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# Characterizing the influence of electron irradiation on scleroglucan

Christian Augsten [a], Wolfgang Knolle [b], Karsten Mäder [a,*]

[a] *Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, D-06120 Halle/Saale, Germany*
[b] *Leibniz Institute of Surface Modification (IOM) e.V., Permoserstr. 15, D-04318 Leipzig, Germany*

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**Abstract**

The electron irradiation effect on scleroglucans was investigated using different energy doses. Electron spin resonance spectra revealed radicals that were stable for several days. $^{1}H$ NMR, $^{13}C$ NMR and Raman spectra indicated no differences in chemical backbone structure due to irradiation. In contrast, lower viscosities of aqueous solutions were received at higher energy doses. This was caused by polymer degradation. The irradiation also decreased the weight average molar masses observed by gel permeation chromatography and multi-angle light scattering. Beginning from raw materials exceeding $4 \cdot 10^{6}$ Da, a number of main chain scissions of approximately $0.3 \cdot 10^{-7}$ mol $J^{-1}$ was found. But for one scleroglucan quality the scission number decreased with higher doses. In addition, the characterization via asymmetrical flow field-flow fractionation proved the presence of low and high molar mass fractions. The electron irradiation led to a preferred scission of the high molar mass chains and increased the lower molar mass fraction. Due to this effect, the broadness of the molar mass distribution decreased.
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*Keywords:* Scleroglucan; Electron irradiation; Flow field-flow fractionation; Gel permeation chromatography; Multi-angle light scattering (MALS); Molecular weight; Molar mass distribution; Raman spectroscopy; Electron spin resonance; Viscosimetry

## 1. Introduction and theory

The aim of our study was to irradiate scleroglucan by electrons and to characterize the influence of different energy doses on molecule structure by electron spin resonance spectroscopy (ESR), $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy (NMR) and Raman spectroscopy. Water soluble scleroglucan was received by a method using gentle conditions what was required to investigate the irradiation influence on average molar mass. Therefore, gel permeation chromatography (GPC) connected to multi-angle light scattering (MALS) and refractive index (RI) detectors was applied. To characterize size distribution changes more in detail, aqueous solutions of scleroglucan were separated by asymmetrical flow field-flow fractionation (aFlow-FFF or AF4) combined with MALS/RI.

### 1.1. Scleroglucan

Scleroglucan belongs to the group of polysaccharides consisting of $\beta(1 \rightarrow 3)$ connected D-glucopyranose units. The production by bacteria, algae and fungi and the properties and various applications of this group are described (Lee, 2005). Scleroglucan is produced in fungi, mainly by those of the genus sclerotium. As shown in Fig. 1, its $\beta(1 \rightarrow 3)$ chains are additionally connected via $\beta(1 \rightarrow 6)$ with one glucopyranose monomer approximately every third chain monomer (Pretus et al., 1991).

In aqueous solution mainly a triple helix molecule structure is described. The helix shows a rigid to flexible behavior, depending on its molar mass (Yanaki, Norisuye, & Fujita, 1980). Only at very high pH values a transition to single chains occurs, described as helix melting. Thus, the molar mass values above and below this pH value differ in the factor of three (Gawronski, Park, Magee, & Conrad, 1999). Furthermore, under special circumstances cyclic spe-

* Corresponding author. Tel.: +49 345 5525167; fax: +49 345 5527029.
*E-mail address:* karsten.maeder@pharmazie.uni-halle.de (K. Mäder).

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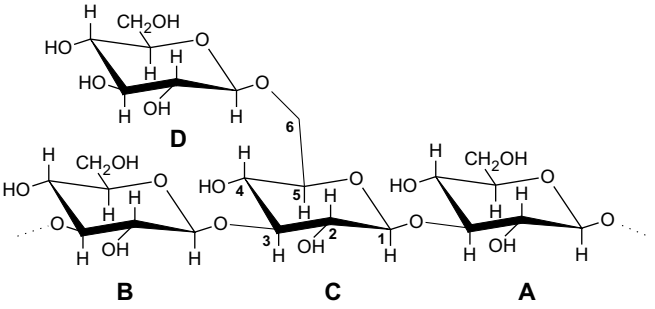


Fig. 1. Scleroglucan polysaccharide consisting of β(1 → 3) connected *d*-glucopyranose (A–C) and β(1 → 6) units (D) approximately every third chain group.

cies were described (Falch, Elgsaeter, & Stokke, 1999; Sletmoen, Geissler, & Stokke, 2006).

The molecular conformation of this polysaccharide in solution is the reason for its exceptionally high viscosity in aqueous solution and its resistance to hydrolysis, temperature and electrolyte changes. Thus, scleroglucan is used as a thickener in oil industry, water color or ink production or adhesive preparation (Halleck, 1969; Pirri, Gadioux, & Rivenq, 1992). In cosmetics, it is included, e.g., in hair control compositions, protective lotions or creams (Halleck, 1972). It also achieved increasing importance in Japanese food industry where product qualities were improved, as e.g. in frozen food (San-Ei Chemical Industries, 1982). In pharmaceutics, the general use as a stabilizer, as a laxative and in modified release dosage forms has been described (Nguyen Cong Duc, 1982; Coviello et al., 2005). Furthermore, it was explored for ophthalmic solutions and tablet coatings (Lachman & Sheth, 1968; Sheth & Lachman, 1967).

Due to their special molecular conformation β(1 → 3) glucans were found to have tumor regression and potential anti-HIV effects (Jamas, Easson, & Ostroff, 1998; Stokke, Elgsaeter, Hara, Kitamura, & Takeo, 1993). In the special case of scleroglucan, similar applications in the fields of immunostimulation, antimicrobial and antiviral activity were described (Mastromarino et al., 1997; Pretus et al., 1991). The increasing interest of scleroglucan in pharmaceutics is also reflected by novel patents about its skin moisturizing and mucoadhesive properties (Farwick, Maczkiewitz, Mecking, Schick, & Wollenweber, 2006; Nystroem, Fransen, & Bjoerk, 2006).

### 1.2. Irradiation of scleroglucan

Irradiation can be used to sterilize raw materials or to change product properties as solution viscosity in a desired way. That is most important for polysaccharides used in pharmaceutics. In the case of final formulations that are unable to be sterilized, pre-sterilized products must be used. The irradiation of polymers and especially polysaccharides has been investigated by many scientists [see reviews by Kasaai (2004); Wündrich (1989)]. The majority of the studies deal with the γ-irradiation of aqueous polysaccharide solutions [see review by Ershov (1998)]. Only few papers describe the irradiation of polysaccharides by electrons (Balazs, Laurent, Howe, & Varga, 1959; Charlesby, 1955; Katayama, Tada, Todoriki, & Nakauma, 2005; Liu & Priou, 2003). To best of our knowledge, no data have been published on electron irradiated scleroglucans.

