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Structure and stability of polynucleotide-(1,3)-β-D-glucan complexes

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ABSTRACT

The structure and stability of complexes formed between (1,3)- β-D-glucans and the polynucletide poly(C) are investigated using differential scanning calorimetry (DSC), atomic force microscopy (AFM) and size exclusion chromatography (SEC). DSC revealed the melting transition of the complexes at 55 °C and an endothermic transition for renatured scleroglucan in the interval 30–40 °C. AFM topographs support the interpretations that the latter transition is due to melting of associated polymer sequences positioned in between structural defects in renatured triplexes. The complexes form between poly(C) and single stranded or renatured scleroglucan, but not with annealed scleroglucan. The complex formation thus seems to require single strands, but the short single stranded stretches present in renatured scleroglucan are sufficient, and full dissociation into single strands is not required. No differences were observed between complexes formed by adding poly(C) to renatured triple helical or denatured single stranded (1,3)- β-D-glucans. Implications of this insight on proposed structural models are discussed.

1. Introduction

Scleroglucan is one of several polysaccharides found in nature having a main chain composed of (1,3)-β-linked D-glucopyranosyl residues, and single (1,6)-linked D-glucopyranosyl residues attached as side chains to every third sugar residue of the main chain (Fig. 1). These extracellular, high molecular weight polysaccharides are neutral and water-soluble. Commercially available (1,3)-β-D-glucans are often named after their biological sources. The compounds lentinan, laminaran, schizophyllan, and scleroglucan are examples of (1,6) branched (1,3)-β-D-glucans extracted from different sources. Even though only produced by a few prokaryotic micro-organisms, (1,3)-β-D-glucans are widely distributed in plants, especially algae, and in fungi, where they have storage, structural or protective roles (Stone & Clarke, 1992).

Most high molecular weight (1,3)-β-D-glucans dissolved in water at room temperature adopt a linear, rigid, right handed, triple helical structure stabilised by hydrogen bonds (Fig. 1). In the accepted model, the hydrogen bonds are present at the center of the helix (Atkins & Parker, 1968). This model was recently re-examined and challenged using semiempirical molecular-orbital package and *ab initio* calculations (Miyoshi, Uezu, Sakurai, & Shinkai, 2004). A model was proposed in which intermolecular H-bonds are formed along the helix. A later molecular dynamics simulation study (Palleschi, Bocchinfuso, Coviello, & Alhaique, 2005) however supports the interstrand H-bonding model proposed by Bluhm and coworkers (Bluhm, Deslandes, Marchessault, Perez, & Rinaudo, 1982). Exposing the scleroglucan triple helixes to denaturating conditions destabilises the hydrogen bonds (Bluhm et al., 1982; Deslandes, Marchessault, & Sarko, 1980; Ensley et al., 1994; Norisuye, Yanaki, & Fujita, 1980). A conformational transition will then take place where the triple helical molecules dissociate into single stranded random coils (Yanaki & Norisuye, 1983). Denaturation of triplexes occurs in alkaline solutions (>0.25 M NaOH), (Bluhm et al., 1982; Bo, Milas, & Rinaudo, 1987; Tabata, Ito, Kojima, Kawabata, & Misaki, 1981), in dimethylsulfoxide (DMSO) (water weight fraction $W_H < 0.13$), (Kitamura & Kuge, 1989; Norisuye et al., 1980; Sato, Norisuye, & Fujita, 1981; Yanaki, Norisuye, & Fujita, 1980) or by increasing the temperature above the triple helix melting temperature, $T_m = 135$ °C (Norisuye et al., 1980; Yanaki, Tabata, & Kojima, 1985). The single stranded random coils are much more flexible than the triple helixes, giving, for high molecular weight samples, lower viscosity compared to solutions containing triple helixes. When denaturated samples are restored to thermodynamic conditions favouring the triple helical structure, circular structures can be observed among the "renatured triplexes" by ultramicroscopy techniques (Fig. 2A) (Stokke, Elgsaeter, Brant, & Kitamura,1991, 1993). The existence of circular structures was unexpected given the stiff nature of the linear triple helix. Linear-, hairpin-, and aggregated structures are also observed in mixtures of renatured molecules. The proportion of circular and other morphologies in renaturated (1,3)-β-D-glucan samples is a function of molecular weight, polymer concentration, as well as the duration of and temperature during the annealing treatment of the renaturated samples (McIntire & Brant, 1998; Stokke et al., 1991). The thermally induced conformational transitions of schizophyllan in water/DMSO mixtures have previously been investi-

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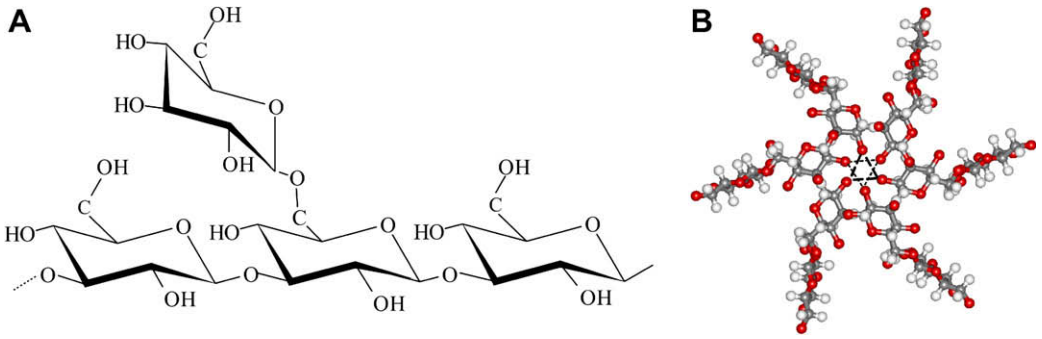

Fig. 1. (A) The repeating unit of scleroglucan. (B) Cross-section of 3-dimensional illustration of triple helical scleroglucan. Interstrand hydrogen bonds (——) are formed between the OH-2 of backbone glucose units, giving a triangular pattern. The two triangles are located at successive levels along the helix axis.

gated over the whole composition range by high sensitivity differential scanning calorimetry (DSC) (Kitamura & Kuge, 1989). That study showed that high sensitivity DSC is potentially as useful in the studies of these biopolymers as it is in the studies of other biopolymers such as proteins and nucleic acids.

The finding that (1,3)-β-D-glucans are recognised by toll like receptors which are key regulators of innate immunity responding to invading microorganisms (Takeda & Akira, 2005; Young, Ye, Frazer, Shi, & Catranova, 2001) marked the start of a series of molecular biology studies performed to understand the molecular mechanism of the observed effects of these macromolecules. Later, several papers have made it clear that (1,3)-β-D-glucans can interact with certain homo-polynucleotides (poly(C), poly(A), poly(dA) and poly(dT)) to form triple stranded and helical macromolecular complexes. Sakurai and co-workers were the first to present experimental evidence of the formation of these novel macromolecular complexes (Sakurai & Shinkai, 2000). The complexes were prepared by mixing a polynucleotide sample with (1,3)-β-D-glucans in the single stranded state by adding a (1,3)-β-D-glucan /DMSO solution to a polynucleotide/water solution (Sakurai, Mizu, & Shinkai, 2001; Sakurai & Shinkai, 2000). Observed differences between circular dichroism (CD) spectra for (1,3)-β-D-glucans renatured in the presence of polynucleotides and spectra obtained for the polynucleotide alone indicated an interaction. The stoichiometry of the complexes formed was investigated through the composition dependence of the signal intensity at the wavelength of maximum CD intensity. Based on the results, the structure of the complex was proposed to be a triple helix consisting of two chains of (1,3)-β-D-glucan and one chain of polynucleotide (Fig. 2B). Sakurai and coworkers later showed that other homo-polynucleotides (poly(G), poly(U), poly(I), poly(dG) and poly(dC)) show no affinity toward single stranded (1,3)- β-D-glucans (Sakurai et al., 2001). These observations have been rationalised by the fact that for the latter polynucleotides, the hydrogen-bonding sites in the bases are occupied by intra- or inter-molecular interactions. On the other hand, poly(C), poly(A), poly(dA) and poly(dT) do not form superstructures, and their hydrogen-bonding sites are therefore unoccupied. This observed correlation between the complexation ability and the presence of free hydrogen-bonding sites on the polynucleotides indicates that the hydrogen-bonding between single stranded (1,3)- β-D-glucans and polynucleotides is essential to induce the macromolecular complex formation. The CD and UV spectroscopy studies also allowed determining the melting temperature of the complexes, being 54 °C for the complex involving poly(C) and 32 °C for the complex involving poly(A). Several papers dealing with the characteristic nature of this interaction have later been published (Bae et al., 2004; Sakurai et al., 2002; Sletmoen & Stokke, 2005), and a molecular model for the complexes formed has been suggested (Fig. 2B). (Sakurai et al., 2001).

