Microscopic Visualization of Nanostructures of Cellulose Derivatives

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Summary: Microscopy has proved to be a valuable tool for the investigation of supermolecular structures of cellulose derivatives. Thus, with AFM it was possible to show that statistically functionalized derivatives (degree of substitution below 3) form specific aggregates called fringed micelles. In contrast, derivatives with a non-statistic distribution exhibit a more network-like structure as known for galactomannans consisting of block-like structures. If samples are prepared by deposition of dilute solutions of polyelectrolytes on mica and drying at elevated temperature, the formation of nanorings is observed. In addition, AFM and REM were applied to investigate the morphology of membranes prepared of cellulose esters with unsaturated substituents. It was shown that cross-linking of the polymer chains in the membranes does not lead to a change in the nanostructure of the surface, i.g., the surface roughness and the pore sizes were not modified.

Keywords: atomic force microscopy (AFM); ester; ether; nanostructures; polysaccharide

Introduction

Native and artificial supermolecular structures of polysaccharides have a decisive influence on the overall properties of these biopolymers.[1,2] The significance of the accessibility factor in affecting their reactivity has been well recognized, on one hand.[3] On the other hand, supermolecular architectures of cellulose and its derivatives play a dominant role in polymer characteristics like rheology and should be crucial features in case of their biological interactions.[4,5] Especially the habitus of specifically functionalized cellulosics in solution and the resulting molecular order controls the chemical[6], physical[7] and biological behavior.[8] Moreover, structure formation on the nanometer scale is a rapidly growing field of research because it opens up new applications, e.g., nanopatterning for nanoreaction systems, divers immobilization methods or new separation techniques. Here the polymer may

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deal as construction material itself or as a template for the construction with inorganic compounds, e.g., nanoparticles.[9-11]

Up to now, the most detailed data concerning association and aggregation in solution or during the interaction of polysaccharide solutions with surfaces were acquired by means of light scattering, angular dependence of the scattered light, transmission electron microscopy, and birefringence measurements.[12,13] The surprising result is that cellulose derivatives with a total degree of substitution (DS) below three form various aggregates, so-called fringed micelles (Figure 1), caused by intermolecular and intramolecular hydrogen bonding of the remaining OH groups independent on the chemical nature of the functional groups introduced. A similar behavior was observed for polyelectrolytes as well as hydrophobically and hydrophilically modified polymer chains.

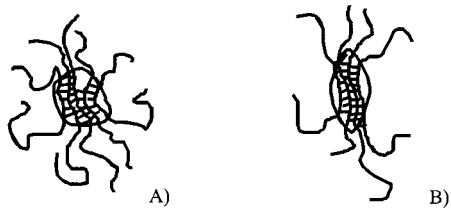

Figure 1. Two stages of fringed micelle (schematic representation). A) starbranched (high aggregation) B) worm-like chain (low aggregation).[12]

A powerful new tool for the investigation of the supermolecular architecture of biopolymers on the nanometer scale is the atomic force microscopy (AFM) in fluids.[14,15] Unmodified polysaccharides like scleroglucan[16], gellan[17], acetan, and xanthan[18,19] were studied using this technique. It was possible to resolve single molecules, network-formation processes, gelation, and even denaturation-renaturation cycles. Even single molecule force spectroscopy using AFM was applied to xanthan and carboxymethyl cellulose.[20] Here we describe the application of AFM and raster electron microscopy (REM) to elucidate superstructural features, e.g., aggregates in solution, nanorings or pore sizes in films of unconventional cellulose derivatives.

Experimental

Materials

Cellulose **1** (Avicel® PH 101, degree of polymerization, DP = 280) and **2** (spruce sulfite pulp, degree of polymerization = 680) was treated in vacuum at 105°C for 2 h prior to use. All other chemicals (Fluka) were used without further purification. The carboxylic acids, *N,N*-dimethylacetamide (DMAc), dimethyl sulfoxide (DMSO), *N,N'*-carbonyldiimidazole (CDI), and LiCl were obtained from Fluka and used without further treatment.

