was to use micro-rheology (MR) techniques in order to give insights on the structure-function relationship of pectin gels. MR, by probing the Brownian motion of microscopic probes of negligible inertia, allows the mechanical characterisation of soft materials over a large frequency range. In this study, two complementary MR techniques were set-up and exploited: a fluorescence microscopy method, multiple particle tracking (MPT), and a light scattering technique called diffusing wave spectroscopy (DWS). By tracking the motion of individual probes, MPT provides plots of how the mean square displacements (MSD) of each particle vary as a function of time. The raw data therefore contains a lot of information about the extent to which mechanical heterogeneities might exist in the sample probed, at different spatial scales. Alternatively, if the material is characterised as being ergodic, DWS can be used to obtain an ensemble averaged MSD plot over a significantly larger time window, and in particular allows the precise determination of the high frequency behaviour. This is especially relevant as the scaling of the MSD with frequency in this region contains essential information about the basic constitutive strands of the polymer network. At the other side of the frequency range, the time dependence, as well as the value of the MSD at longer times, will give insights on the nature and density of the cross-links responsible for the gelling of the sample.

It is possible to make pectin gels using different processes (acidification for example) depending of the fine structure of the used pectin, but we decided to focus the present thesis on calcium induced gels, which are known to play various important mechanical functionalities *in-vivo* in the plant cell wall and be of great interest for the food or pharmaceutical industries. Conventional macro-rheology teaches us that the viscoelastic properties of calcium induced gels are obviously largely dependant of polymer and calcium concentration, as well as of the fine structure of the used polymer. It is accepted that pectin of a high DM cannot gel with calcium, and that the strength of a calcium induced gel will increase with a lower DM and/or with a blockier distribution of the charged residues. However, little is known about how the pectin chains arrange themselves in the gels in order to build a network and the results of the microrheolgical studies described herein provide useful data to be considered when addressing such questions.

Main results:

After setting up the apparatus for the two MR techniques and verifying their validity and range of application on model systems of varying spatial and temporal complexity, ranging from simple fluids to mechanically disrupted biopolymer gels, MR was first applied to gels made with pectin samples that had been modified *in-vitro* by pectinesterase (PME), a plant enzyme which deesterifies the methylated galacturonic residues. These pectins where characterised as having a relatively low DM with a very blocky distribution of their acidic residues. The subsequently induced gels were initiated with a slow release of calcium and were found to exhibit the main signatures of *semi-flexible networks*, suggesting that perhaps pectin matrices may be used as a stress-hardening material by the plant, in the same fashion of F-actin networks found in the cell's cytoskeleton.

However, it could be argued that these strain-hardening roles are already likely fulfilled by other rigid filaments present in the plant cell wall, such as cellulose or hemi-cellulose, and such considerations lead to the idea of using MR to study more biomimetic samples. In these experiments the PME deesterification happens in the presence of the cross-linker agent (rather than substrates being pre-engineered and then introduced to calcium), which is forming a gel. It turned out that such systems exhibit a strikingly different behaviour than that shown by the first studied gels where the deesterification was occurring without calcium, and in fact display the characteristics of *cross-linked flexible networks*. MR, and capillary electrophoresis investigations on pectin extracted from the gel formed in this manner, provided evidence for an intramolecular distribution made of short blocks of charged residues, with a block length of about the minimum number of consecutive residues needed to from a stable calcium junction zone. We argued that the strength of gels made with pectin of this specific pattern is maximized, consistent with the fact that is it possible to make calcium gels with pectin of a very high DM when the pattern is made of such short blocks.

While the presence of these short binding zones seemed to be linked in a logical manner to the presence of a flexible network, later MR experiments also showed that gels could be made with the same pectin chains but with different calcium and polymer concentration, that exhibited semi-flexible signatures. To explain this, we proposed a framework borrowing ideas of other studies regarding the binding of calcium to alginate (another anionic polysaccharide with many similarities to pectin) which have suggested that under certain conditions simple dimeric association is favoured, while at higher calcium concentrations a lateral association of these

dimmers follows. In addition, we hypothesise this latter effect would be possible only when the primary network mobility is low enough to allow the reptation and rearrangement of the dimers, which could yield the formation of a network of relatively long and rigid filaments. When the primary network mobility is stronger, such lateral associations are dynamically constrained, resulting in a chemically cross-linked flexible network where the cross-links are the single egg-box junction zones, and the bridging basic strands, single pectin chains. This network mobility is obviously linked to the polymer and calcium concentration, and a state diagram of the different regimes of association has been proposed, where the governing parameters are the polymer concentration and a newly proposed parameter describing the effective cross-link generating calcium concentration. The influence of the pectin fine structure on this state diagram is finally discussed.