### 1.3. Molar mass determination using Flow-FFF/MALS

A correct characterization of molar mass distribution is most important for industrial and pharmaceutical purposes due to the impact of the molecular weight on functional properties. In pharmaceutics this is especially important when hydrogel based modified release dosage forms are considered (Coviello et al., 2005). Furthermore, for scleroglucan a molar mass dependent chain conformation was described (Yanaki et al., 1980). For several β(1 → 3) glucans molar mass dependent pharmacological effects were found (e.g. antitumoral activity in mice, Yanaki et al., 1983). Smaller single-stranded molecules seem to have no immunostimulatory effect (Stokke et al., 1993).

Polysaccharides are often polydisperse with respect to their molar mass. Additionally, raw scleroglucan is known to achieve average molar masses in the GDa range (Pretus et al., 1991). The main advantage of Flow-FFF/MALS combination is the broad characterization scope, ranging for polymers from $10^3$ to above $10^7$ Da. Due to the better separation of polymers greater then a theoretical value of $5 \cdot 10^4$ Da, Flow-FFF/MALS has become a recent alternative to GPC/MALS (Hansen & Klein, 2001). The principles of asymmetrical Flow Field-Flow Fractionation are given elsewhere (Wahlund & Giddings, 1987). In brief, molecules or particles are separated with regard to their hydrodynamic sizes. Small sample components are eluting first from the separation channel. Therefore they can be easy separated from bigger ones. The hydrodynamic diameter can be calculated from the retention time using Flow-FFF theory (Dondi & Martin, 2000; Schure, Schimpf, & Schettler, 2000).

Nevertheless, this calculation can be difficult due to various sample-membrane interactions, zone spreading or overloading phenomena inside the channel (Cölfen & Antonietti, 2000). Thus, coupling FFF to molar mass detectors is an optimal option. The most common and successfully used detectors within this field are MALS detectors that allow an absolute molar mass calculation for each slice eluting from the separation channel. A detailed overview of basic theories for molar mass and radius calculation using MALS detectors can be found elsewhere (Wyatt, 1993, 1998). This combination of both techniques was already successfully applied to various macromolecular substances for pharmaceutical purposes (Augsten & Mäder, 2006; Fraunhofer & Winter, 2004). But also several polysaccharides were separated in the past. Examples include pullulans and dextrans (Wittgren & Wahlund, 1997) or κ-carrageenan and xanthan (Viebke & Williams,

2000). However, no articles are available yet that describe the Flow-FFF/MALS characterization of scleroglucan.

## 2. Experimental methods

### 2.1. Electron irradiation

Scleroglucan CS11 GR lot 20009584 and Scleroglucan CS6 lot 13299 from Degussa GmbH (Germany) were used where the first was mycelium purified by the producer. The weighed powder was filled in a 10-ml glass vial and covered air tight. The solid samples were electron irradiated with energy doses of 5, 11, 24, 51 and 102 kGy at room temperature with vessels containing air. The electron-beam irradiation was done at the 10 MeV linear accelerator Elektronika (Toriy, Russia) of the Leibniz-Institut für Ober-flächenmodifizierung, Leipzig. The accelerator operated at a 50 Hz repetition rate with 4 μs pulses, using a scanning horn (scanning width up to 40 cm, scanning frequency 1 Hz) and a movable table for irradiating samples. The dose was determined with a graphite calorimeter; the typical error in dose determination is ~5%.

The average dose rate, taking the whole irradiation time as a reference, was calculated to be ~0.1 kGy $s^{-1}$, which is 2 orders of magnitude higher compared with typical γ-irradiation (up to ~1 Gy $s^{-1}$). However, due to the special mode of pulsed operation, the dose rate during one single pulse is even higher, namely $5 \times 10^5$ kGy $s^{-1}$. This has to be taken into account especially in irradiated solutions, where fast bimolecular radical reactions (recombination) may occur; in solid, dry samples the probability of cross-linking may be slightly enhanced, which does not play a significant role in the present case.

### 2.2. ESR

The raw materials and 102 kGy irradiated powders were characterized by electron spin resonance (ESR) using a Miniscope MS 200-10 (Magnettech GmbH, Germany). The samples were measured with 5 mW microwave power using a field of 336 mT with a sweep of 10 mT and a modulation of 0.1 mT. When radical signals were not detectable anymore, further characterizations by NMR, Raman, viscosimetry or Flow-FFF were performed.

### 2.3. $^1H$ and $^{13}C$ NMR

DMSO-$d_6$ 99.8% (Chemotrade GmbH, Germany, PS 06185) was used as a solvent. Concentrations of 10 mg/ml were used for $^1H$ NMR and 30 mg/ml for $^{13}C$ NMR, respectively. The samples were heated to 80 °C to dissolve the powder and also during measurement to degrade triple-helical structures (Falch et al., 1999) and to receive sufficient NMR spectra (Nardin & Vincendon, 1989). The measurements were performed using a Varian Inova 500 (Varian Inc., USA) with 500 MHz for $^1H$ NMR and 125 MHz for $^{13}C$ NMR.

### 2.4. Raman spectroscopy

The raw materials and irradiated powders were characterized using a FT apparatus RFS 100/S (Bruker Optik GmbH, Germany). The equipment contained a Nd:YAG laser with a wave length of 1064 nm. The samples were measured between 10 and 3500 $cm^{-1}$ with a resolution of 4; 200 scans with a power of 340 mW were applied.

### 2.5. Production of water soluble scleroglucan

A clear solution was achieved within 60 min by solving scleroglucan in 0.4 M NaOH at room temperature under gentle stirring. This was followed by a neutralization step with HCl, where after dilution with water a 0.12 M NaCl solution was achieved. Prior to dilution, sodium azide was added to achieve a concentration of 0.2 g/l in the final solution to prevent bacterial growth. The final concentration of scleroglucan was 4 mg/ml in aqueous solution. If necessary, further dilutions were performed with the same solution medium to prevent a refractive index change of the solvent. The Scleroglucan CS6 samples were centrifuged for 10 min at 4000 U/min (Heraeus Laborfuge 300 with angle rotor, Germany) to remove unsolved mycelium components. The supernatant was used for further characterizations.

### 2.6. Viscosimetry

The dynamic viscosities of 1 mg/ml solutions were determined in 0.12 M NaCl containing 0.2 g/l sodium azide using an Ubbelohde viscosimeter (Schott type 0a, Germany) at a temperature of 25 °C. The outlet time results were adjusted via Hagenbach correction to consider the amount of potential energy that was needed to accelerate the liquid. The liquid densities were determined using a Mohr-Westphal balance (Johannes Hammer, Germany). Each of the measurements was performed at least three times.