Dissolution of cellulose in DMAc/LiCl (3)

For a typical preparation, 1.0 g (6.2 mmol) of dried cellulose and 40 mL DMAc was kept at 130°C for 2 h under stirring. After the slurry has been allowed to cool down to 100°C, 3 g of anhydrous LiCl were added. By cooling down to room temperature under stirring, the cellulose dissolved completely.

Synthesis of conventional CMC

It was prepared by conversion of cellulose (**2**) with sodium monochloroacetate in aqueous NaOH/isopropanol according to reference.[21] The sample was free of low molecular weight impurities (NaCl, sodium glycolate).

IR (KBr): 1631 cm^{-1}, 1416 cm^{-1} (C=O, carboxylate group); HPLC-analysis according to[21] revealed the following mole fractions: glucose: 0.039; 2-; 3-; and 6-mono-*O*-carboxymethylglucose: 0.271; 2,3-; 2,6-; and 3,6-di-*O*-carboxymethylglucose: 0.453; 2,3,6-tri-*O*-carboxymethylglucose: 0.237 with a resulting DS = 1.89. Partial degrees of substitution determined by means of ^{1}H-NMR analysis[22] gave: O-2: 0.649; O-3: 0.278; O-6: 0.816 (DS = 1.75).

Carboxymethylation in reactive microstructure

A solution of 1 g cellulose (**2**) in DMAc/LiCl (**3**) was treated with a suspension of 2.47 g pulverized NaOH in 20 mL DMAc and a suspension of 3.59 g sodium monochloroacetate in 20 mL DMAc under vigorous stirring. The temperature was raised to 70°C. After 48 h the

mixture was cooled to room temperature and was precipitated into 300 mL methanol. The precipitates were filtered off, dissolved in 75 mL distilled water, neutralized with acetic acid and reprecipitated into 300 mL ethanol. After filtration, the products were washed with ethanol and dried in vacuum at 50°C. The sample was dialyzed against running water for 5 days.

IR (KBr): 1620 cm^{-1}, 1410 cm^{-1} (C=O, carboxylate group); HPLC-analysis according to[21] revealed the following mole fractions: glucose: 0.1135; 2-; 3-; and 6-mono-*O*-carboxymethylglucose: 0.3235; 2,3-; 2,6-; and 3,6-di-*O*-carboxymethylglucose: 0.2909; 2,3,6-tri-*O*-carboxymethylglucose: 0.2721 with a resulting DS = 1.72. Partial degrees of substitution determined by means of ^{1}H-NMR analysis[25] gave: O-2: 0.525; O-3: 0.394; O-6: 0.828 (DS = 1.75).

Preparation of cellulose furoate

A solution of 7.5 g (46.5 mmol) *N,N'*-carbonyldiimidazole was added to a solution of 5.3 g (46.5 mmol) furane-2-carboxylic acid in 20 mL DMAc. The clear solution was stirred 24 h at 40°C and was then added to the solution of 1.0 g (6.2 mmol) cellulose dissolved in DMAc/LiCl (**3**). The homogeneous reaction mixture was stirred for 24 h at 60°C. Product was isolated by precipitation into 300 mL ethanol and filtration. After washing three times with 100 mL ethanol, the polymer was dried at 45°C under vacuum.