Implications of these results, and future work:

In the case of the punctually cross-linked flexible networks, the main fundamental idea suggested by this work is that in order to maximize the strength of calcium induced pectin gel, the junction zone length has to be adapted to the minimum number of consecutive charged residues necessary to form a stable bond. In other words, the cross-link density has to be maximized, which will subsequently maximize the elastic strength of the cross-linked flexible network. This can be adapted to any kind an ionic pectin gels where the minimum bond length will differ from calcium induced gels, and even adapted to the gel formation due to weaker interaction such as hydrogen bonding where the minimal junction zone length to form a significant bond would be much greater (explaining why it is possible to make acid induced gels only with pectins having very long blocks of acidic residues). The implications of this idea are vast: the fact that the pollen tube cell wall uses pectin gels with optimal properties tells us than Mother Nature is still unbeaten in building soft materials, and that copying it would help to design food or pharmaceutical matrices in a more efficient way.

Obviously, this idea implies that semi-flexible pectin networks are not an efficient way to make strong gels, as a great part of the calcium is used inefficiently in building the semi-flexible filaments instead of network cross-links. But these semi-flexible matrices might be used by the plant cell wall in some functionalities, in order to increase its porosity for example. It may also be used as an adhesive and/or a stress-stiffening material in the cell inter-space.

In order to investigate these hypotheses a lot of biophysical work remains to be done in the mechanical characterisation of the *in-vivo* pectin gels present in the plant cell

wall, in addition to the characterisation of *in-vivo* pectin fine structures. On the *in-vitro* side, studying gels made of intact branched pectin molecules instead of the linear homogalacturonan used in this study would be of a great interest in the sense that they are more biologically relevant. Will the reconstituted gels made with intact branched pectin chains exhibit the same behaviours, i.e. be able to form both kinds of networks?

On a more physical point of view, the idea that some pectin gels may used as a model system for the latest theories is developed in the Annexe 2 of this thesis, based on preliminary results. These are very promising and further MR investigations are definitely worth doing, but a good understanding of these gels and of the interactions, which form them, is mandatory before claiming them as a model system for the glassy wormlike chain theory. This will require determining a way of measuring experimentally the energy barrier to cross-links sliding, which may be pursued perhaps by pulling them with AFM or with optical tweezers.

Annexe 1 – Studying the local micro-environment by multiple particle tracking

Multiple Particle Tracking (MPT) was first developed for studying the spatial in-homogeneities that might exist in a soft materials' mechanical properties [1, 2], where the first motivation was probing the rheological properties existing in microenvironments inside cells. While MPT microrheology requires probing many particles displacements individually, considerably more effort than a scattering experiment if it is simply being carried out in order to determine the averaged mechanical properties from the ensemble averaged mean square displacement (MSD); the use of statistical tools can provide information regarding the local micro-environment by analysing the motion of individual particles. These tools will be detailed first, and subsequently demonstrated by application to pectin gels, assembled by a slow release of calcium into the system [3, 4]. In particular these results provide a way to understand how the gel phase is growing with the concentration of polymer. These results have been originally presented as a poster at the AMN3 International Conference in Wellington (New Zealand, 11-15/02/2007).

1. The tools

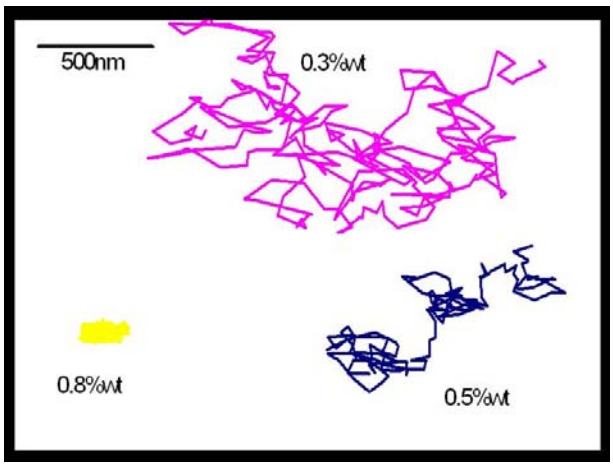

Figure 1: Direct plot of the tracks of fluorescent 548nm latex spheres from MPT experiments, for calcium induced pectin gels made of the commercial sample LM12 (DM 35%, random distribution). The used Ca^{2+} concentration for the 3 different polymer concentrations is equivalent to R=0.3.

The general 'shape' of the motion of tracer beads already conveys much information about the mechanical properties of its surroundings, as shown in figure 1. For the lowest concentration, the tracked bead explores a large spatial region and its motion is probably barely affected by any induced network. On the contrary, at the highest

concentration, a more restricted motion is exhibited, as the bead is confined to a small spatial region and probably embedded in a relatively elastic network. At an intermediate concentration of 0.5% w/w, a more unexpected effect is observed where the particle's motion appears restricted for a time before jumping to another restricted state, as if the particle was jumping from one "cage" to another. This sort of motion is referred as Lévy flights, and has been the subject of an extensive literature in many fields, with applications ranging from financial mathematics to applications in biology. On a more related subject, Lévy flights are seen in soft materials made of hard spheres where the spheres are jumping between cages or in polymeric networks such as F-actin [5] where a Brownian probe is jumping from one network mesh to another.