### 2.7. GPC- and Flow-FFF/MALS/RI

The GPC- and aFlow-FFF/MALS/RI measurements were performed in 0.12 M NaCl containing 0.2 g/l sodium azide. The detector volume flow was constant at 1 ml/min for both methods. For GPC/MALS/RI, 100 μl of 0.4 mg/ml sample solution was injected with 0.2 ml/min. The equipment consisted of an Eclipse F and an 18 angle scattering detector Dawn EOS (Wyatt Technology Europe, Germany). An RI detector RI-101 (Shodex, Japan) was used. The GPC column was a HEMA 40 50 8.0 (MZ-Analysentechnik, Germany). For aFlow-FFF/MALS/RI, 100 μl of 1 mg/ml sample solution was injected into the channel with 0.2 ml/min. With exception of the column, the same equipment combination mentioned above was used. The system was tested with Dextran 65 kDa (Wyatt Technology Europe) to check separation and molar mass

determination capability. After several experiments to optimize separation, the following method was used. After injection, the sample was focused for 1 min with 2 ml/min focus flow. During elution, the cross flow decreased within 20 min from 2 ml/min linearly to 0 ml/min. A channel spacer of a height of 190 μm was used. The membrane consisted of polyether sulfone with a cut-off of $10^4$ g/mol (Nadir P010 F, Microdyn-Nadir GmbH, Germany). For GPC and aF-FFF, data were evaluated using the Astra 4.9 software (Wyatt Technology Europe) and Origin 6.1 (OriginLab Corporation, USA). Weight average molar masses were calculated using the Debye equation with a detector fit degree of 2. Given error bars correspond to the statistical uncertainties from at least three single measurements.

The refractive index increment of scleroglucan CS11 was determined from peak area by direct injection of the neutralized solution into the calibrated RI detector at 1 ml/min detector flow rate and 0.2 ml/min injection flow rate. Considering the water amount, a value of $0.146 \pm 0.001$ ml/g resulted for six single measurements. This was in good agreement with the literature value of 0.145 ml/g (Pretus et al., 1991) in 0.5 M sodium nitrate and 0.144 ml/g (Stokke et al., 1993) in 0.01 M NaOH. The water content was determined by thermogravimetry (Netzsch TG 209, Germany) where values of 10.3% resulted for scleroglucan CS11 and 9.6% for CS6. The undissolvable mycelium amount was determined from the relationship of total RI recovery of scleroglucan CS6 and CS11. The values were received by direct detector injection of scleroglucan CS11 and the supernatant of the neutralized centrifuged solution of scleroglucan CS6, as described above for CS11. The measurement was performed in triplicate.

## 3. Results and discussion

### 3.1. Production of water soluble scleroglucan

Despite the harsh thermal treatment of the neutral aqueous scleroglucan solutions by autoclaving at 121 °C for 15 min, no complete solubility was achieved. This can be explained by occurrence of rigid triple helices and the intermediate polysaccharide solubility class scleroglucan belongs to (Maache-Rezzoug, Rezzoug, & Allaf, 2001). By alkaline treatment it is possible to create soluble scleroglucans where the helix/single chain transition can be found between 0.2 and 0.3 M NaOH (Pretus et al., 1991). Other authors described a region between 0.01 M and 0.1 M NaOH (Bluhm, Deslandes, & Marchessault, 1982). Some authors also used 0.43 M NaOH or 0.4 M NaOH and 0.15 M NaCl (Gawronski et al., 1999) to avoid a helix occurrence; after this procedure a molar mass of 0.85 GDa was found. Scleroglucan was also made soluble by 3-h treatment in 10 g/l NaOH at 100 °C, followed by a detection of 1.56 GDa in neutral medium (Pretus et al., 1991). Thus, a rapid degradation due to high pH treatment was not indicated.

However, we tested various pH values and temperatures to find a possibly gentle method to receive non-degraded and soluble scleroglucan. According to the literature, a high pH value was necessary. Finally, we decided to use a treatment with 0.4 M NaOH for 1 h at room temperature. Lower pH values resulted in gels or solutions containing gel particles. Higher temperatures in combination with higher pH values caused a solution color change indicating degradation. Due to the additional mycelium amount, a part of the scleroglucan CS6 precipitated. Mycelium of fungi of the genus sclerotium mainly consists of soluble and insoluble carbohydrates, proteins, lipids or fatty acids, ergosterol, ribonucleic and desoxyribonucleic acid and oxalic acid (Gottlieb & van Etten, 1966; Punia, 1985). The solubilized CS6 amount was quantified as $83.9 \pm 0.8\%$. Thus, approximately 16% of CS6 consisted of undissolvable mycelium. This relatively small amount can be explained by precleaning procedures by the producer.

### 3.2. ESR

To detect irradiation induced radicals, ESR spectra of the powders were recorded the same day electron irradiation was performed and during storage time. Due to low signal intensities, the spectra in Fig. 2 are recorded using highest possible amplification. Broad signals of approximately 1.0–1.5 mT peak-peak-width were received. Scleroglucan CS6 exhibited a slightly higher intensity than CS11,

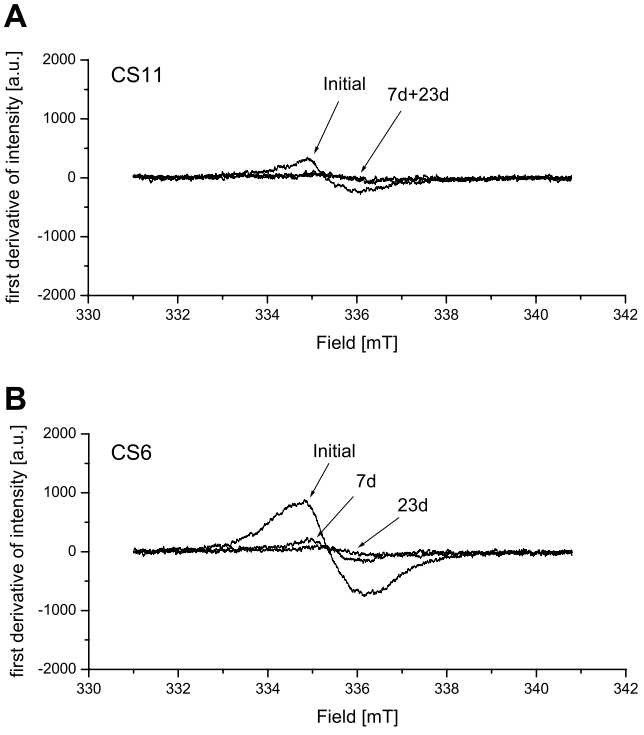


Fig. 2. ESR x-band spectra of both scleroglucans after 0, 7 and 23 day of post-electron irradiation storage, CS11 (A) and CS6 (B).

indicating a higher irradiation sensitivity or a higher radical stability due to its mycelium components. During storage the signal intensities rapidly decreased for both scleroglucans. Nearly no radicals were detectable after 7 day, after 23 day the signals completely disappeared. Reference spectra for electron irradiated scleroglucans are not available. Depending on the type, short-lived EPR signals were also received for several electron- and γ-irradiated carbohydrates (Dilli & Garnett, 1963; Esteves, Andrade, & Empis, 1999; Stachowicz et al., 1992). Further product characterizations were performed after 23 day of storage after disappearance of the EPR signals. For the raw materials no radical spectra were detectable.