The ability of (1,3)-β-D-glucans to form complexes with polynucleotides have already proved to be useful. It has opened for the use of these polysaccharides as potential carriers for various functional polynucleotides, and experiments have been carried out to suggest a new method to deliver DNA by using this complex (Matsumoto et al., 2004; Minari et al., 2007; Sakurai et al., 2005; Shimada et al., 2006). Understanding the specific interaction

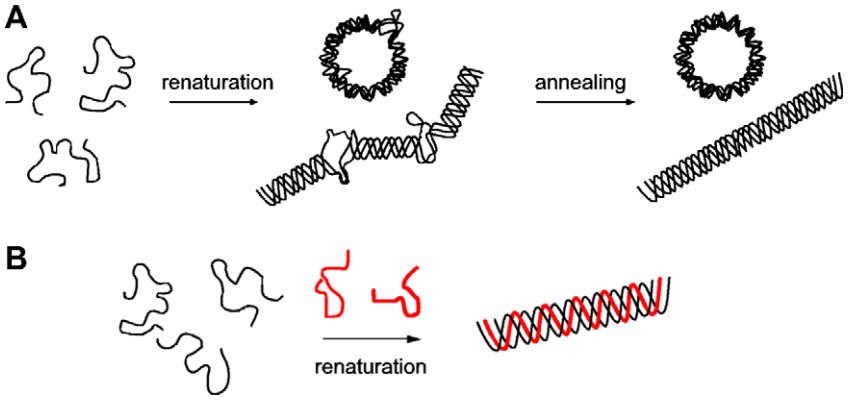

Fig. 2. (A) Schematic illustration of the species formed upon renaturation and subsequent annealing of scleroglucan (Scl). Denatured scleroglucan (black lines) exists as single stranded random coils. Under renaturation, these single strands recombine to unperfect triple helixes (t-Scl). Circular structures can also be observed in the renaturated sample. Under annealing the structural defects originally existing in the renatured sample are removed. (B) Sakurai and coworkers were the first to report that if (1,3)- β-D-glucans renature in the presence of poly(C) (red lines), triple stranded helical complexes form consisting of two polysaccharide strands and one polynucleotide strand (Sakurai et al., 2001).

between polynucleotides and other materials and developing such functional compounds are of great importance in the gene technology. One current limitation of the use of this ability is related to the fact that these complexes only form under conditions where the (1,3)-β-D-glucan exists in the single stranded form. In the present study we have investigated the requirements for complex formation between triple helical (1,3)-β-D-glucan and poly(C). The structure and thermal stability of these complexes as well as complexes formed between single stranded (1,3)-β-D-glucan and poly(C) are determined and compared. The properties of the complexes as well as the properties of pure scleroglucan exposed to different pretreatment procedures are investigated using differential scanning calorimetry (DSC), atomic force microscopy (AFM) and size exclusion chromatography (SEC) coupled with refractive index (RI)-, ultraviolet (UV)- and multi-angle laser light scattering (MALLS) detectors.

2. Materials and methods

2.1. Sample preparation

2.1.1. Biopolymer samples

The scleroglucan used in this study was Actigum CS11 provided by Sanofi Bioindustries, France. It was dissolved in water to a concentration of 2.5 g/L, left under stirring at 55 °C for ~4 h then at room temperature for 3 days. The scleroglucan was ultrasonically depolymerised (Braun Labsonic 1510, power 150 W, total 200 ml) for a duration of 35 min. We have previously reported on applications of sonication to give a weight average molecular weight (M_w) within the interval yielding a large fraction of cyclic topologies upon denaturation–renaturation of the scleroglucan (Falch, Elgsaeter, & Stokke, 1999) and also complex formation between the scleroglucan and poly(C) (Sletmoen & Stokke, 2005). The 35 min sonicated sample prepared in the present study was freeze-dried. Samples prepared according to this procedure and dissolved in water are in the following referred to as sonicated scleroglucan. Using SEC-MALLS the M_W obtained when using this treatment in the present study was determined to be 5.7×10^5 g mol^{-1} and the polydispersity index of the sample was determined to 1.5. Poly(C) with a degree of polymerisation (dp) equal to 485 was obtained from Sigma.

2.1.2. Denaturation and renaturation of scleroglucan

The freeze-dried scleroglucan was dissolved in DMSO to induce the dissociation of the triple helixes into single stranded scleroglucan (s-Scl). Scleroglucan samples of 0.9 or 1.8 mg were dissolved in 75 μl DMSO giving concentrated polymer in DMSO solutions. Renaturation, i.e. regeneration of the triple helix, occurred upon dilution with an aqueous solution containing 0.09 M NaCl to a final volume of 1.5 mL.

2.1.3. Preparation of scleroglucan-poly(C) complexes

A solution containing single-stranded scleroglucan (s-SCL) was obtained by dissolving freeze-dried scleroglucan in DMSO as described above. The poly(C) was brought in contact with the single stranded scleroglucan chains by adding an aliquot of the s-SCL/DMSO solution to poly(C) dissolved in aqueous 0.09 M NaCl. The final volume fractions of DMSO and H_2O in the polynucleotide/scleroglucan mixtures were equal to 5% DMSO and 95% H_2O. The concentrations of the s-SCL /DMSO and the poly(C)/water solutions were chosen to give the desired s-SCL and poly(C) molar concentrations after mixing. The scleroglucan concentration in the final poly(C)/scleroglucan mixture was 1.2 mg/ml, whereas the poly(C) concentration was 0.24 mg/ml. Samples of scleroglucan renatured before the addition of the poly(C) were also prepared.

2.1.4. Treatment of renatured scleroglucan samples and of poly(C)-scleroglucan complexes

Some samples were subjected to high temperature treatment before further analysis. Aliquots of 2–3 mL polymers in aqueous 0.09 M NaCl were annealed, i.e. heated in pressurised vessels to a temperature equal to 90 °C in an oil bath for 2 h. These experimental conditions were chosen based on previous investigations of different annealing procedures (Falch et al., 1999; McIntire & Brant, 1998). After this annealing treatment, the samples were left at room temperature in order to cool. In some experiments, the annealing process was performed in the calorimeter by leaving the sample at 95 °C for 1 h after the first up-scan.

2.1.5. Differential scanning calorimetry

The calorimetric experiments were performed using a differential scanning calorimeter (Nano DSC series III from Calorimetry Sciences Corporation, Lindon, USA). The scans were carried out at a rate of 1 °C/min. A lower scan rate, 0.5 °C/min, did not increase the resolution of the thermograms. The thermal stability of the aqueous samples was monitored in the temperature interval from 1 or 10 °C to 40, 60, 75, 90 or 95 °C. An equilibration period of 10 min was used between up- and down-scans, except for the annealing treatment where an equilibration period of 1 h was used. All solutions were degassed in a low pressure chamber prior to the measurements. The calorimetric data were corrected for the calorimetric baseline (by subtracting scans with buffer both in the sample and reference cell). The area under the transition peaks was calculated by using two-point setting peak integration in the instrument software.