In order to quantify the mechanical homogeneity of a material the Van Hove correlation function of an ensemble of tracked beads is a commonly used tool [5, 6] where the probability of a particle to move of a distance r between the times t and $t+\tau$ (τ is the time lag) can be plotted against r for the ensemble of beads. A Van Hove correlation function for an ensemble of beads following a Gaussian distribution indicates that this probe or ensemble of probes is behaving as a Brownian particle with a random motion, where all the possible position of the available space are explored. That is: even if the mechanical properties of the surrounding medium are homogeneous then not all beads will move the same distance in the same time; simply owing to the statistical nature of thermal activation. The Van Hove plot provides a way of assessing whether or not mechanical inhomogeneity is required to explain the distribution of different displacements measured. For homogeneous system the extraction of the mechanical properties of the embedding material from the motion of the micro-spheres is subsequently possible, for the time lags where this condition is verified. This could be seen as an equivalent to the ergodicity condition for light scattering techniques. Thus a system where the Van Hove correlation does not follow a Gaussian law would be considered as non suitable for the MR analysis based on ensemble averages.

As the Van Hove analysis for all the accessible τ would be very laborious, a parameter can be used to quantify how Gaussian is a Van Hove distribution. Many such parameters exist, but we decided to use the following non-Gaussian parameter [5, 7]:

$$N(\tau) = \frac{\left\langle r^4(\tau) \right\rangle}{3\left\langle r^2(\tau) \right\rangle^2} - 1 \qquad \text{(A1.1)}$$

where $r(\tau)$ is the displacement at a time lag τ. This factor is equal to 0 when the Van Hove correlation is a Gaussian, positive if it is a sharper distribution, and negative

otherwise. Plotting $N(\tau)$ allows discriminating the range of 'ergodicity' of the studied system, for the used MPT apparatus.

Another parameter which is useful in the characterisation of 'caged' systems, i.e. for determining if the particle's motion is effectively restricted by a polymeric structure, is a degree of correlation between subsequent particle displacements [8], defined as:

$$\langle x_{12} \rangle = \langle \bar{r}_{01} \bullet \bar{r}_{12} \rangle \qquad \text{(A1.2)}$$

with $\bar{r}_{01}$ the displacement between *t* and $t+\tau$ and $\bar{r}_{12}$ the displacement between $t+\tau$ and $t+2\tau$. $\langle x_{12} \rangle$ plotted against $\bar{r}_{01}$, it will show a linear behaviour with a negative slope if the bead is elastically caged. Indeed, a particle which moves from the centre of the 'stiff' cage will tend to come back to its central position. The stiffness of the cage can be related to the slope of the linear correlation. If this parameter appears un-correlated with $\bar{r}_{01}$, the particle's motion is not elastically restricted, but undergoes a motion in a Newtonian or quasi-Newtonian complex fluid. The degree of correlation between subsequent particle displacements can help to characterize systems exhibiting a Lévy flight, which are inherently non-ergodic. In such systems, the correlation is initially linear until a critical value of $\bar{r}_{01}$ where it starts to become uncorrelated. This critical value could be used to estimate the cage size [9, 10].

2. Application to calcium induced pectin gels

The conventional way of making pectin gels is by adding $CaCl_2$ to a pectin solution heated to 80°C, where the initially high temperature prevents significant calcium binding. Subsequently a gel is formed when the solution is cooled down. For MR, this technique is difficult to apply mostly owing to the geometry and the small volume of the samples (1 ml), and could introduce artefacts such as micro-sphere aggregation. In the present thesis, a slow Ca^{2+} release method has been used routinely by first adding non-soluble $CaCO_3$ particles (1μm) to the pectin solution, and subsequently adding glucono-δ-lactone (GDL), which slowly hydrolyses, releasing protons which solubilise calcium from the carbonate particles, subsequently triggering the gelation of a sample *in-situ* in a measurement cell. While this method is routinely used to produce macroscopically homogeneous samples that do not exhibit signs of precipitation (problematic in the temperature method described above) the details of the gelation induced by this method are complex: the gel starts to form around the $CaCO_3$ beads, as seen in fluorescent microscopy images where embedded latex micro-spheres seem to be pushed at the boundary of 'gellified' aggregates.

The tools previously presented were first applied to elastic gels, at a reasonably high pectin concentration of 0.8 %w/w, where the gel appears visually elastic. The Van Hove correlation is very close to be a narrow Gaussian distribution (figure 2a), which is the case for all the studied time lags as the non-Gaussian parameter *N* remains close to zero for all the accessible τ (figure 2b). The degree of correlation between subsequent particle displacements (D_C) is linearly correlated with the first displacement (figure 2c), all demonstrating the presence of a restricted Brownian motion of a particle embedded in an elastic and isotropic network. The bead diameter is definitely larger than the mesh size, and the MR method can be applied safely to analyse the MSD of [7].