### 3.3. $^{1}H$ NMR

The $^{1}$H NMR spectra in Fig. 3 are received before (A) and after (B) irradiation. Only a few reference spectra for raw material can be found in literature (Nardin & Vincendon, 1989; Vlachou, Politou, Dais, Mazeau, & Taravel, 2001). The reference spectra recorded at various temperatures and solvents differed from each other and the allocation of all peaks was not exactly the same. Thus, the given spectra were mainly described according to the authors who also used DMSO-$d_6$ at 80 °C (Nardin & Vincendon, 1989). The spectrum of irradiated scleroglucan CS11 conformed to this reference. H atoms 1A,B,C and D could be referred to several peaks at high chemical shifts. The peaks of the 12 OH groups above 4.1 ppm could only be found for the irradiated substance. For the raw material, a baseline curvature indicated broadened OH group peaks

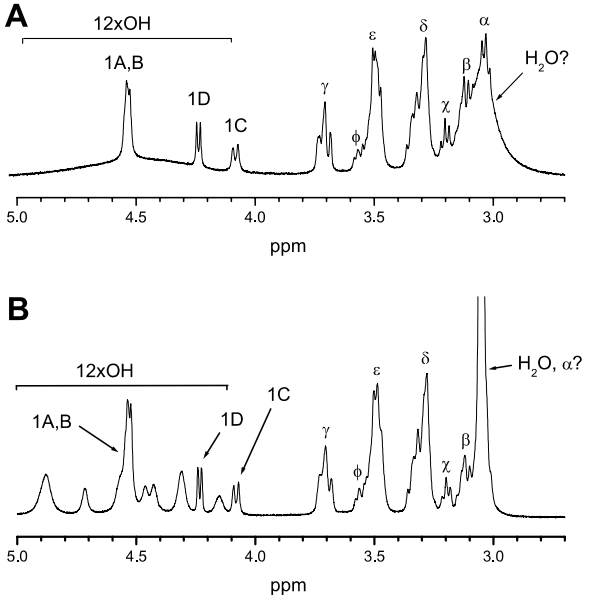


Fig. 3. $^{1}$H NMR spectra of scleroglucan CS11 raw material (A) and 102 kGy irradiated powder (B), peak allocations given within the text, Latin letters represent saccharide unit as shown in Fig. 1.

between 4 and 5 ppm. For the remaining $H_2O$ peak at approximately 3.05 ppm the same effect could be seen. A similar effect was observed for several carbohydrate solutions as for sucrose (Martin, Ablett, Darke, Sutton, & Sahagian, 1999). The authors found separate resonances for hydroxyl protons in slow exchange and only a single broad resonance for the hydroxyl protons in faster exchange with water molecules. Thus, the effect in Fig. 3 was related to a transition between fast and slow exchanges of hydroxyl protons with water molecules and could not be related to covalent bond changes of the chemical structure. Due to the overlapping OH group peaks and the baseline curvature, a correct quantification of the amount of $\beta(1 \rightarrow 6)$ connected glucopyranose units was impossible. However, the peaks 1C and 1D of raw and irradiated material represented each 0.5% of the whole spectrum intensity between 5 and 4 ppm including the OH and 1 A,B signals. Thus it was indicated that the irradiation had no detectable effect on the amount of $\beta(1 \rightarrow 6)$ covalent bonds. All peaks below 3.8 ppm are also given in reference spectra (Nardin & Vincendon, 1989). But they could not be allocated definitely due to overlapping peaks. Thus, the individual peaks were numbered with greek letters to allow a comparison of the spectra. All signals in that range could be found before and after irradiation, if not covered by the water signal.

Thus, electron irradiation led to a spectral change. This change was caused by a narrowing of the OH peaks and could not be related to differences in chemical backbone structure due to electron irradiation.

In contrast to scleroglucan CS11, the CS6 powder was not completely dissolvable in DMSO-$d_6$ due to its mycelium part. Thus, the $^{1}$H NMR spectra of CS6 did not have the same high resolution as given for CS11. But all previously detected peaks could also be found and no additional peaks could be detected indicating dissolved mycelium components.

### 3.4. $^{13}C$ NMR

As can be seen in Fig. 4, for the irradiated sample of CS11 (B) a spectrum of high quality is achieved. This was in contrast to the raw material (A). A similar effect was observed by other authors after the aggressive treatment of the scleroglucan backbone (Pretus et al., 1991). The received irradiated spectra matched the detailed spectrum found by other authors and all the peaks could be allocated by using this reference (Vlachou et al., 2001). As can be seen in Table 1, the main chain peaks (A,B,C) and also peaks of the $\beta(1 \rightarrow 6)$ residues (D) could be detected. For CS6, further peaks characterizing potential solved mycelium components were not detected, indicating that the mycelium components completely precipitated. However, for both substances due to the electron irradiation no additional unknown peaks were received. No changes in the chemical structure could be detected by $^{13}$C NMR.

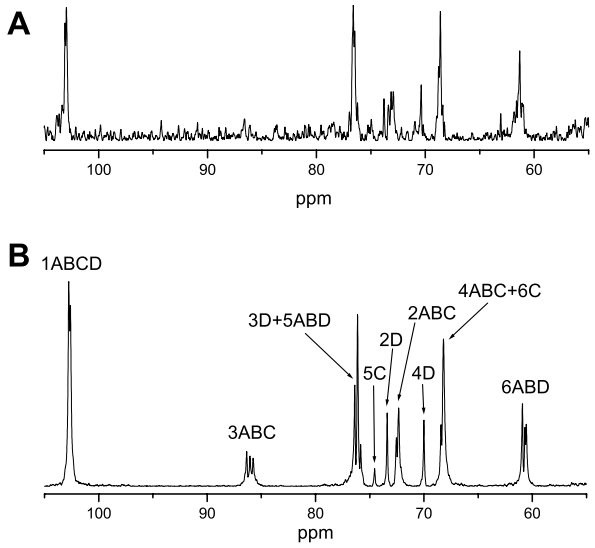


Fig. 4. $^{13}C$ NMR spectra of scleroglucan CS11 raw material (A) and 102 kGy irradiated powder (B), peak allocations for all C-atoms with Latin letters representing saccharide unit as shown in Fig. 1.