2.1.6. SEC-MALLS

The properties of the polynucleotide/polysaccharide samples were investigated at room temperature by size exclusion chromatography (SEC) coupled with serially connected refractive index (RI)-, ultraviolet (UV)- and multi-angle laser light scattering (MALLS) detectors. Inter-detector volumes were corrected for in the data analysis. The refractive index increment of scleroglucan (dn/dc) was taken to be 0.15 ml/g (Yanaki, Kojima, & Norisuye, 1981). For poly(C), the value determined for DNA (0.188 ml/g) was used (Reichmann, Rice, Thomas, & Doty, 1954). All the samples were diluted prior to injection with a solution containing 0.05 M Na_2SO_4/0.01 M EDTA adjusted to pH 6 and subjected to filtration (pore-size equal to 800 nm). The injected volume varied from 100 to 250 μl. Light scattering was monitored employing a DAWN DSP multi-angle laser light scattering detector. The wavelength of the laser light was 632.8 nm. The RI-detector used was a DAWN Optilab 903. UV detection was carried out at the wavelength $\lambda = 270$ nm (Schimadzu SPD – 10 A). This is the wavelength reported to give rise to the maximum UV absorption of poly(C) (Sakurai & Shinkai, 2000). The system was eluted with 0.05 M Na_2SO_4 / 0.01 M EDTA adjusted to pH 6 at a flow rate of 0.5 ml/min. Two serially connected HPLC-SEC columns TSK 6000 and 5000 were used.

2.1.7. Atomic force microscopy

The samples were prepared for atomic force microscopy (AFM) following a procedure reported elsewhere (Stokke, Falch, & Dentini, 2001). Aliquots of the samples were mixed with 60% aqueous glycerol to a final polysaccharide concentration of 2–4 μg/ml and a final weight fraction of glycerol equal to 50%. A small volume of these solutions were sprayed on freshly cleaved mica discs and vacuum dried at 1.3×10^{-3} Pa for at least two hours. Previous analysis of polysaccharide chain flexibility from the trajectories of specimen's prepared involving vacuum drying from aqueous glycerol is reported to preserve quantitative information about chain stiffness. The vacuum drying also appears to be less harmful in inducing aggregation of some polysaccharides than a method of

air drying. AFM topographs of scleroglucan and poly(C)-scleroglucan complexes were obtained using a Digital Instruments Multimode IIIa atomic force microscope equipped with an E-scanner. Silicon nitride cantilevers (PPP-NCH-W, Pointprobe plus, Nanosensors) with nominal spring constants of 10–130 N/m and nominal resonance frequencies of 200–500 kHz were employed. The instrument was operated in tapping mode as described previously (Stokke et al., 2001).

3. Results

3.1. DSC studies of structure and thermal stability of scleroglucan

3.1.1. Transition at low temperature, i.e. ~ 8 – 10 °C

When heating or cooling samples of linear scleroglucan dissolved in water using DSC, a reversible endothermic transition is observed in a temperature interval centered around 8–10 °C (Fig. 3A and B). This signal has previously been observed in DSC investigations of (1,3)-β-D-glucans (Kitamura & Kuge, 1989).

3.1.2. Transition at intermediate temperature, i.e. ~ 35 °C

A second endothermic transition is observed for renatured scleroglucan. This transition gives rise to a partial reversible signal in the DSC up-scans in the temperature interval 30–40 °C, centered at 35 °C. When solutions of renatured scleroglucan are monitored up to 40 °C (Fig. 3E) and 90 °C (Fig. 3C and D), the transition peak is reduced by approximately 46% and 84%, respectively, in the second up-scans compared to the first up-scan. This transition is not observed when inspecting samples containing linear scleroglucan dissolved in water (Fig. 3A) and was therefore not observed by Kitamura and Kuge in their study of linear schizophyllan dissolved in water / DMSO mixtures (Kitamura & Kuge, 1989). Furthermore, this transition is suppressed following the annealing (95 °C, 1 h) of the renatured scleroglucan (Fig. 3F, second up-scan).

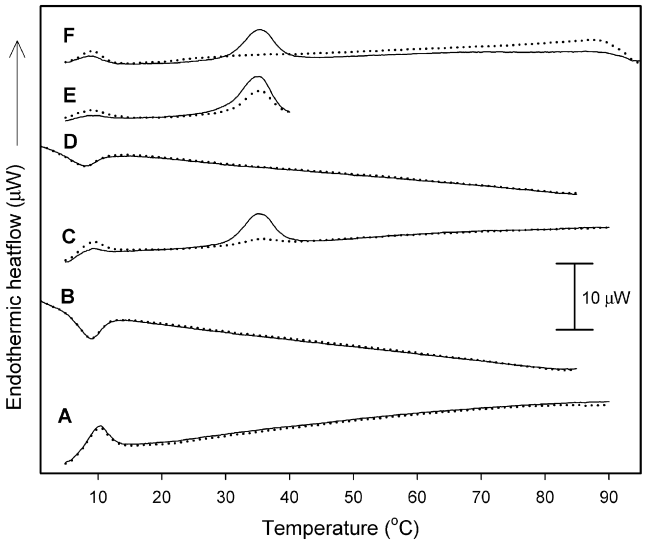

Fig. 3. DSC thermograms of scleroglucan solutions. The scleroglucan concentration is 1.2 mg/ml. The curves have been shifted in the vertical direction to make them distinguishable. A small temperature interval has been ignored in the beginning of up- and down-scans due to instrument relaxation time. Solid - and dotted lines denote the first and second up- or down-scan, respectively. (A) Up-scans and (B) down-scans of linear scleroglucan solutions monitored in the interval 1–90 °C. (C) Up-scans and (D) down-scans of renatured scleroglucan solutions monitored in the interval 1–90 °C. (E) Up-scans of renatured scleroglucan solutions monitored in the interval 1–40 °C. (F) Up-scans of scleroglucan solutions annealed in the calorimeter by leaving the samples at 95 °C for 1 h after the first up-scan.

3.2. DSC studies of structure and thermal stability of poly(C)-scleroglucan complexes

3.2.1. The transition temperature of the poly(C)-scleroglucan complex

Fig. 4A and B shows DSC thermograms obtained for solutions of scleroglucan renatured in the presence of poly(C). The peak observed at 35 °C is also present for solutions of renatured scleroglucan (Fig. 3C). An additional endothermic transition is observed in the temperature interval 44–58 °C, centered at 55 °C. Poly(C) alone does not give rise to any signal detected by the calorimeter (Fig. 4C).

3.2.2. The reversibility of poly(C)-scleroglucan complex melting processes

In the present study, the transition peak centered at 55 °C of the complexes is fully or partially reversible depending on the temperature history of the solution. When the solution of complexes is monitored up to 60 °C, the transition occurring at the same temperature and with the same excess heat for subsequent scans indicates a reversible process (Fig. 4B). However, when the solution of complexes is monitored up to 90 °C, the transition peak is reduced by approximately 63% and 76% in the second and third up-scan, respectively, compared to the first up-scan (Fig. 4A). In addition to this reduction of signal, the shape of the peak is altered in the second and third up-scan compared to the first up-scan (Fig. 4A). The peak is sharpened and most of the peak is within the high temperature part of the signal observed in the first up-scan. After scanning the sample to 60 °C, the signal centered at 35 °C in the first up-scan is shifted towards lower temperatures in the second and third up-scan, and a broad signal is observed in the interval 20–40 °C (Fig. 4B).

3.2.3. Requirements for poly(C)-scleroglucan complex formation

Complexes between (1,3)-β-D-glucans and poly(C) have previously been prepared by allowing single stranded (1,3)-β-D-glucans to renature in the presence of poly(C) (Sakurai & Shinkai, 2000; Sakurai et al., 2001; Sletmoen & Stokke, 2005). We investigated the ability of triple stranded scleroglucan to interact with poly(C).