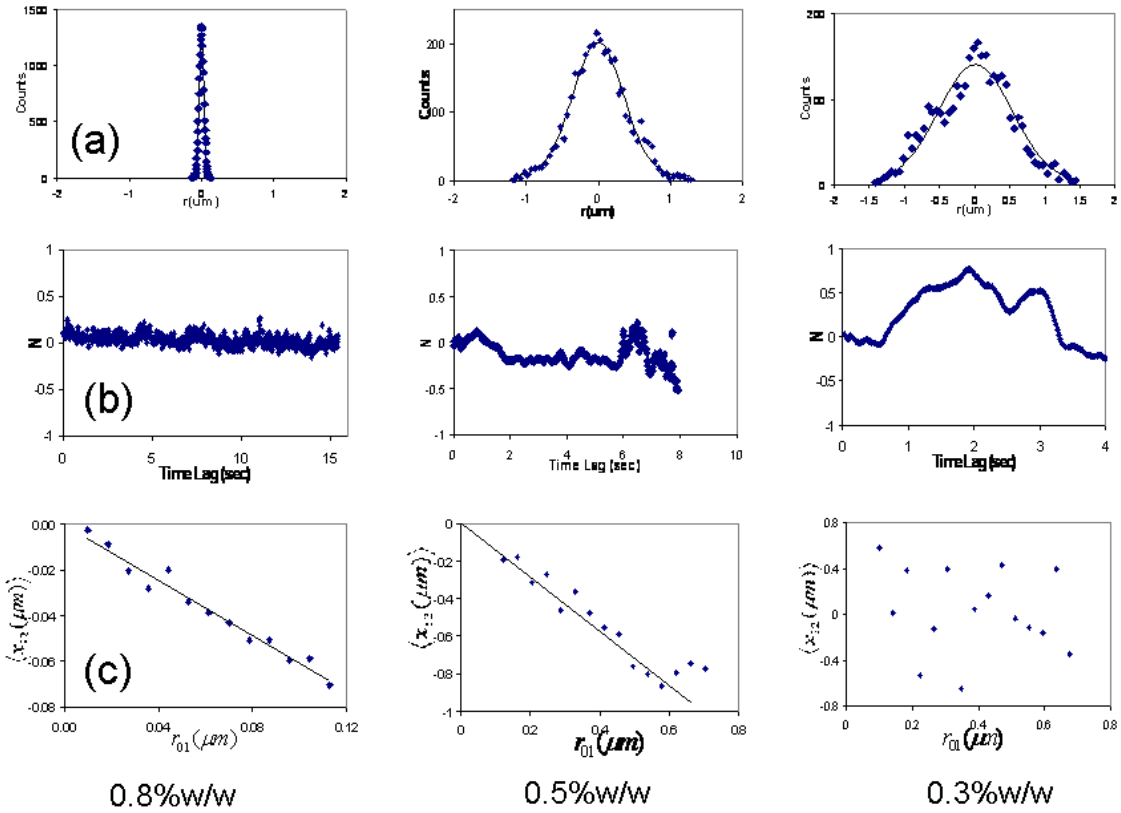

Figure 2: Van Hove correlation for a single particle at τ=0.3s with the best Gaussian fit (a), non-Gaussian parameter (b) and degree of correlation between subsequent particle displacements for τ=3s. The three different gels studied are the as in for figure 1.

At a lower polymer concentration of 0.3% w/w, when no bulk gel is physically observed, the Van Hove correlation again fits reasonably well with a Gaussian function for a small τ values (figure 2a), but with a wider shape, obviously linked to the low viscosity of the material. D_C is completely uncorrelated with the first displacement (figure 2c), which could be expected as the particle diffuses freely in a near-Newtonian fluid. But strangely, N seems to remain far from 0 at time lag higher

than 1second (figure 2b), which would be the sign that the studied particle is moving in a non-isotropic medium. It is suggested that this system should be investigated further in future experiments.

In order to explain this unexpected effect, the same analysis was performed at an intermediate concentration where Lévy flights were seen to occur, i.e. at C_P=0.3% w/w (figure 1). The Van Hove correlation seems to stay close to a Gaussian distribution all over the accessible time lags, despite of the presence of uncorrelated jumps. In fact, only a few jumps occurred during the MPT experiment, and their contribution to the Van Hove correlation is negligible as a total of 900 frames were used for the statistical analysis. More interestingly, D_C appears correlated with the first displacement $\bar{r}_{01}$ until a certain $r^* \geq \bar{r}_{01}$ from where it is uncorrelated, agreeing with presence of jumps between some cages. From r^* we can estimate a pore or cage size as $p = 2a + r^* = 1\mu m$ [9, 10] for the studied particle, which is in good agreement with the direct imaging of "jumps", such as shown in figure 1. The correlation between this lengthscale and the characteristic mesh sizes [11] and entanglement lengths in these systems would be a further aspect for future study.

Here, we simply propose the following model depicted in figure 3: during the slow release method, the gel starts to form around the $CaCO_3$ particles when GDL is added, and the latex spheres are excluded into the remaining solution. For lower concentrations (figure 3a), such micro-gels don't occupy sufficient phase volume in order to percolate and the probes can move almost freely in the interstitial space, where their diffusion is limited at long times by the presence of gel aggregates. This can explain the fact that the non-Gaussian factor N remains far from 0 at long times. For a concentration of 0.5 %w/w (figure 3b), the gelled microspheres start to percolate, but again owing to limited concentration leave small pores where some Brownian probes can jump in between, explaining the observed Lévy flights. At higher concentration (figure 3c), the gel is fully formed and the latex spheres are embedded in an isotropic polymer network, and quantitative MR is possible.