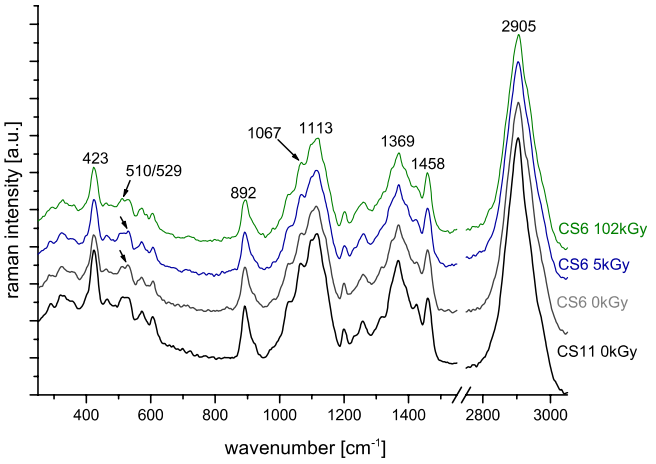


Fig. 5. Raman spectra of both scleroglucans including examples of irradiated samples.

### 3.5. Raman spectroscopy

In contrast to high resolution NMR, Raman spectroscopy advantageously allowed a fast characterization of the powders and did not require appropriate solutions. No articles are available yet describing the Raman spectra of scleroglucan. Even for other polysaccharides, only limited information is available, e.g., in collections from Vasko, Blackwell and Koenig (1971), Workman (2001b).

Comparing scleroglucan CS6 and CS11 from Fig. 5, no obviously differences could be detected. Thus, the following assignment can be used for both substances. The meanings of the different spectra ranges for saccharides are described, e.g., by Dauchez, Derreumaux, Lagant and Vergoten (1994), Sekkal and Legrand (1993). The range between 200 and 600 $cm^{-1}$ characterizes the individual backbone vibrations. For scleroglucan, here several bands could be found with the most characteristic at 423 $cm^{-1}$. The range between 2800 cm and 3050 $cm^{-1}$ with the global maximum at 2905 $cm^{-1}$ could be assigned to C–H-valence vibrations (Workman, 2001a). The middle range between 950 and 1500 $cm^{-1}$ as a part of the "fingerprint signals" showed a profile similar to other polysaccharides as dextrin or corn starch (Hendra & Agbenyega, 1993). According to Winter and Noll (1998), besides C–H-valence vibrations this range also includes signals caused by C–C stretching and OH group signals. The range between 800 and 950 $cm^{-1}$ is also called the "anomeric region" (Dauchez et al., 1994). The single peak at 892 $cm^{-1}$ was characteristic for scleroglucan. For other oligo- or polysaccharides a similar peak at 900 $cm^{-1}$ indicated a $\beta(1\rightarrow4)$ or at 940 $cm^{-1}$ an $\alpha(1\rightarrow4)$ linkage (Sekkal, Dincq, Legrand, & Huvenne, 1995). Due to the lack of literature, it is not safe but probable that the detected peak was caused mainly by the $\beta(1\rightarrow3)$ connections of scleroglucan. The peak tail to higher wave numbers could be explained by the additional $\beta(1\rightarrow6)$ linkages.

Example spectra of electron irradiated material are also given in Fig. 5. Obvious changes as disappearing or creation of new peaks were not visible. Only slight variations in the 200–600 $cm^{-1}$ region seemed to appear, but they were too small to be sufficiently detected. Besides these slight differences, the irradiation had no significant effect on Raman spectra and thus on herewith detectable molecule vibrations.

### 3.6. Viscosimetry

As can be seen in Table 2, even for the used low concentrated solutions the received dynamic viscosities of raw materials showed values of approximately the 1.9-2.6fold

Table 1
$^{13}C$ NMR peak allocations of both scleroglucans in comparison to the literature values[a] from (Pretus et al., 1991), all in DMSO-$d_6$ solution ([b]insufficient signal quality)

| C atom | Ref.[a] | CS 11 (0 kGy) | CS 11 (102 kGy) | CS 6 (0 kGy) | CS 6 (102 kGy) |
|---|---|---|---|---|---|
| 1ABCD | 103.0 | 102.6 | 102.8 | 102.7 | 102.8 |
| 2ABC | 72.5 | 72.3 | 72.3 | 72.4 | 72.3 |
| 3ABC | 86.3 | (86.2)[b] | 86.1 | (86.3)[b] | 86.3 |
| 4ABC,6C | 68.5 | 68.4 | 68.2 | 68.2 | 68.2 |
| 3D + 5ABD | 76.2 | 76.2 | 76.1 | 76.2 | 76.1 |
| 6ABD | 60.9 | 60.9 | 60.9 | 60.7 | 60.7 |

Table 2
Dynamic viscosities ($\eta$) of both scleroglucans depending on the electron irradiation energy dose ($D$)

| $D$ (kGy) | $\eta$ CS11 (cP) | $\eta$ CS6 (cP) |
|---|---|---|
| 0 | $1.705 \pm 0.005$ | $2.350 \pm 0.008$ |
| 5 | $1.375 \pm 0.004$ | $1.560 \pm 0.003$ |
| 11 | $1.359 \pm 0.006$ | $1.526 \pm 0.008$ |
| 24 | $1.245 \pm 0.002$ | $1.200 \pm 0.003$ |
| 51 | $1.113 \pm 0.003$ | $1.084 \pm 0.003$ |
| 102 | $0.988 \pm 0.006$ | $1.015 \pm 0.002$ |

of water. These relatively high viscosity values in aqueous solution could be explained by scleroglucans extended chain conformation what is known to be relatively independent of various solvent parameters (Sletmoen et al., 2006). Raw scleroglucan CS6 exhibited higher viscosities than CS11 what was also the case for all irradiated samples. With higher energy doses lower values were received. This was already shown for other polysaccharides where it was described that the main influence of ionizing irradiation was a decrease in viscosity (Balazs et al., 1959). Furthermore, for both scleroglucans a relatively large viscosity difference could be seen between raw and 5 kGy radiated samples. That implied a considerable effect of even small energy doses.

To check the effect of alkaline storage used to produce the solutions, unirradiated CS11 was stored for a longer time of 3 day in 0.4 M NaOH before neutralization. A dynamic viscosity of $1.676 \pm 0.002$ cP resulted what was only slightly different from the value for short time alkalized CS11 in Table 2. In addition this demonstrated the extraordinary stability of the scleroglucan molecule.

### 3.7. GPC- and aFlow-FFF/MALS/RI

Injecting unirradiated samples, a gelation inside the aFlow-FFF channel occurred during the focusing step. This was attributed to reaching the critical concentration needed for gelation (Sletmoen et al., 2006). Thus, to receive average molar mass values, all samples were characterized by GPC/MALS/RI. The irradiated samples were characterized by aFlow-FFF/MALS/RI to receive more detailed information about their molar mass distributions.