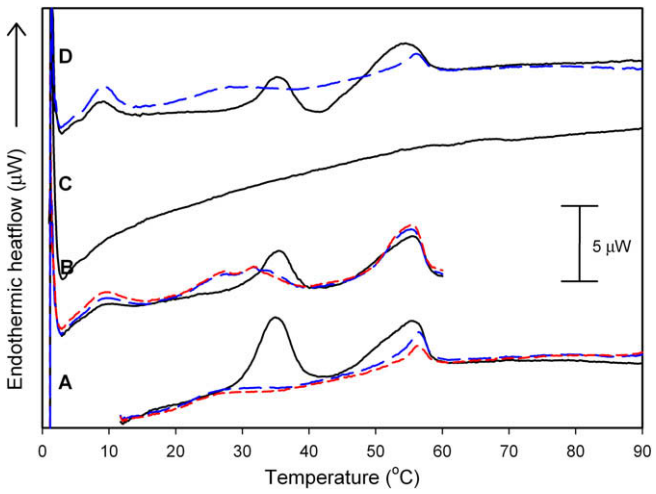

Fig. 4. DSC thermograms of solutions containing poly(C)-scleroglucan complexes and poly(C) alone. The scleroglucan and poly(C) concentration is 1.2 mg/ml and 0.24 mg/ml, respectively. The curves have been shifted in the vertical direction to make them distinguishable. Solid lines, long dashed lines and dotted lines denote the first, second and third up-scan, respectively. (A) Up-scans of solutions containing poly(C)-scleroglucan complexes monitored up to 90 °C. (B) Up-scans of solutions containing poly(C)-scleroglucan complexes monitored up to 60 °C. (C) Up-scans of poly(C) alone. (D) Up-scans of mixtures containing renatured scleroglucan and poly(C).

Table 1
Requirements for poly(C)-scleroglucan complex formation.

Sample	Scleroglucan	Sample preparation	Complex formation
A	Single stranded	Poly(C) added prior to Renaturation	Yes
B	Triple helical	Addition of poly(C)	No
C	Renatured	Addition of poly(C)	Yes
D	Renatured and annealed	Addition of poly(C)	No

Table 1 summarizes our observations concerning the requirements for poly(C)-scleroglucan complex formation. Linear native scleroglucan triple helixes or annealed scleroglucan do not show any interaction with poly(C). On the other hand the mixture of renatured scleroglucan and poly(C) gives rise to a signal in the temperature interval 44–58 °C (Fig. 4D). The behavior of this sample is identical to the behavior observed for mixtures of scleroglucan renatured in the presence of poly(C) (Fig. 4A).

3.3. AFM observations of poly(C)-scleroglucan complexes

The AFM topographs shown in Fig. 5 depict the structures present in a sample obtained by allowing scleroglucan to renature in the absence (Fig. 5A) and presence (Fig. 5B) of poly(C). The topograph presented in Fig. 5C presents the structures present in a sample where poly(C) has been added to a sample containing renatured scleroglucan. A comparison of the images obtained for scleroglucan samples renatured under the same experimental conditions but in the absence or presence of poly(C) (Fig. 5A and B, respectively) reveal that the fraction of circular structures decreases if adding poly(C) prior to scleroglucan renaturation. Furthermore, the variability of molecular shapes observed in the sample containing the poly(C)-scleroglucan complexes is large. In addition to the linear, circular and racket shaped forms, aggregates are also observed (Fig. 5B). The structural content of samples prepared by adding poly(C) to renatured samples of scleroglucan indicate that the addition of poly(C) does not lead to a significant disruption of the circular structures formed during scleroglucan renaturation. Irrespective of the preparation method, the structures present in the samples containing both scleroglucan and poly(C) show structural imperfections (Fig. 5B and C). Fig. 6 presents a magnification of some of the structures observed in samples containing poly(C)-scleroglucan complexes prepared by addint poly(C) prior to the scleroglucan renaturation. Bumps or loops are observed along some of the linear structures (Fig. 6A and C), probably giving rise to a non-homogenous linear mass density along the chain. In some of the linear structures, the bending stiffness seems to change abruptly at a certain position along the linear structure, giving rise to one stiff structure with a significantly more flexible end (Fig. 6B).

Fig. 7 shows the structures present in a sample containing poly(C)-scleroglucan complexes heated to 56 °C and cooled. This temperature is close to, but above, the peak of the melting transition of the complexes (Fig. 4). The AFM topographs reveal the presence of predominantly linear structures. The observed relatively constant height along the structures indicates that they have a relatively homogenous mass density. Only a few structures containing visible bumps indicating accumulated mass are observed.

3.4. Elution profile of samples containing poly(C)-scleroglucan complexes determined by SEC-MALLS

The change in melting behavior of the complexes after exposure to a melting and regeneration cycle occur concomitant with small changes in the SEC-MALLS elution profile. The elution profile of the complexes can be deduced from the elution profile of the poly(C) content in the samples, as detected by the UV detector (Fig. 8). Complexes that had been heated to 60 and 90 °C for two hours (Figs. 8E and F) eluted in a less broad interval of elution times than

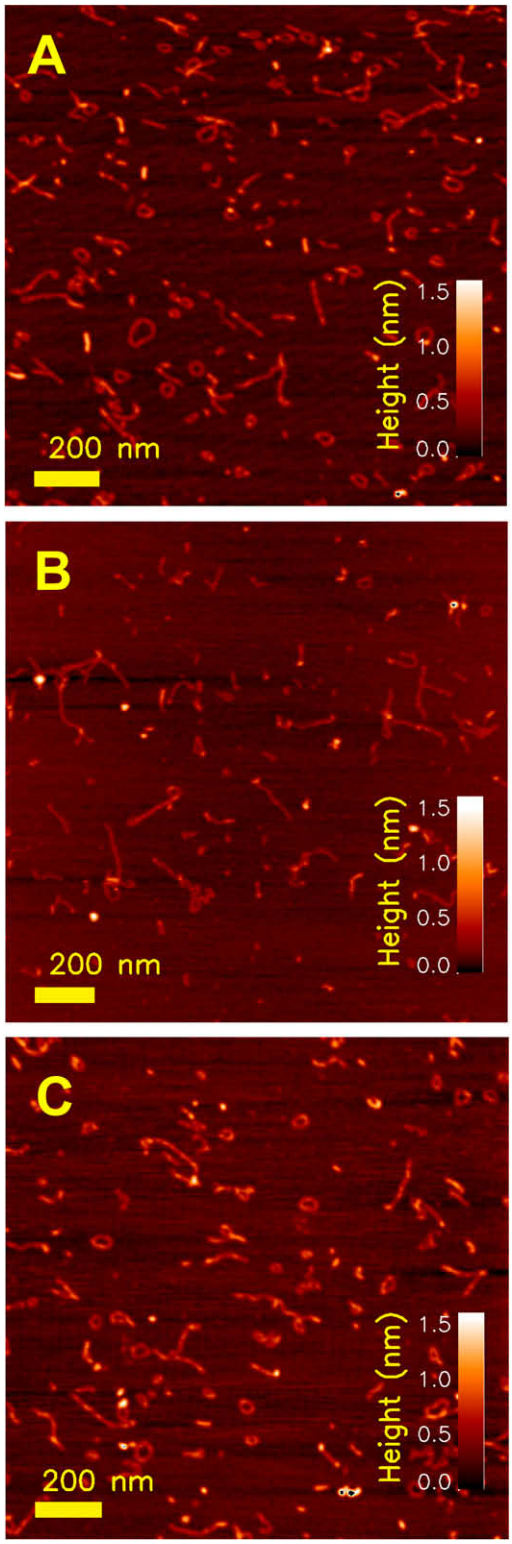

Fig. 5. Tapping mode AFM topograph of scleroglucan and poly(C)-scleroglucan complexes. (A) Scleroglucan exposed to a denaturation-renaturation cycle; (B) poly(C)-scleroglucan complexes obtained by allowing scleroglucan to renature in the presence of poly(C). (C) Poly(C)- scleroglucan complexes obtained by adding poly(C) to a sample of renatured scleroglucan.