DS (determined by ^{1}H-NMR after perpropionylation): 1.49; FTIR (KBr): 3493 ν (OH), 3142 (C-H furan), 2892 ν (C-H), 1233 ν (C-O-C_{Ester}), 1579 ν (furan ring), 1728 ν (CO_{Ester}) cm^{-1}; ^{13}C NMR (DMSO-d_6): δ (ppm) =157.3 (CO), 102.9 (C-1), 99.8 (C-1`), 72.3 (C-2), 73.9 (C-3), 80.2 (C-4), 76.3 (C-5), 63.1 (C-6), 143.4 (C-8), 118.8 (C-9), 112.1 (C-10), 147.6 (C-11); ^{1}H NMR (of the perpropionate dissolved in $CDCl_3$): δ (ppm) = 5.00 (H-3), 4.85 (H-2), 4.38 and 4.08 (H-6), 3.66, 3.63 (H-4, 5), 6.50 (H-10), 7.20 (H-9), 7.56 (H-11), 2.04 (CH_2-propionate), 0.77, 0.93 (CH_3-2, 3-propionate).

Membrane Preparation

A solution of 8.7 g cellulose furoate (DS = 1.97) in 50 mL DMSO (14.8%, w/w) was cast with a doctor on a glass plate with a velocity of 2 cm/s. A film of 120 μm thickness was

obtained. This film was immediately treated with ethanol for 2 min and stored in ethanol. The resulting membrane has a thickness of 50 μm.

AFM-studies of CMC

A stock solution of CMCs with a concentration of 1 mg/mL was diluted to various concentrations in the range 1-10 μg/mL. Drops (about 2 μl) of these solutions were deposited onto freshly cleaved mica and allowed to dry 15 min on air. The samples were then imaged in a liquid cell under butanol in both contact and tapping mode. The AFM measurements were carried out with a MultiMode SPM and Nanoscope IIIa control system (Digital Instruments, Santa Barbara, CA). The tip used was a NP-STT type (Nitride Probes Oriented Twin Tip) with a spring constant (nominal) of 0.1 N/m. The force during contact mode measurements was in the range of 0.5-1 nN and the amplitude in tapping mode experiments was about 4 nm (damping 1%). For the nanoring formation, the samples were dried at 70°C for 5 min and then measured under butanol under the same conditions.

AFM-studies of the membranes

The membranes were carefully transferred in the wet state onto the freshly cleaved mica surface and measured in the wet state. The AFM measurements were carried out with a DualScope C-21 (DME, Denmark). The tip used was a silicon nitride tip. The force constant was 60.0 N/m and the force 0.28 nN. The frequency applied was 254 kHz.

REM-investigation of cellulose furoate membranes

The wet membranes were critical point dried using a critical point dryer CPD030 (BAL-TEC, Balzers, Lichtenstein) and mounted on stubs for REM. Mounted samples were subsequently coated with gold using a sputter coating device BAL-TEC-SCD005. The measurements were carried out with LEO-1450 VP (LEO, Oberkochen, Germany) with a Cryo-transfer BAL-TEC-VCT 100. An EHT potential of 15.00 kV was applied. The micrographs were taken at a magnitude of 5.00k X and a working distance of 11 mm. For the cross-linking, the membrane was kept in ethanol and treated by UV irradiation for about 5 hours before critical point drying and coating with gold.

Results and Discussion

An important topic of our own work in the field of polysaccharide chemistry is focused on the determination of substitution patterns of cellulose derivatives on the level of the repeating unit as well as along the polymer chain. In combination with new synthesis pathways, attempts were made to establish structure-property relationships. A remarkable influence on the polymer behavior was observed by introducing concentration gradients of functionalization along the chain applying reactions in reactive microstructure. Thus, an important approach to new cellulosics was found to be the reaction via an induced phase separation with solid alkali hydroxide particles. Starting from a solution of cellulose in *N,N*-dimethylacetamide (DMAc)/LiCl or from organo-soluble cellulose derivatives dissolved in dimethyl sulfoxide (DMSO), subsequent etherification and esterification yield products of unconventional functionalization patterns and properties.[23-25]

To elucidate if AFM is usable for the investigation of superstructural features of such modified cellulosics, first carboxymethyl cellulose (CMC) was chosen. Because of its anionic nature sample preparation from aqueous stock solutions and AFM measurements under butanol are possible as successfully applied for gellan.[17]