In Fig. 6, the weight average molar masses ($M_w$) from GPC/MALS/RI are given. Values of $4.8 \pm 0.4 \cdot 10^6$ Da were received for scleroglucan CS11 and $4.6 \pm 0.2 \cdot 10^6$ Da for CS6 raw material. They were within the range of known literature values between approximately $8.5 \cdot 10^5$ Da (Gawronski et al., 1999) and $5.7 \cdot 10^7$ Da (Yanaki et al., 1980) for scleroglucan native material. Lower $M_w$ values were received for both substances with increasing energy dose (D). This $M_w$ decrease was caused by polymer degradation what also explained the decrease in viscosity in Table 2, the better $^{13}C$ NMR spectra quality in Fig. 4 and the avoidance of gelation inside the aF-FFF channel due to irradiation.

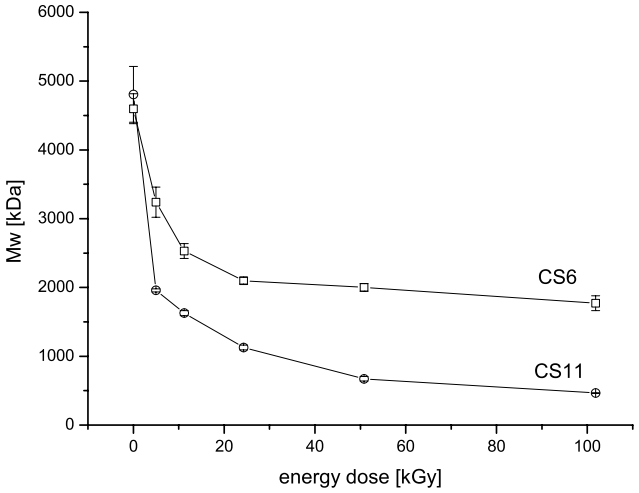


Fig. 6. Weight average molar masses ($M_w$) from GPC/MALS/RI of both scleroglucan raw materials and irradiated powders.

We used the following formula to describe the molar mass changes of both scleroglucans (Schnabel, 1978):

$$\frac{1}{M_w} - \frac{1}{M_{w_0}} = \frac{\left(\frac{G_{(S)}}{2} - 2G_{(X)}\right)D}{100N_A} \quad (1)$$

or for SI units

$$\frac{1}{M_w} - \frac{1}{M_{w_0}} = 5.18 \cdot 10^{-8}\left(G_{(S)} - 4G_{(X)}\right)D \quad (1a)$$

$M_{w0}$ and $M_w$ are the weight average molar mass before and after irradiation with respective energy dose D (kGy or eV/g for SI units) where $N_A$ is the Avogadro number, $G_{(S)}$ describes the number of main chain scissions and $G_{(X)}$ the number of intermolecular crosslinks produced per 100 eV ($=1.602 \cdot 10^{-17}$ J) absorbed by the irradiated polysaccharide. Both values can be used to characterize the relative importance of scission (S) or crosslinking (X) processes. The decrease of molar mass of both scleroglucans with increasing D already indicated mainly chain scission in a relationship of $G_{(S)} > 4G_{(X)}$. Furthermore, dry polysaccharides are known to undergo primarily chain scission due to the relatively weak glycoside linkages between the single units (Schnabel, 1978). Therefore, the amount of crosslinks occurring due to irradiation is negligible and the Eq. (1) or 1a are simplified to:

$$\frac{1}{M_w} - \frac{1}{M_{w_0}} = \frac{G_{(S)}D}{200N_A} \quad (2)$$

or for SI units

$$\frac{1}{M_w} - \frac{1}{M_{w_0}} = 5.18 \cdot 10^{-8}G_{(S)}D \quad (2a)$$

In Fig. 7a, the corresponding plot of CS11 is given. For a linear description the value of the unirradiated powder had to be excluded. This was done also for other polysaccharide irradiations, e.g., by Ulanski and Rosiak (1992). It is attributed to the fact that in the Charlesby–Pinner

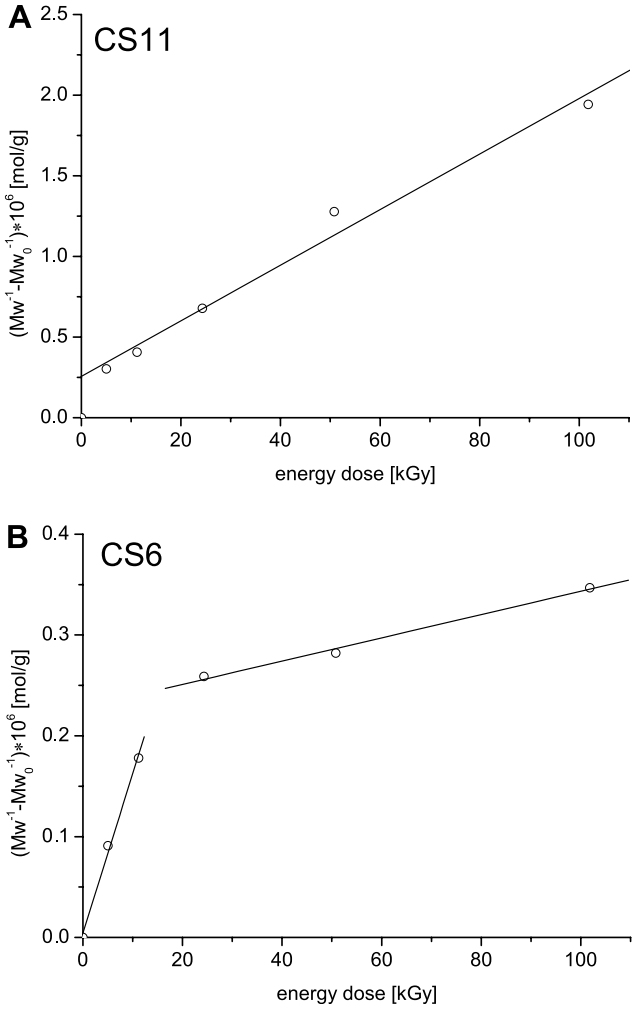


Fig. 7. The dependence of $1/(M_w - M_{w0})$ from energy dose for scleroglucan CS11 (A) and CS6 (B), received from GPC/MALS/RI, linearization: $R^2 = 0.992$ for CS11, 0.997 (0–11 kGy) or 0.996 (24–102 kGy) for CS6.

approach (Eq. (2)) the most probable molecular weight distribution ($M_w/M_n = 2$) of the initial sample is assumed, which is probably not exactly true in the present case. However, it was shown theoretically und experimentally that such a distribution is obtained in any case with increasing irradiation time (Schnabel, 1978), justifying the use of Eq. (2) for the linear part. Another explanation which has to be taken into account is the contribution of the oxygen dissolved in the polymer to the scission process. This influence on the irradiation of polymers is most significant at low energy doses (Wündrich, 1989), and limited at higher doses due to the restricted (slow) diffusion of oxygen into the polymer. This could also explain the similar effect observed for viscosity data between 0 and 5 kGy in Table 2.