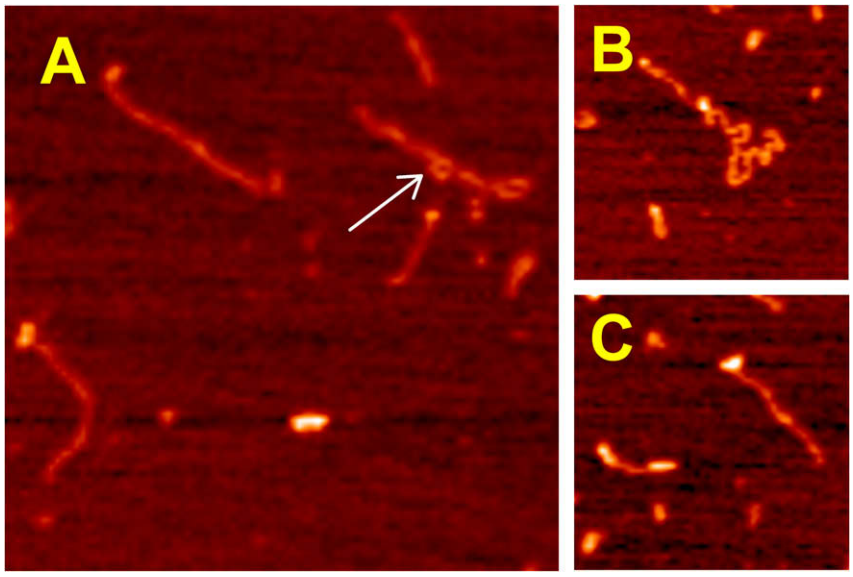

Fig. 6. Tapping mode AFM topograph of poly(C)-scleroglucan complexes prepared as described in Fig. 5. The images shown are magnifications of selected areas and depict different categories of structural defects observed in the images. These structural imperfections include dots (image A and C) or circles (identified by an arrow in image (A) formed along linear structures as well as chains of high flexibility extending from stiff linear structures (image B). The size of the scan-areas shown is for image A-556 nm, for image B-390 nm and for image C-360 nm.

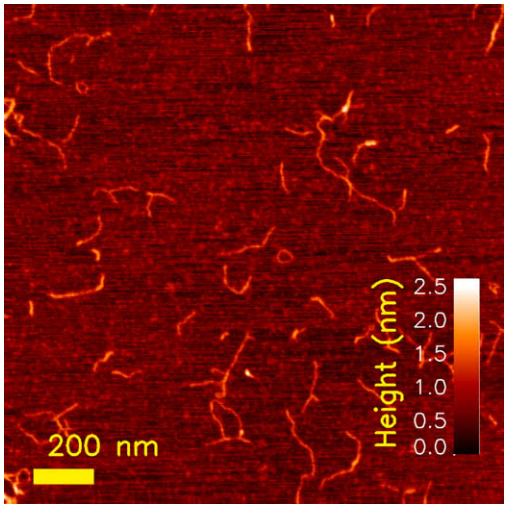

Fig. 7. Tapping mode AFM topograph of poly(C)-scleroglucan complexes heated to 56 °C. The amount of structural defects seems to be reduced compared to the images shown in Figs. 5B and 6. The number of circular structures is significantly lower than observed for renatured samples of scleroglucan (Fig. 5A).

complexes not exposed to high temperatures (Figs. 8C and D). Whereas the complexes originally elute in the interval 12–19.5 ml (Fig. 8C and D), the complexes that had been heated to 60 °C for 2 h eluted in the interval 13–19 ml (Fig. 8E), and those that had been heated to 90 °C for 2 h eluted in the interval 13 – 18 ml (Fig. 8F). The M_w of the samples decreases as a result of the temperature treatment from 5.7×10^5 g mol^{-1} for the sample containing complexes previous and not exposed to any thermal treatment, to 4.1×10^5 g mol^{-1} for the sample heated to 60 °C for 2 h and further to 3.9×10^5 g mol^{-1} for the sample heated to 90 °C for 2 h. The polydispersity index ($I = M_w/M_n$) for these samples decreases from 1.7 for the starting sample, to 1.4 when heated to 60 °C and further to 1.3 when heated to 90 °C.

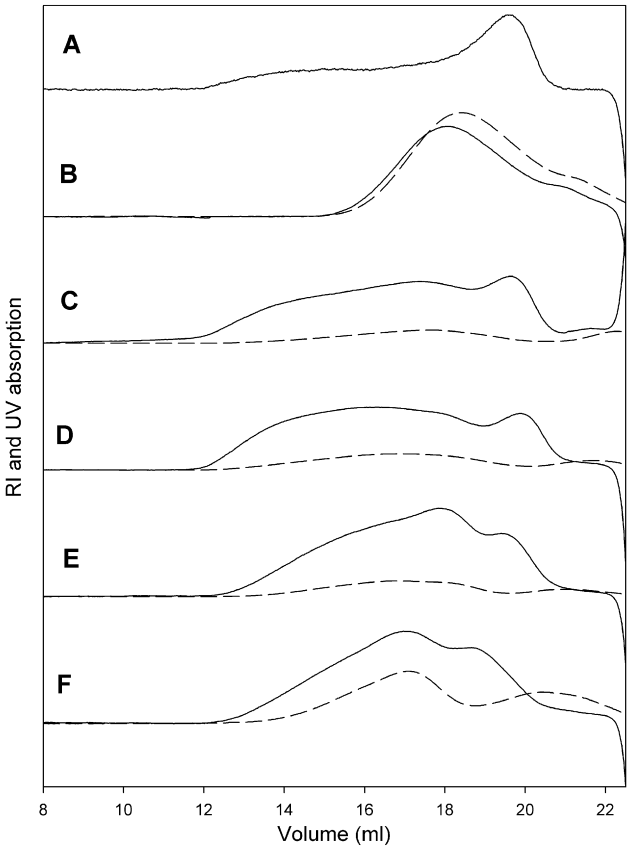

Fig. 8. Chromatograms obtained when analysing mixtures containing poly(C) and / or scleroglucan with SEC-MALLS. The detected refractive index (continous lines) as well as the UV absorption at $\lambda = 270$ nm (discontinous lines) is plotted as a function of elution volume. (A) Renatured scleroglucan. (B) Poly C, (C) poly(C)-scleroglucan complexes obtained by adding poly(C) to renatured scleroglucan, (D) poly(C)-scleroglucan complexes obtained by allowing scleroglucan to renature in the presence of poly(C). These complexes were subsequently heated to 60 °C (E) and 90 °C (F) for 2 h and cooled prior to the analyses.

3.5. Hydrodynamic properties of poly(C)-scleroglucan complexes formed under different preparation conditions

The hydrodynamic properties of the poly(C)-scleroglucan complexes formed under different preparation conditions were investigated using SEC-MALLS. Fig. 9 presents the rms z-average radius of gyration (R_g) as a function of M_w for a sample of the linear scleroglucan and for samples containing poly(C)-scleroglucan complexes. The complexes were prepared by allowing scleroglucan to renature in the presence of poly(C) (Table 1 sample A) or by adding the poly(C) after renaturation of the scleroglucan (Table 1 sample C). Both samples were investigated before and after heating to 80 °C for 1 h. The sample containing linear scleroglucan molecules shows a hydrodynamic behavior consistent with earlier studies of (1,6)-branched (1,3)-β-D-glucans (Kashiwagi, Norisuye, & Fujita, 1981; Sletmoen, Christensen, & Stokke, 2005). In a log–log plot (Fig. 9), the relationship between M_w and R_g conforms to a linear relationship with slope equal to 0.94 in the low M_w part of the distribution. At higher M_w, the chain flexibility of the polymers and possible aggregation of the molecules causes a less steep increase in R_g with further increase in M_w. The poly(C)-scleroglucan complexes show a different behavior. The structures are denser. In the low M_w part of the curve a steeper increase in R_g with increasing M_w is observed for the heated samples.