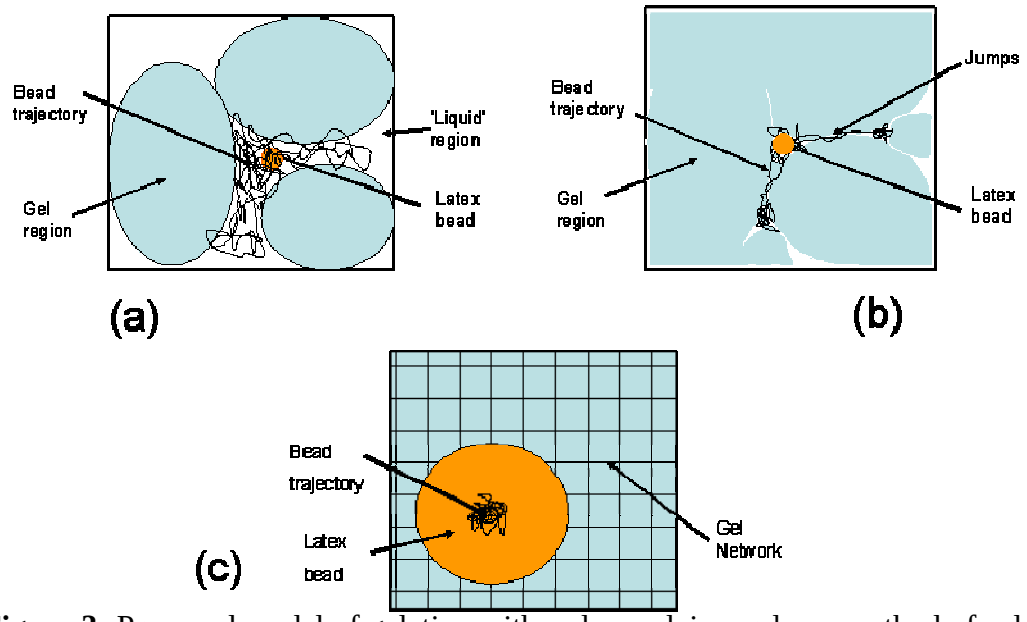

Figure 3: Proposed model of gelation with a slow calcium release method of calcium where the gel start to grow around the $CaCO_3$. (a) At a low calcium concentration of 0.3%w/w, the gel parts do not overlap, pushing the latex probes in the space between the gel aggregates. (b) For a concentration of 0.5%w/w, these gel particles start to overlap, leaving some small pores where some latex spheres are entrapped. (c) At higher concentration (0.8%w/w), the micro-beads are embedded in an isotropic gel.

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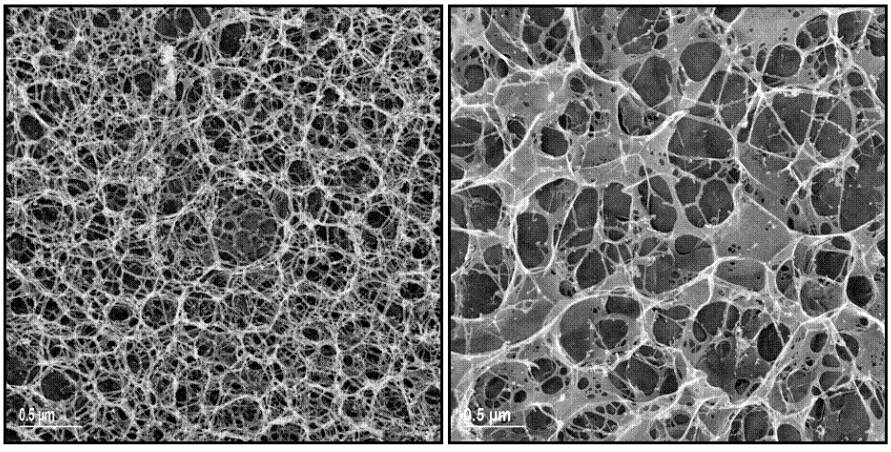
Annexe 2 – Comments on Chapter III - Pectin gels as a model system for the Glassy Wormlike Chain model

Background

In [1], we reported the existence of a regime where pectin gels behave as semi-flexible networks, widespread in the biological gels arena, whose main example and indeed model systems are solutions of the protein F-actin. Believed to be the major load-bearing element in the cell cytoskeleton, the study of these systems led to tremendous advances on both the experimental and theoretical sides, mainly through the development of micro-rhelogy (MR) [2, 3], which served to validate semi-flexible viscoelastic models [4, 5] on *in-vitro* reconstituted F-actin solutions [6, 7]. Furthermore, there appears to be interesting similarities between living cells and *in-vitro* systems, as they both exhibit stress-stiffening following the same scaling behaviour [8, 9], in agreement with recent theoretical developments [10]. However, this widely accepted view as been challenged by experiments on living cells [11], and a "soft glassy rheology" model [12] has also been proposed in order to explain the mechanical properties of the cytoskeleton [13]. To accommodate these two conflicting paradigms, the semi-flexible nature and the soft glassy behaviour, a unified theory was needed. A glassy version of the wormlike chain model [14] has been proposed by [15]. The principal idea of this model is to induce sliding cross-links between the wormlike filaments, which accommodates naturally both the single filaments and the stress-stiffening properties. This model appears to be relevant experimentally [16], but for further validation requires the measurement and control of the energy involved in the yielding of the sliding cross-links.