As given in Table 3, for CS11 a $G_{(S)}$ of $0.34 \pm 0.03 \cdot 10^{-7}$ mol J$^{-1}$ resulted. This value was smaller compared to other literature values of polysaccharides as cellulose with $G_{(S)} = 3.4 - 7.0 \cdot 10 - 7$ mol J $- 1$ or amylose with $1.3–2.8 \cdot 10^{-7}$ mol J$^{-1}$ (Schnabel, 1978). This indicated a relatively high radiation resistance of the material. For

Table 3
Half values dose of $M_w$ ($HWD_{Mw}$) and the parameters $G_{(S)}$ and $G_{(X)}$ from the linear fits in Fig. 6 ([c]assumed values)

| Parameter | CS11 | CS6 |
|---|---|---|
| $HWD_{Mw}$ | 0–5 kGy | 11–24 kGy |
| $G_{(S),5–102\ kGy}$ | $0.34 \pm 0.03\ 10^{-7}$ mol J$^{-1}$ | – |
| $G_{(S),0–11\ kGy}$ | – | $0.32 \pm 0.02\ 10^{-7}$ mol J$^{-1}$ |
| $G_{(S),24–102\ kGy}$ | – | $0.023 \pm 0.002\ 10^{-7}$mol J$^{-1}$ |
| $G_{(X)}$ | $\sim 0^c$ | $\sim 0^c$ |

CS6, the plot in Fig. 7b could be divided in two parts of different slopes. From 0 to 11 kGy a $G_{(S)}$ of $0.32 \pm 0.02 \cdot 10^{-7}$ mol J$^{-1}$ resulted, a value similar to CS11. But at higher D values the $G_{(S)}$ decreased down to only $0.023 \pm 0.002 \cdot 10^{-7}$ mol J$^{-1}$. A decrease of the scission number with higher energy dose was already found for other polysaccharides as, e.g., cellulose (Sekurada, Okada, & Kaji, 1971). This effect was not completely clear. Probably some radiolysis products were formed that counteracted degradation. The main difference between CS11 and CS6 was their mycelium amount. Therefore, this small amount was probably the reason for the degradation protection of CS6. This protection effect can also be seen in the final $M_w$ values at 102 kGy. While the initial molar masses were similar, for CS11 resulted only $465 \pm 4$ kDa where in contrast for CS6 resulted $1771 \pm 107$ kDa.

One other simple parameter to characterize polymer irradiation resistance is the half values dose (Wündrich, 1989), here especially of $M_w$ ($HWD_{Mw}$). The $HWD_{Mw}$ was between 0 and 5 kGy for CS11 or 11 and 24 kGy for CS6, indicating a higher radiation resistance of CS6. The corresponding D values can be found at an ordinate value of $0.21 \cdot 10^{-6}$ mol/g for CS11 or $0.22 \cdot 10^{-6}$ mol/g for CS6. As it can be seen for both values in the plots in Fig. 6, the discrepancy from theoretical description in the respective regions permitted a calculation of the exact values from the fitted models. Furthermore, the comparison of HWD properly demands polymers of the same raw material molar mass. The corresponding values of both unirradiated scleroglucans were nearly the same ($4.8 \pm 0.4 \cdot 10^6$ Da for CS11 and $4.6 \pm 0.2 \cdot 10^6$ Da for CS6). So the $HWD_{Mw}$ values support the findings from $G_{(S)}$ values but should be estimated lower in their significance than $G_{(S)}$.

The first aFlow-FFF/MALS/RI measurements were performed using regenerated cellulose membranes that caused sample adsorption, proved by a negligible RI signal and obvious gelation on membrane surface. The difficulties were solved by the use of polyether sulfone membranes. RI recovery rates between 81% and 95% were achieved for CS11, suggesting a small amount of sample molecules below approximately $10^4$ Da that was able to cross the membrane pores. The recovery rates of CS6 between 67% and 74% were smaller with respect to the removed mycelium amount.

The fractograms of both scleroglucans are given in Fig. 8 with respect to the energy doses. For both substances resulted graphs covering the range of molar masses