Thus, conventionally prepared CMC (conversion of cellulose in isopropanol/aqueous NaOH) with a degree of substitution (DS) of 1.89 was selected, which shows a totally statistic distribution of substituents along the polymer chain.[21] Aqueous solutions of the CMC with a polymer content between 1 and 10 μg/mL were studied. The AFM-samples were prepared by deposition of droplets (about 2 μL) of CMC solutions on freshly cleaved mica. Well resolved images were obtained using concentrations below 2 μg/mL. If AFM-samples of these statistically functionalized CMC samples are prepared by drying on air at room temperature for 15 min and AFM images are acquired under butanol, the dominant superstructures observed are aggregated systems (Figure 2). Separate aggregates of a number of polymer chains are visible. In analogy to the model of fringed micelles, they consist of a hard core and flexible chains.[12,13] The size of the aggregates varies from 200 to 350 nm, the hard cores are in the range of 60 to 100 nm. Chains with up to 150 nm were determined. This would be in agreement with the estimated molecular dimensions. We are convinced that these aggregates are the predicted fringed micelles. Because of limited resolution, the lateral dimensions of the "individual dangling chains" characteristic for these fringed micelles are too large.

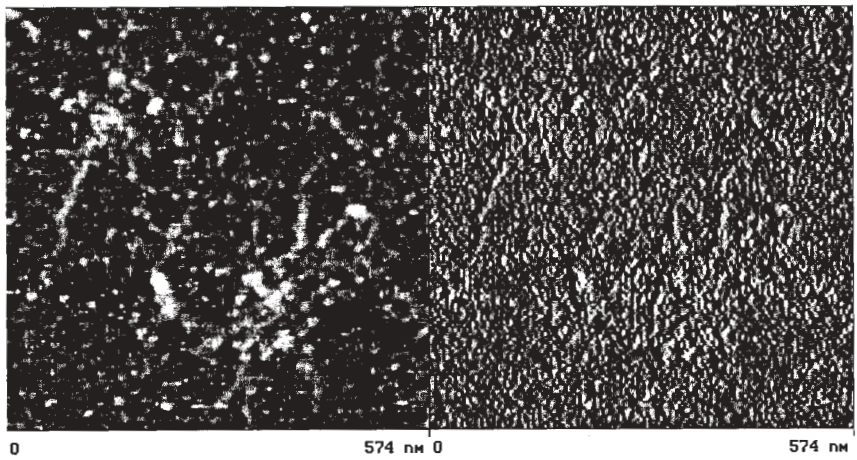

Figure 2. Atomic force microscopy image (contact mode) of carboxymethyl cellulose (degree of substitution = 1.89, statistic distribution of carboxymethyl groups) on mica under butanol, left: topographic image, right: deflection image. Sample dried at room temperature.

In comparison, naturally occurring polysaccharides are either dissolved as single molecules and helices or form patterns of perfectly aligned chains as well as perfect networks.[17-19] No evidence for the formation of micelles in these systems were published. The micelle-formation in case of polysaccharide derivatives is usually explained by strong hydrogen bonding of low or unmodified regions along the polymer backbone. However, it is questionable if this is really the major reason and universally true.

To elucidate this question, we studied CMC samples prepared in reactive microstructures, i.e., conversion of dissolved cellulose or cellulose derivatives via an induced phase separation with solid alkali hydroxide (see Experimental). These samples show a block-like distribution of substituents along the polymer chain as can be concluded from HPLC measurements of completely depolymerized samples and comparison with statistic calculations,[21] as well as from results of enzymatic degradation studies.[26] An AFM-image of such CMC polymers obtained from an aqueous solution containing 2 μg/mL sample and drying at room temperature for 15 min is shown in Figure 3.