a) b)

Figure 1: TEM picture of calcium induced gels made of a) a blocky pectin with a DM of 48, and b) a random pectin with a similar DE [17], with R=0.4

Can pectin gels be an alternative model system for these theories? MR experiments [1] can indeed show the semi-flexible network signatures, and TEM micrographs do appear to show the presence of filaments, despite the sample preparation involved and the fact that the imaged gels were made with different pectins than those used herein. It seems clear that in the presence of calcium under certain conditions, pectin chains tend to agglomerate in meso-scale filaments, which appear rigid as shown on figure 1.

Although these filaments may be rigid, they would not in any case be long enough to entangle to make a semi-flexible network, or be visualised as in figure 1. Indeed, even for very blocky pectins made by PME deesterification, only few very long blocks (>40 residues) of charged residues are found [18]. This would mean than the egg box junction zones would only have a length of 10 to 40 nm! The meso-filaments evident in the micrographs are then most likely made with a different self-assembled structure.

Some insight can be gained by revisiting the few papers published regarding the calcium binding of alginate [19, 20]. Using two very different techniques, these two studies show that basic dimers are formed below a certain calcium concentration, followed by a second lateral association of these dimers at higher Ca^{2+} concentration. As discussed in Chapter I, this is also likely to be the case for calcium induced blocky pectin gels, and this would explain the pectin chains self-assembly in filaments. Indeed, the calcium concentrations used in Chapter III (as well as for the TEM pictures of figure 1) are all above this limit for lateral association. Subsequently, multiple dimer associations should occur, and may result in the formation of relatively long filaments.

Using pectin gels as a model system for the very promising glassy wormlike chain (GWLC) model developed recently [15] will require controlling the cross-link's energy barrier profile. In the case of F-actin networks, one can act on the binding strength by varying different physiological parameters [16]. In the case of the calcium induced pectin gels, the cross-links are formed from strong chemical bonds, and are subsequently unlikely to slide. Studying a broader range of bond energy profiles requires the use of weaker cross-linking agents, such as hydrogen bonds or even simple cross-links due to depletion forces [21].

As depicted in the first chapter of this thesis, it is possible to make pectin gels with some polymer fine structures using monovalent ions [22]. It is furthermore possible to make acid induced gels from some pectins, where the main cross-linking agent would be weak hydrogen bonds, possibly associated by hydrophobic interactions

[23]. Indeed, when the pH of a pectin solution is decreased below its pKa, weak hydrogen bonds are formed between two galacturonic acid residues. Because of this weak interaction, it is easy to imagine that the required number of consecutive galacturonic acid residues to form a stable junction zone would be larger than in the case of calcium-mediated connections (8-16 residues). This is in agreement with the fact it is only possible to induce a gel by decreasing the pH for pectins presenting many long unesterified galacturonic acid blocks [24].

Investigations on acid induced pectin gels

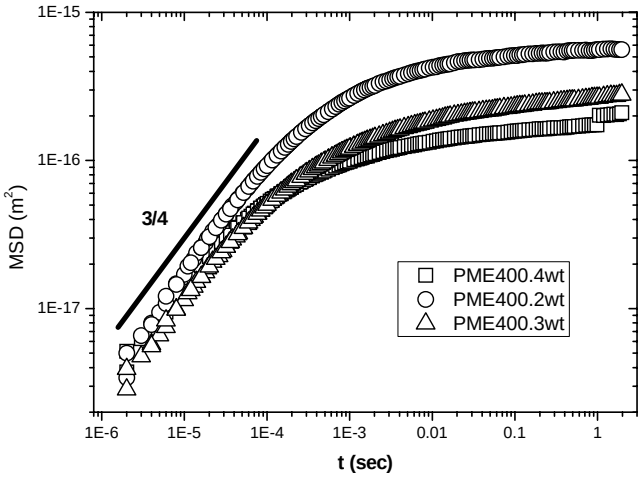


Figure 2: MSD plots from DWS for PME40 acid gels at different polymer concentrations. The pH has been decreased by GLD to an ending pH of 3. The tracer beads were 465nm latex micro-spheres with a concentration of 0.8%.

We started some micro-rheological investigations on acid-gels, with the aim of establishing whether these systems exhibited a semi-flexible regime. Very blocky pectins with a DM of 40% (named PME40) were engineered by PME action on a buffered solution of apple pectin of DM 78, as detailed in [25]. Stock solutions with a polymer concentration of 1.5%wt are made by dissolving the home-made pectin in Milli-Q water. Three different methods for decreasing the pH (down to pH 3) were tested: direct addition of HCl, use of a buffered solution, and addition of GDL to decrease slowly the pH. The latter method was found to be the only suitable one, providing homogeneous looking gels, while the former were unable to provide a gel even after melting / resetting these supposed reversible gels [23]. This reversibility has been tested by MR studies carried out on GDL-induced acid gels of PME40, described above, and of a commercial blocky pectin B37 (DM 37%). For both pectin

samples, the DWS results give very similar MSD plots before and after the melting of the gels, consistent with the reversibility of these gels.