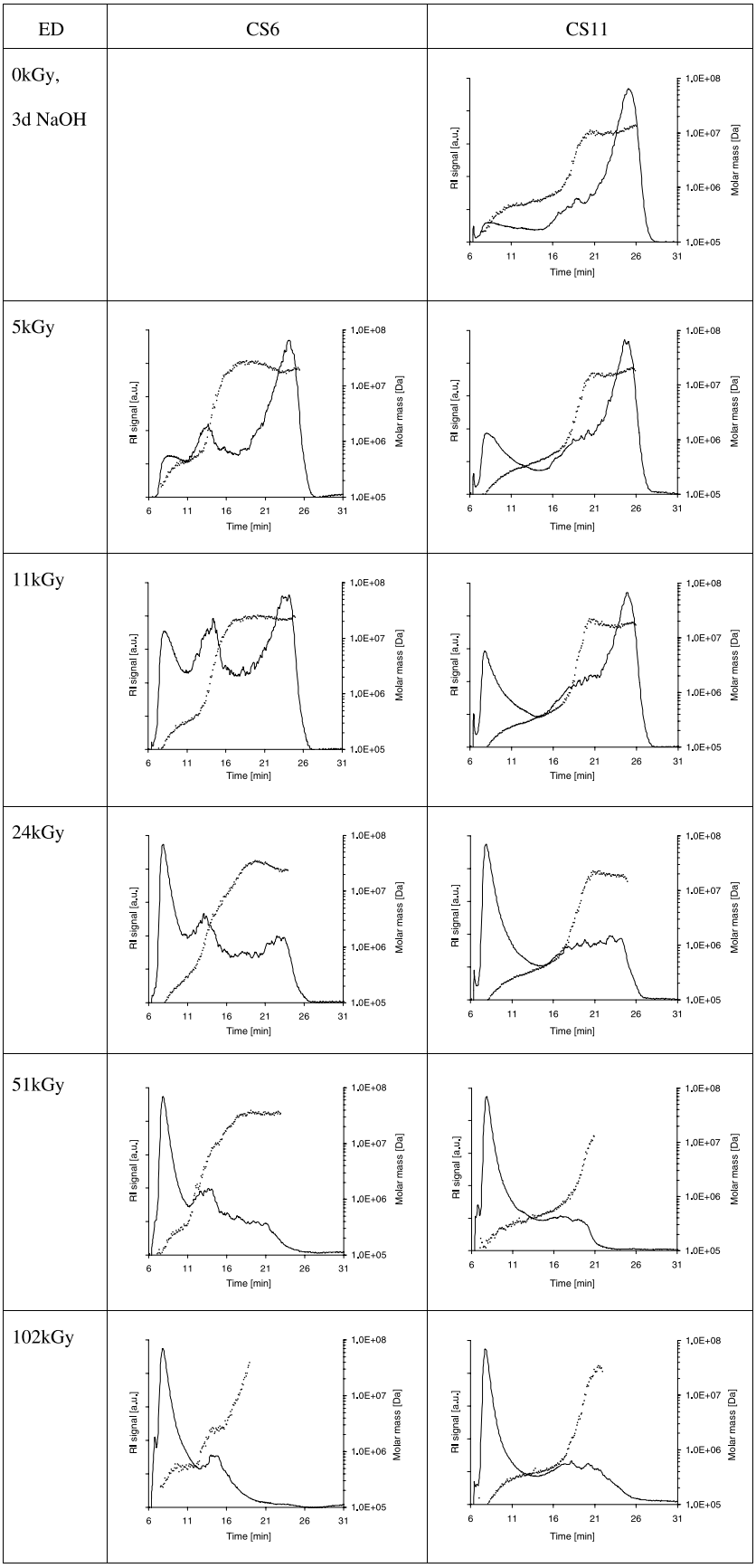


Fig. 8. aF-FFF Fractograms of both scleroglucans with respect to energy dose, peaks (full line) represent RI concentration signal, dotted line shows the corresponding molar masses from MALS/RI.

between approximately $10^5$ and above $10^7$ Da, indicating a broad distribution. In the case of CS11, two main fractions have been recognized. One eluted below 15 min shown by the peak with molar masses between approximately $10^5$ and $10^6$ Da. The second fraction eluted above 20 min with molar masses between approximately $10^7$ and $10^8$ Da. With higher energy doses the peak above 20 min was decreased and the fraction below $10^6$ Da increased. Thus, the electron irradiation caused a preferred scission of the high molar mass chains. Due to his effect, the broadness of the molar mass distribution decreased. Similar effects were also found for other polymer materials of high polydispersity. With increasing energy doses a PDI $= M_w/M_n$ value of approximately 2 was predicted, independently if the raw material had a broader or narrower molar mass distribution before (Schnabel, 1978). This was also found for polysaccharides, e.g., for chitosan, (Ulanski & Rosiak, 1992).

For CS6 an analogous effect was detected in Fig. 8. But an additional third peak around 14 min could be seen, covering a molar mass range between approximately $5 \cdot 10^5$ and $10^7$ Da. The amount of this additional fraction decreased due to the irradiation. In contrast, the low molar mass fraction eluting before 11 min increased. But this third peak could be recognized up to 102 kGy, indicating a higher resistance of these average molar mass chain lengths.

Additionally, the 3 day in 0.4 M NaOH stored scleroglucan was characterized by both methods. The $M_w$ of $2006 \pm 56$ kDa was lower than the value for the raw material but slightly above the one of 5 kGy. Comparing the aF-FFF fractograms, the high molar mass amount above approximately 20 min was more distinctive in comparison to 5 kGy results. That means the irradiation with 5 kGy had more influence on the material compared to long time alkaline storage.

Extrapolating all these findings, the raw scleroglucans mainly consisted of high molar mass material eluting from the channel within the peak in other fractograms above approximately 20 min. With higher energy doses of electron irradiation, for both scleroglucans mainly high molar mass chains were cleaved. This resulted in higher amounts of low molar mass material, eluting below approximately 15 min. As a consequence, average molar masses and the broadness of the molar mass distributions significantly decreased.

## 4. Conclusion

Due to their special molecular conformation $\beta(1 \rightarrow 3)$ glucans achieved increasing interest in the fields of immunostimulation or antimicrobial and antiviral activity. Furthermore, especially scleroglucan is an attractive stabilizing and bioadhesive polymer due to its high viscosity in aqueous solution and its resistance to hydrolysis, temperature and electrolyte changes. Besides the influence of the molecular weight on viscosity, molar mass dependent pharmacological effects have been described. Thus, the molar mass is especially critical in the field of pharmaceutics. Therefore, in addition to GPC- we applied aFlow-FFF/MALS/RI to characterize two scleroglucans with respect to their molar mass distributions. This combination is already known to be advantageously for other polysaccharides due to its broad separation scope.

Electron irradiation can be used to sterilize raw materials or to change the molecular weight. Only limited information and in the case of scleroglucan no literature can be found on the impact of electron irradiation on polysaccharides. Thus, scleroglucan powders were irradiated with different energy doses. ESR spectra revealed radical species of intermediate stability (several days). $^1$H NMR, $^{13}$C NMR and Raman spectra showed no relevant changes, indicating no detectable differences in chemical backbone structure due to electron irradiation. Chemical modifications, especially the formation of carbonyl and/or carboxyl functions are expected to occur to a certain extent, see, e.g., Lim, Khor and Koo (1998); but it can estimated that even at the highest dose of 100 kGy the number of all modified glucopyranose units will be in the range of 0.1% at maximum. Such a small amount of byproducts will not be detected by both methods and therefore we conclude that no major changes of the polymer backbone took place, but we do not exclude traces of byproducts. The spectra of CS6 did not contain further information concerning the additional mycelium amount.

In contrast, with higher energy doses a strong decrease of aqueous solution viscosity was observed for both substances. Furthermore, an averaged weight molar mass decrease was detected by GPC/MALS/RI. Thus, both parameters were changed due to irradiation induced polymer degradation. The influence on molar mass could be described mathematically as a chain scission effect. One $G_{(S)}$ value could be applied to the whole dose range of Scleroglucan CS11. In contrast, the $G_{(S)}$ value of low doses of irradiated CS6 was similar to CS11 but showed a decrease of the scission number at high doses. This irradiation protection effect was probably due to the mycelium amount in CS6. Also final $M_w$ values at 102 kGy as well as the half values doses of $M_w$ supported a higher radiation resistance of CS6.

In addition to GPC, for both substances aFlow-FFF/MALS/RI fractograms proved the presence of a lower molar mass fraction between approximately $10^5$ and $10^6$ Da and a second fraction between approximately $10^7$ and $10^8$ Da. For CS6 even a third fraction between $5 \cdot 10^5$ and $10^7$ Da was recognized. Thus, the samples were broadly distributed with respect to their molar mass. The electron irradiation mainly affected the high molar mass chains. They were cleaved and higher amounts of the low molar mass fraction were observed. Due to this effect, the broadness of the molar mass distribution decreased.

Thus, electron irradiation of scleroglucans did not change their chemical backbone structure, but can be used advantageously to decrease and to adjust the molar mass.

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