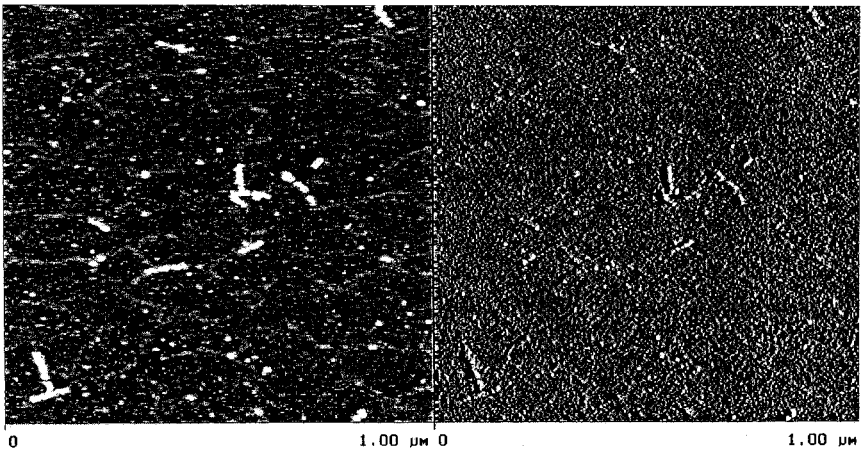

Figure 3. Atomic force microscopy image (tapping mode) of carboxymethyl cellulose (degree of substitution = 1.72, non-statistic block-like distribution of carboxymethyl groups) on mica under butanol, left: topographic image, right: amplitude image.

The amazing result was that no separate aggregates were detected. Instead, the polymers form an extended network with domain-like areas. The low concentration applied avoids the formation of network-like aggregation during AFM-sample preparation. As shown for the gelation of the polysaccharide gellan[17], it is inhibited by reducing the bulk polymer concentration to 2 µg/mL.

In comparison with naturally occurring polysaccharides like galactomannans consisting of block-like structures, which exhibit the same behavior (model of network formation, see Figure 4[27]) and concluding from the structural features of the CMC samples investigated, it seems obvious that this domain formation is due to the interaction of non- or low-carboxymethylated chain segments. Thus, further evidence was found for the block-like structure of the alternatively synthesized CMC samples.

In a separate series of experiments, we dried the samples at elevated temperatures. Thus, droplets (about 2 µL) of an aqueous solutions (2 µg/mL) of a conventionally prepared CMC (DS = 1.89) were dried at 70°C for 5 min on freshly cleaved mica.

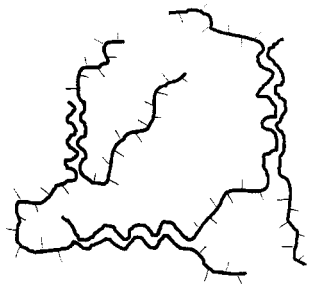

Figure 4. Schematic model of the network-formation of polysaccharides with a block-like structure.[27]

Tapping mode as well as contact mode measurements were carried out under butanol using a fluid cell. Surprisingly, in this case the formation of well defined rings was observed (Figure 5). These rings differ in size from 40 nm to about 120 nm. The chains that built up the rings are up to 17 nm thick.

In comparison to results obtained for scleroglucan[16], a polysaccharide of fungal origin, we first believed that this ring formation is due to the denaturation of the polysaccharide backbone. However, this seems unlikely since the functionalized cellulosics investigated (DS < 3, i.e., not all the accessible OH groups were functionalized) do not form highly ordered superstructures in solution like helices under ambient conditions. Vossmeyer et al.[28] discussed that the symmetries of superstructures are often determined by the shape of the subunits, on one hand. On the other hand, the authors have found and revealed by means of scanning electron microscopy that ring superstructures appear for different subunits such as spheres, disks, and rods. Thus, we are now convinced that the phenomenon we have observed is comparable to the structure forming processes of organically passivated metal nanocrystals and porphyrin molecules as described.[28] In addition, Nolte and co-workers[29] observed the assembly of porphyrin molecules into ring-shaped superstructures under comparable conditions, i.e., dilute solutions of the porphyrin in chloroform were allowed to evaporate, followed by draining the remaining solution. The ring formation was explained by nucleation in a liquid film. Nanoring structures on the basis of polyelectrolytes may be used as ligands for the immobilization of proteins, as calcium phosphate nucleation centers as recently proposed by Flores et al.[30] or may be exploited as templates for the formation of nanoreactor systems.[31]