Figure 2 shows the MSD plots of acid induced PME40 gels, with concentration ranging from 0.2 to 0.4 %wt. For all the concentrations, the MSD scale with the time lag with a power law of ¾, agreeing well with the expected behaviour of a semi-flexible regime, even if at 0.4 %wt the slope seems slightly smaller than ¾. In addition, the concentration dependence of the low frequency plateau scales reasonably well with $c^{-1.4}$ (figure 3) also as predicted by semi-flexible network theories [26]. The existence of a semi-flexible regime appears realistic for PME40 acid gels, even if the concentration dependence should be studied more precisely at the light of the GWLC model. Indeed, the presence of 'sticky' cross-links is predicted to induce a logarithmic behaviour of the long time response, which is found in the case of pectin's acid gels; and this stickiness, named ε in the GWLC model, will modify slightly the concentration dependence of the plateau elastic modulus.

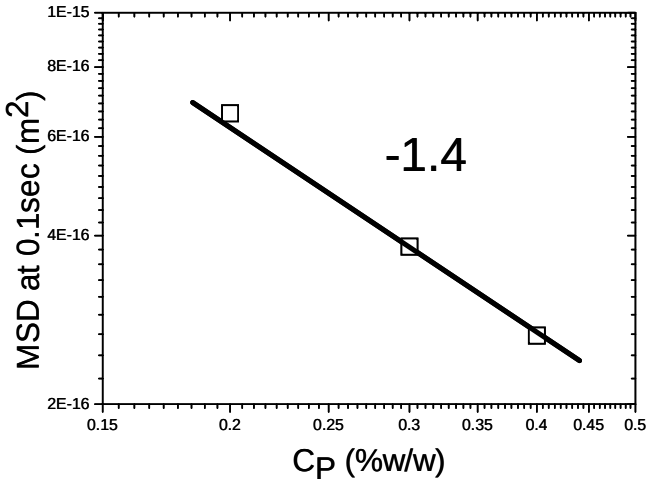


Figure 3: Concentration dependence of the plateau of MSD at long times, for PME40 acid induced gels. The slope is equivalent to a power law fit with a power of -1.4.

Now a semi-flexible regime has been identified, we can try to destabilize these cross-links in order to study their nature. As hydrogen bonds have been proposed to play an important role in the assembly of acid induced gels, the first idea was to try and weaken these with urea, which is a well-known hydrogen bond breaking agent. We found that adding concentrations lower than 0.1 M of urea did not change the MR behaviour, but above this concentration the MSD is noticeably modified (although the gel is not completely broken). In figure 4 the DWS results with a low and a high concentration of urea are shown, and it can be seen that the high frequency behaviour

is conserved, a sign that there is the same concentration of semi-flexible filaments. The long time behaviour however is modified, the destabilized gel being weaker. As the gel is not completely destroyed at these concentrations of urea it is possible that hydrophobic interactions may also play a role in the assembly process and may be sufficient to induce cross-links in this pectin system to make a gel, which is not surprising in fact considering the interaction strength of the interactions: the hydrophobic interaction strength typically ranges from 5 to 10 kJ/mol, while the strength of hydrogen bonding is typically 21 kJ/mol. When the system is not disturbed by urea, i.e. when both interactions may be present, the hydrogen bonds dominate, providing fairly strong cross-links to the network while, when the hydrogen bonds are broken, 'hydrophobic' cross-links may play a more significant role. As seen on figure 4, the disturbed gel is weaker with a more marked logarithmic behaviour at long time. This would be explained in the GWLC model by a smaller stickiness parameter.

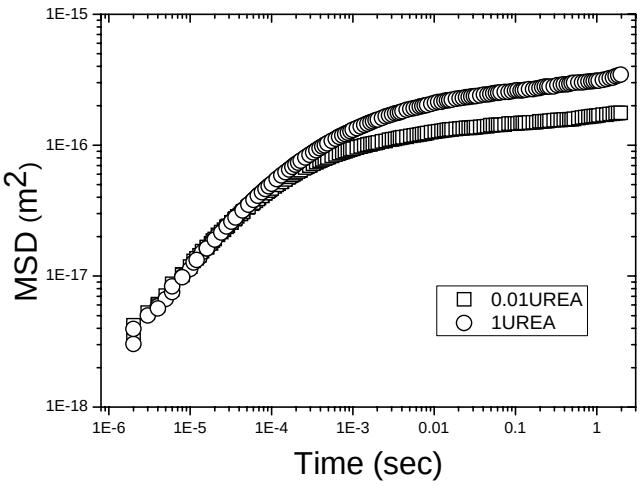


Figure 4: MSD from DWS for PME40 acid induced gels with c_{pectin}=0.3%wt, with 0.01%w/w Urea (squares) and 1M Urea (circles).

This section of work in particular leaves many issues to be addressed by future work, where the leading question will be: is it possible to control this "stickiness parameter" more precisely by changing environmental conditions? If the interactions can be understood better and controlled in order to select different "stickiness", this will make pectin gels a good model for these advanced glassy / semi-flexible theories.