The estimated the mass per unit length from the experimentally determined parameters represent an additional set of information to deduce structural features of the complexes. The experimentally determined R_g and M_w in the rodlike limit forms the basis for such an analysis using the fact that the radius of gyration of rigid rods of length L is given by the expression $R_g = L \times 12^{-0.5}$. The ratio $R_g \times 12^{0.5}/M_w$, is presented in Fig. 10 as a function of M_w. The limiting rod-like behavior is evident as a plateau at low M_w. The uncertainties in the obtained values of M_L are expected to be similar to the values previously determined (Sletmoen et al., 2005), i.e., about 11% of M_L The data obtained for linear scleroglucan are consistent with earlier results and indicate a mass per unit length of scleroglucan equal to 2270 ± 250 g mol^{-1} nm^{-1}. This is consistent with the predicted value of triple helical structures of scleroglucan, being equal to 2150 g mol^{-1} nm^{-1} (Chuah, Sarko, Deslandes, & Marchessault, 1983). The structures formed when allowing scleroglucan to renature in the presence of poly(C), or when adding poly(C) to a solution containing renatured scleroglucan, have a M_L equal to 2860 ± 310 g mol^{-1} nm^{-1}. After heating these samples to 80 °C for 1 h the position of the plateau shifts to a niveau indicating a M_L equal to 3570 ± 390 g mol^{-1} nm^{-1} (Fig. 10).

4. Discussion

4.1. Scleroglucan studied by DSC

The existence of the endothermic peak at 8–10 °C of aqueous scleroglucan (Fig. 3) has previously also been reported and suggested to originate from structural transition involving the side chains of the triple helix (Kitamura & Kuge, 1989). This structural transition, observed at 8–10 °C in the present study, has previously been reported to occur at 7 °C for aqueous solutions of (1,3)-beta-D-glucans (Bluhm et al., 1982; Bo et al., 1987). The position of this peak has, however, been shown to shift to higher temperatures with increasing amounts of DMSO added to the aqueous solution. The discrepancies of 1–3 °C between our observations and the

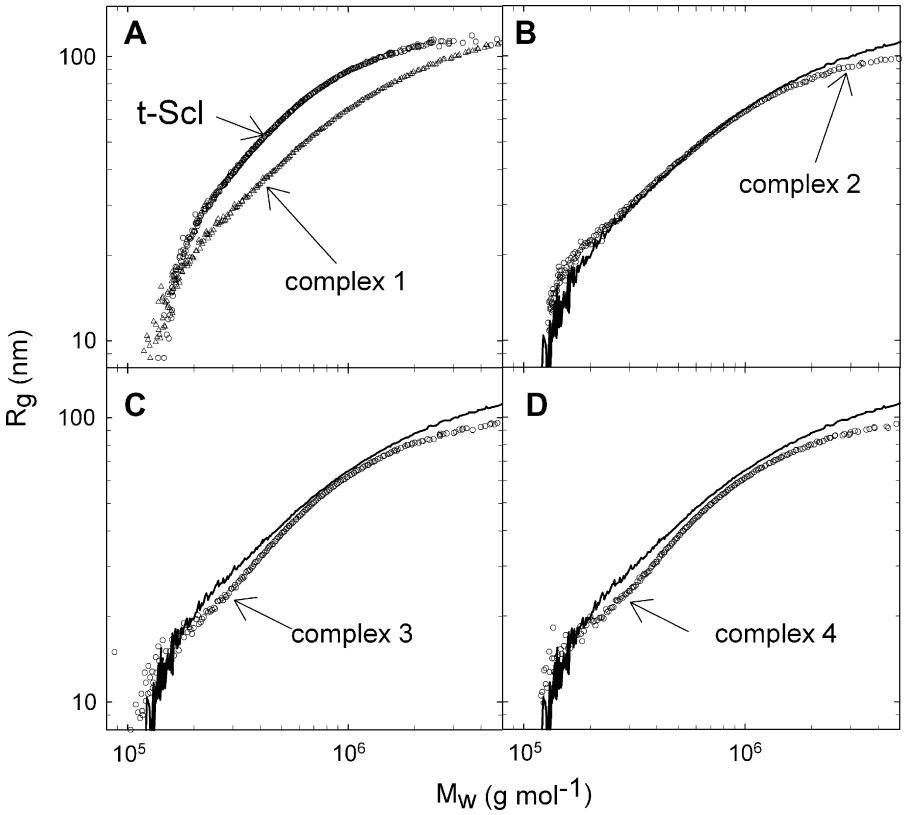

Fig. 9. Radius of gyration versus weight average molecular weight (M_w) for samples containing linear scleroglucan as well as for samples containing poly(C) – scleroglucan complexes prepared using different procedures. (A) Linear scleroglucan (t-Scl) and complexes formed by allowing scleroglucan to renature in presence of poly(C) (Complex 1). (B) Complexes formed by allowing scleroglucan to renature prior to addition of poly(C) (Complex 2). In order to facilitate the data-comparison the data obtained for complex 1 is added as a continuous line in figure B, C and D. (C) Complex 3 is formed by heating complex 1 to 80 °C for 1 h. (D) Complex 4 is formed by heating complex 2 to 80 °C for 1 h.

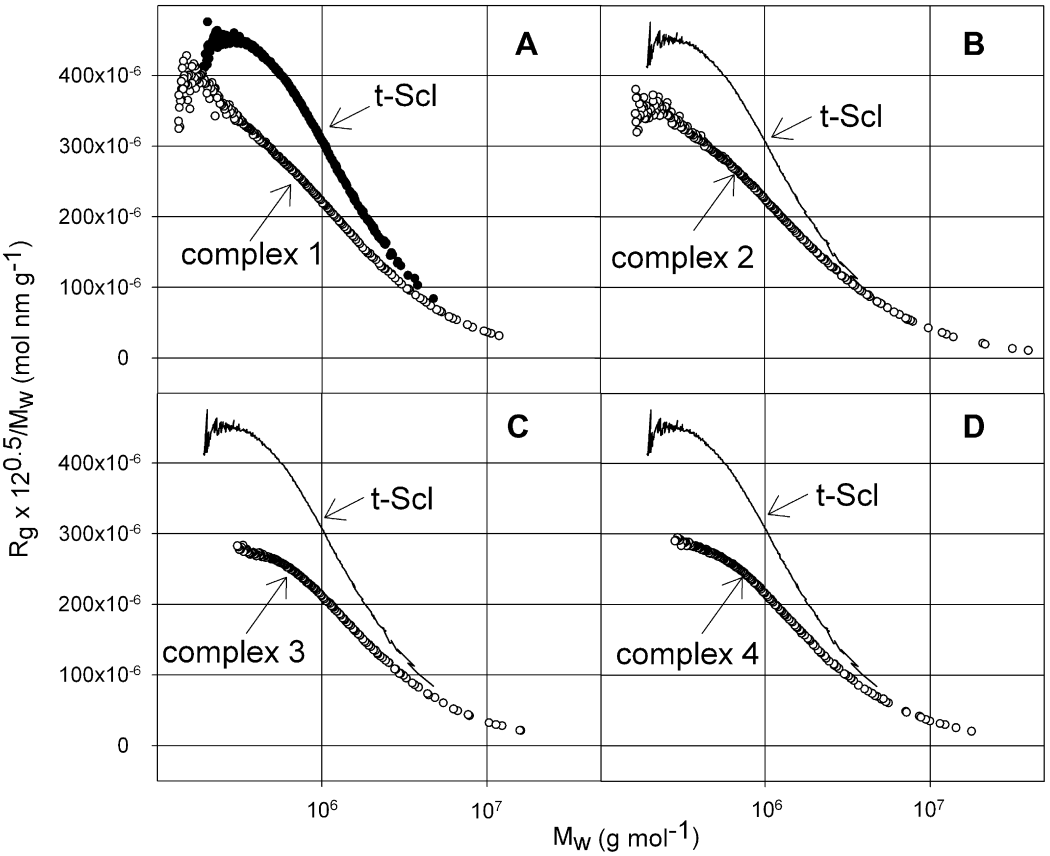

Fig. 10. The inverse of the mass per unit length (calculated as the experimentally determined radius of gyration multiplied by $12^{0.5}$ and divided by the molecular weight) versus weight average molecular weight. An explanation of the sample preparation giving the samples designated complex 1, 2, 3 and 4 are given in the legend to Fig. 9. In order to facilitate the data-comparison the data obtained for linear scleroglucan is added as a continuous line in figure B, C and D.

observations reported for (1,3)-beta-D-glucans in aqueous solutions are thus likely to be due to the small amounts of DMSO present in the samples. The influence of DMSO on the position of the signal observed at low temperature does not influence on the conclusions presented in the paper.