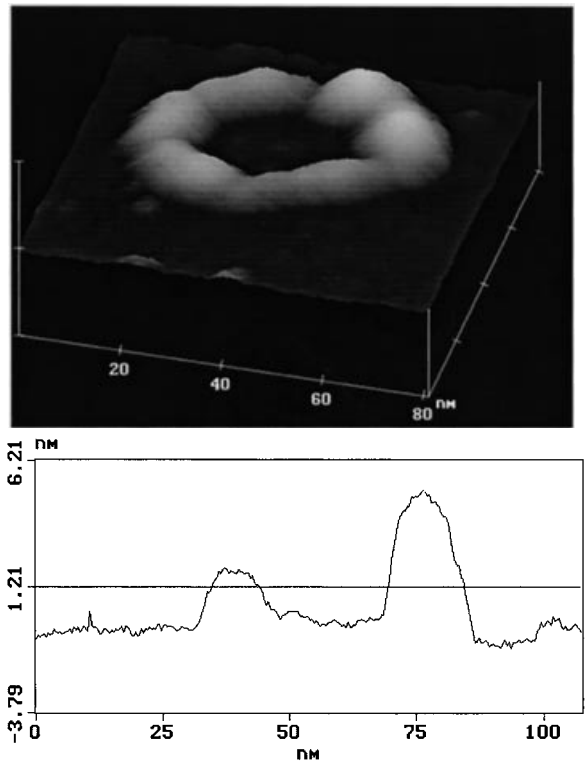

Figure 5. Atomic force microscopy image (tapping mode) of carboxymethyl cellulose (degree of substitution = 1.89, statistic distribution of carboxymethyl groups) on mica under butanol. Sample dried at 70°C. The formation of nanoring structures was observed.

Besides the observation of self-assembly processes and the nanostructure formation during deposition on surfaces, AFM was applied to investigate the structural features of membrane materials, which will be used for protein separation. Furan-2-carboxylic acid esters (furoate) of cellulose were prepared via *in situ* activation of the carboxylic acid with *N,N'*-carbonyldiimidazole (CDI). These esters were synthesized as membrane material because they are biocompatible and can be modified specifically by UV irradiation yielding solvent-resistant, stable films. Cellulose furoates with DS values up to 1.97 were accessible both by

homogeneous conversion in DMAc/LiCl[32] and via reaction in the new cellulose solvent DMSO/tetrabutylammonium fluoride (TBAF).[33]

Membranes were obtained from these samples using a doctor. A solution of cellulose furoate (DS = 1.97) in DMSO with an appropriate viscosity was prepared containing 14.8% (w/w) of the cellulose derivative. It was cast with a doctor on a glass plate with a constant velocity to give a homogeneous film of 120 μm thickness. This film was immediately treated with ethanol for 2 min and finally stored in ethanol.

For REM measurements, samples of the membrane were prepared by critical-point-drying and gold sputtering, which should preserve the pore structure. A rather high surface roughness was observed (Figure 6). The pore size determined by this method was smaller than 800 nm. In case of AFM studies, the membrane can be investigated without any pretreatment. It was simply transferred onto mica and measured in wet state. The surface roughness determined by AFM was in the range of 70 to 100 nm (see Figure 7), which is in agreement with results for membranes of cellulose acetate and cellulose acetate butyrate.[34] The smallest pores determined by this method were in the range of 40 nm (Figure 7).