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Annexe 3: Vegetable gummies – A short essay

Who does not love gummy bears? These delicious lollies are generally made of gelatine, a protein extracted from animal tissues, and so their enjoyment is restricted to non-vegetarians (see recipe in Figure 1). This constitutes a major barrier to me ever becoming a real vegetarian; despite using all my will power to resist the temptation, I simply cannot stop eating gummies once I start a packet! Stronger minded vegetarians manage not to eat gummies, even if they desperately wish they could, and many people are looking for alternative vegetarian compounds to make them with. One such compound could be the carbohydrate pectin, which is a ubiquitous biopolymer present in the cell walls of all land plants, where it plays a major mechanical role. You are perhaps more likely to know the name of pectin through your mum, who used it, knowingly or otherwise, when she was preparing delicious jams, whose gel aspect arises from the formation of relatively rigid polymer matrices.

Figure 1. Classic Recipe for gummy bears. The presence of a large amount of gelatine forbids vegetarians to eat them.

To illustrate the sorts of networks that can be formed of pectin molecules, we can make an analogy with a plate of spaghetti: when tomato sauce is added, the spaghetti pieces can glide in between each other easily and the resulting system acts like a viscous solution. But when you leave the spaghetti drying and cooling down with no sauce, sticky junctions are created in between the filaments forbidding the individual pieces to slide past one another. The subsequent network, which is not really appetizing I concede, will have mechanical properties closer that of a solid material. In the same way, a polymer network can be seen as a solution of micro-spaghetti, whose properties can range from those of a viscous liquid to more jelly materials when the constitutive filaments are linked together.

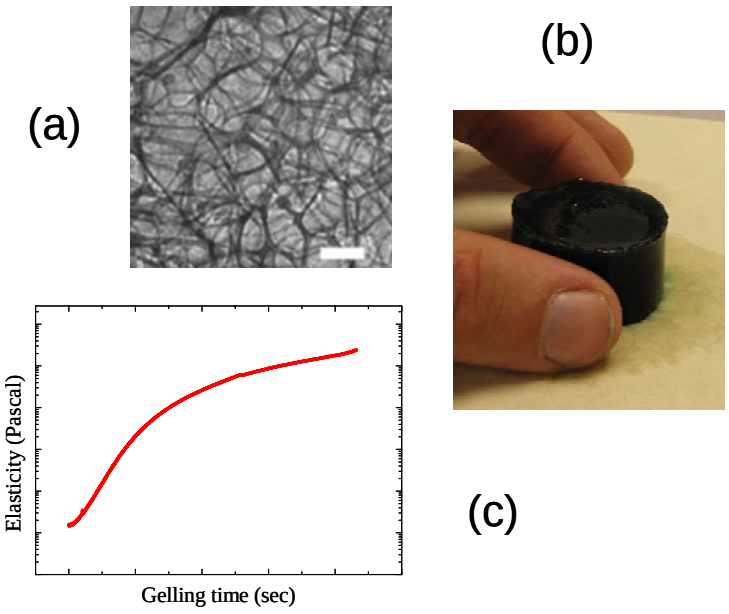

Figure 2: (a) Example of a polymer network (Scale bar 10nm), (b) Evolution of the strength of an enzyme made pectin gel when gelling. The final strength is higher than 5000Pascals, which is around 5times bigger than classic pectin gels. The sample content is 99% of water and 1% of pectin, (c) Aspect of the final gel.

In our laboratory, we are studying the mechanical properties of pectin gels. Our goal is to understand the structure-function relationship of these gels, in the aim of designing new materials with complex properties. We are using pectin extracted from apples and citrus fruits, similar to that which your mum is buying for making jams. But we modify its chemical structure in order to change its gelling properties. Indeed, traditionally making jams needs to use a high concentration of both pectin and sugar, indicating that the initial polymer has relatively poor gelling properties. This is one of the main drawbacks for making relatively strong food materials such as yummy chewy gummies. We have been copying nature and are using a natural plant enzyme to modify the pectin structure and thereby improve the strength of pectin gels.

Indeed, Nature's design rules provide impressive optimised solutions to engineering the properties of soft materials. As an example, pinch your cheek: this soft tissue, composed of at least 75% water, is soft when you grab it but becomes very stiff when you pull on it. These unique properties arise from the properties of the incoporated biopolymers, and how these polymers self-arrange in order to form soft materials with properties of both the liquid and solid kind. In order to study this, we use micro-scale techniques for determining the mechanical properties, whose linked to physics theories provide information about the basic structure of the polymer network. By mimicking the material building process occurring in the growing pollen cell, we

have maximized the mechanical properties of pectin gels and engineered very strong gels with a relatively low concentration of pectin (Figure 2).

As well as improving our fundamental understanding of biomaterials the possible applications of these optimized gelling polymers are vast in the food and pharmaceutical industries, where pectin is more and more often used as a healthy alternative for gelling or conservation properties. But the biggest advance would be obviously for the gummy-bear lover, with the possible manufacture of yummy and healthy gummies bears.