Renatured and annealed (1,3)-β-D-glucans have to our knowledge not previously been studied by DSC. The signal observed at 35 °C is specific for renatured scleroglucan, and not observed for the linear sonicated nor the renatured and subsequently annealed scleroglucan (Fig. 3). The findings that no transition is observed in the down-scan and significant reduction in signal intensity is observed in the second up-scan, suggests that the transition is irreversible. Furthermore, the signal disappears when the renatured scleroglucan is annealed. A possible origin of this signal is the melting of rather short triplex stretches between structural defects in the renatured structures (Fig. 2A). Another possible origin is the existence of short duplex segments in 1,3-β-D-glucans, but the existence of such duplex segments remain to be explicitly proved. In the following we therefore focus on the explanation based on the melting of the triple helical segments even though the existence of double helical stretches can not be ruled out. The finding that the endothermic peak is located at the same temperature but with reduced intensity in the second up-scan suggests that the length of the regenerated triple helical sequences are unchanged, but the content is reduced following the heating-cooling cycle. These triple helical stretches, l_{triple}, are further characterized by low stability compared to the untreated scleroglucan due to their reduced length. During renaturation the single strands do not always manage to regenerate perfect triple helixes. Structural defects have previously been suggested (Falch & Stokke, 2001) and observed (Bae et al., 2004; Sletmoen & Stokke, 2005; Stokke et al., 1991) in these preparations. The imperfections can take the form of loops formed by one strand not being perfectly in register with the other strands making up the triplex. It has previously been reported that annealing removes structural defects (Falch & Stokke, 2001). This can be envisioned to occur by elongation of the triplex regions that are stable at the annealing temperature (Fig. 2). Such a process would facilitate the reduction of both the fraction of imperfectly assembled regions as well as the fraction of shorter triplex regions giving rise to the transition observed at 35 °C. Annealing of a scleroglucan sample with M_w of about $5x10^5$ g mol^{-1}, renatured from high NaOH conditions by decreasing the pH, was found to yield an increase in the fraction of cyclic species and a decrease in the relative abundance of globular and short linear species (Kitamura et al., 1996). These observations indicate that the globular and short linear species formed upon renaturation are kinetically trapped and do not exist under equilibrium conditions.

The suggested model for removal of the structural features giving rise to the transition observed at 35 °C is also supported by the following data. In the samples heated to 90 °C, the signal observed at 35 °C almost disappears in the second up-scan (Fig. 3C) whereas in the samples heated to lower temperatures (i.e. 40 °C, see Fig. 3E), a signal is observed also in the second and subsequent up-scans. The intensity of the signal is however gradually decreasing in the subsequent scans. This suggests that the duration needed for removal of the structural defects are longer at the lower temperatures. At 90 °C, duration of 2 h is sufficient for the growth of the longest triple helical regions, l_{triple}, and associated removal of the non-perfect regions. The kinetics is slower at i.e. 40 °C compared

to 90 °C thus allowing topologically separated triplex regions to be regenerated to a certain extent at even lower temperatures. Previously, an annealing condition of 90 °C with 2 h duration was chosen in order to induce the structural rearrangements associated with annealing (Falch et al., 1999). The present data provide calorimetric evidence that this is a sufficient condition to remove the structural defects thus yielding a more stable structure.

4.2. Melting of poly(C)-scleroglucan complexes observed by DSC

The signal observed at 55 °C in the DSC scans obtained for the poly(C)-scleroglucan samples (Fig. 4A, B and D) is not attributable to poly(C) alone since free poly(C) does not possess any conformational transition in this temperature range (Fig. 4C). The process giving rise to this signal occurs at the same temperature as the melting transition of the complexes, as previously determined by CD (Sakurai and Shinkai, 2000). It is therefore reasonable to assume that this DSC signal corresponds to the melting transition of poly(C)-scleroglucan complexes.

Structural reorganisations are thermally activated processes, with a time constant that is expected to be influenced by the organisation of the polysaccharide segments and the temperature distance between the melting temperature of the growing ordered sequences and annealing temperature. The observations concerning the signal observed at 35 °C are in line with the observations obtained for solutions containing renatured scleroglucan: For samples monitored to 90 °C, the signal observed at 35 °C disappears in the second up-scan (Fig. 4A). For samples heated to lower temperatures (Fig. 4B), a signal is observed also in subsequent up-scans, even though significantly broadened and shifted to lower temperatures. The strength of the signal observed at 55 °C also depends on the thermal history of the sample (Fig. 4). These processes are not observed in samples only containing scleroglucan, so they are expected to be due to scleroglucan – poly(C) interactions. The reduction in intensity in the second and subsequent scans can be due to either reduced availability of scleroglucan conformational states that can form complexes with poly(C), or reduction of chain length of poly(C) due to thermally induced degradation, making the poly(C) chains too short to form stable co-operative interactions with the scleroglucan.

4.3. Properties of the poly(C)- scleroglucan complexes prepared using different procedures

The hydrodynamic and calorimetric investigations of the complexes do not reveal any differences between complexes formed by allowing scleroglucan to renature in presence of poly(C) and complexes formed by adding poly(C) to renatured scleroglucan (Fig. 10). In the present system, the amount of poly(C) relative to scleroglucan present in the mixture eluting from the column is varying throughout the elution profile, and also possibly among co-eluting species. The dn/dc value will thus gradually change through the elution profile, a fact which has implications on the accuracy of the determined M_L. A more precise M_L value for the complexes can be obtained if analysing samples in which the poly(C)-scleroglucan complexes have been separated from the triple helixes of scleroglucan. Such separation has not yet been performed on these complexes. The differences in slope observed at high molecular weight (Fig. 9) might be due to different fractions of aggregates formed in the samples and does not necessarily reflect local structural differences in molecular organisation within the complexes. The thermal stability of the complexes formed between renatured scleroglucan and poly(C) also appears to be identical to the stability observed for the complexes formed between denatured (1,3)-β-D-glucans and poly(C), both dissociating in the interval 44–58 °C.

4.4. Effect of the temperature treatment on the structure of the complexes

The hydrodynamic studies suggest that the structure of the complexes changes as a result of the temperature treatment (Sletmoen and Stokke, 2005) (Figs. 9 and 10). The observations therefore indicate a different structure of the two types of polymers within these complexes compared to those not subjected to annealing. The steeper increase in R_g with increasing M_w observed at low M_w for samples exposed to the temperature treatment is possibly due to the reduction of structural imperfections within the complexes. A value close to 1 was obtained in a previous study of the complexes (Sletmoen and Stokke, 2005). Deviations from this value might be due to the presence, type and amount of structural defects. Whether the poly(C) is added prior to or after the renaturation of the scleroglucan does not seem to influence on the density of material in the complexes formed, nor on the relationship between R_g and M_w (Figs. 9 and 10).