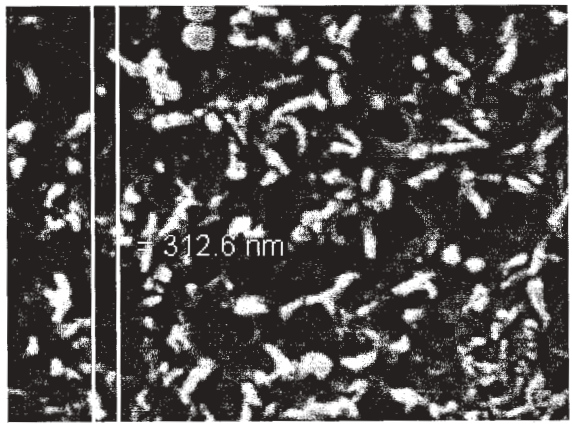

Figure 6. REM image of a membrane of furan 2-carboxylic acid esters (furoate) of cellulose (degree of substitution = 1.97) showing a high surface roughness and pore structures.

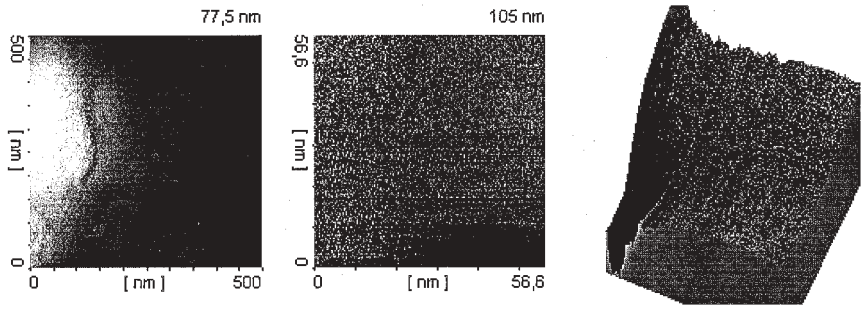

Figure 7. Atomic force microscopy image (tapping mode) of a membrane of furan 2-carboxylic acid esters (furoate) of cellulose (degree of substitution = 1.97) showing a surface roughness in the range of 70-100 nm and pore sizes as small as 40 nm.

For cellulose furoates, cross-linking by UV irradiation is possible, which can be exploited for subsequent modification of the solubility behavior of the derivative in membrane-shape.[32] If samples are treated with light having a wave length in the range of the absorption maximum of the furan moiety (290 nm), cross-linking succeeds presumably via a pericyclic reaction and yields insoluble polymers. By means of REM it was confirmed that the irradiation does not lead to a change in the surface roughness or the pore size (see Figure 8).

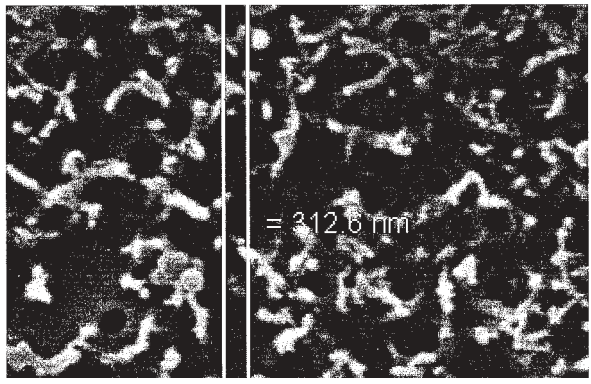

Figure 8. REM image of a membrane of furan 2-carboxylic acid esters (furoate) of cellulose (degree of substitution = 1.97) after irradiation with UV light showing no changes in pore structures and surface roughness (compare Figure 6).

The cross-linking succeeds without changes in the nanostructure and has no influence on the separation properties of the membrane as will be published elsewhere. Therefore, optimization of the membrane structure, e.g., by different casting, partial drying or solvent exchange can be carried out in the first stage and the modification of the solubility behavior of the membrane in a second step.

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