The M_w and polydispersity of samples containing complexes exposed to different temperature treatments reveal that the temperature treatment has not only lowered the molecular weight, but it has also resulted in reduced size heterogeneity within the sample. This might explain the less broad signal for the melting transition observed in the DSC spectra (Fig. 4). The structural defects may be of many forms, ranging from small mismatches between the strands making up the complex, to larger mismatches leading to i.e. loop formation of one of the strands involved in the complex formation. The latter type of mismatch was in this study revealed in AFM images of poly(C)-scleroglucan complexes and included loops and dots observed along the linear structures as well as flexible chains protruding from the end of the linear rod-like complexes (Fig. 6). Small bumps of material along the complexes, interpreted as being due to structural defects, have previously been observed using ultramicroscopy (SEM (Bae et al., 2004) or AFM (Sletmoen and Stokke, 2005)).The AFM topographs depicting complexes exposed to a temperature treatment (Fig. 8) do indicate a reduced fraction of structural defects compared to the situation prior to the treatment (Fig. 5 and 6).

4.5. Requirements for complex formation between scleroglucan and poly(C)

Sakurai and Shinkai were the first to demonstrate that single stranded (1,3)-β -D-glucans can form a macromolecular complex with poly(C) and poly(A) during renaturation (Sakurai and Shinkai, 2000). Since then many studies of such complexes have been published and the first review paper summing up the results obtained within this field has appeared (Sakurai et al., 2005). In all these previous studies, the glucans have been in the single stranded form when allowing the complex formation to take place. This starting situation has been regarded as a requirement for the complex formation to take place. The proposed model for the structure of the complex, were the dissociation of the scleroglucan triple helix is a requirement, is in accordance with this assumed requirement for the scleroglucan to be in the single stranded state. The results obtained in this study (Figs. 4 and 8–10) show that (1,3)-β-D-glucan-poly(C) complexes do not require that the entire glucan is in the single stranded state when introducing the poly(C). Complexes also form between poly(C) and renatured (1,3)-β-D-glucans. These renatured structures are believed to predominantly exist in the triple helical state, with interspersed regions of unpaired single strands (Falch and Stokke, 2001). No complexes are formed between native scleroglucan and poly(C), and the renatured scleroglucan looses the ability to form complexes with poly(C) upon annealing. The requirements for complex formation, as determined in this study, are summarised in Table 1.

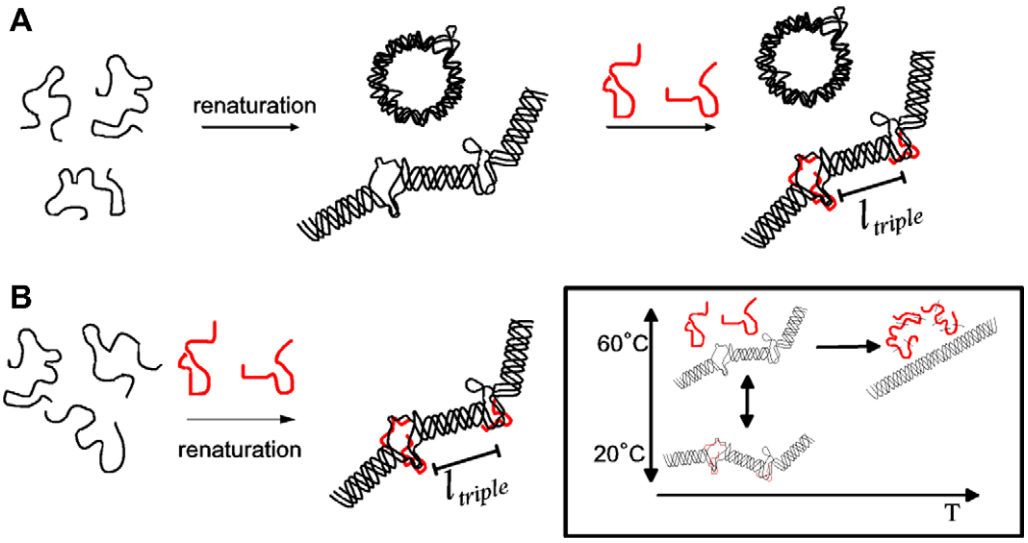

Fig. 11. Schematic illustration of scleroglucan-poly(C) complex formation. (A) The scleroglucan (black lines) is allowed to renature before the addition of poly(C) (red lines). Under certain conditions circular structures form in addition to the linear renatured triple helixes. Complexes are formed between the triple helixes of scleroglucan and poly(C). Renatured structures of scleroglucan contain structural defects. The lengths of the triple helical segments existing between two regions of structural defects are designated l_{triple}. (B) The scleroglucan is allowed to renature in the presence of poly(C). Under renaturation conditions, the single stranded scleroglucan coils recombine to triple-helixes (t-scl). Previous studies have shown that circular structures including poly(C) are not formed, and the fraction of circular structures present in the renatured mixture is therefore decreased compared to the fraction of circular structures formed in the absence of poly(C). No differences in structure or behavior between the complexes formed using the preparation protocols depicted in A and B are detected. Inset: The complexes melt in the temperature interval 44–60 °C, but reform upon cooling. This reformation is hindered by prolonged exposure to high temperatures due to either poly(C) degradation or removal of structural defects.

These results are interesting since they open for polynucleotide-(1,3)-β-D-glucan complex formation in aqueous solutions, with a pH and ionic strength close to physiological conditions. Such complexes can thus be formed in biological systems, unlike the complexes previously described by Sakurai and others, which were all made from single stranded (1,3)-β-D-glucans. The observations presented in this paper also contain clues concerning the requirements for complex formation. The structural defects giving rise to the observed endothermic peak at 35 °C in DSC in the scleroglucan samples are essential for its ability to make complexes with poly(C). The function of these defects in the complexation process may be to expose small fragments of single strands. Such single strands are free to hydrogen bond with the poly(C), and have also in previous studies been regarded as essential for the complex formation process (Sakurai et al., 2001; Sakurai et al., 2005).

4.6. Structural implications of the present findings

A revised model for scleroglucan-poly(C) complex formation, consistent with all the observations presented in the present paper, is schematically illustrated in Fig. 11. The present findings appear not to be in line with a homogenous mixed triple helix structure, including two chains of (1,3)-β-D-glucan and one chain of poly(C) (Fig. 2B) (Sakurai et al., 2001) as the only structural organisation of the complex (Sakurai et al., 2001). The observations presented in this paper open for such structural organisation only in the regions of the (1,3)-β-D-glucan molecules where perfect triple helixes are not regenerated prior to renaturation, i.e. in regions where single strands are exposed. Further investigations are needed in order to conclude concerning the detailed structure of these complexes.

5. Conclusions

We have presented new information concerning the thermal stability and properties of poly(C)-(1,3)-β-D-glucan complexes obtained using DSC, SEC-MALLS and AFM investigations. The results reveal that the thermally induced melting transition of the complexes is at least partially reversible. This finding has practical implications in the sense that it opens up for the use of these complexes also in situations where the hydrogen bonds between scleroglucan and poly(C) are transiently broken. Furthermore, the results reveal that complexes also form between poly(C) and triple helical (1,3)-β-D-glucans provided that the glucan has been exposed to a denaturation–renaturation treatment and not annealed. The complexes can thus be formed in aqueous solutions, with a pH and ionic strength close to physiological conditions. The complexes formed under these conditions have identical melting temperature, chain stiffness and mass per unit length as the complexes formed from (1,3)-β-D-glucans in the single stranded state. It therefore appears likely that the complexes obtained when mixing renatured (1,3)-β-D-glucans with poly(C) are structurally identical to the complexes formed when allowing single stranded (1,3)-β-D-glucans to renature in the presence of poly(C). This new insight into the requirements for complex formation sheds new light on the formation mechanism and structure of the complexes. Structural defects along the scleroglucan triple helixes seem essential for their ability to form complexes. The findings presented in this paper call for a revision of the proposed structural model which affirms that the complexes are mixed triple helices of homogenous structure, comprising two strands of (1,3)-β-D-glucan and one strand of poly(C). Further experiments should be performed to reveal the exact role of the structural irregularities in the complex formation process.

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