

Electrostatically driven modulation of the reinforcement of agarose hydrogels by cellulose nanowhiskers

Kevin J. Le Goff,¹ Cédric Gaillard,² Catherine Garnier,² Thierry Aubry¹

¹LIMATB—Equipe Rhéologie, Université De Bretagne Occidentale, 6 Avenue Victor Le Gorgeu, CS 93837, Brest Cedex 3, 29238, France

²INRA—UR 1268 Unité Biopolymères, Interactions, Assemblages, Rue De La Géraudière—BP 71627, Nantes Cedex 3, 44316, France

Correspondence to: T. Aubry (E-mail: thierry.aubry@univ-brest.fr)

ABSTRACT: The modulation of the reinforcement effect brought by the addition of tunicate cellulose nanowhiskers with different surface charge densities to an agarose hydrogel matrix has been studied using linear viscoelastic measurements. The cellulose nanowhiskers were characterized using transmission electron microscopy, and their surface charge was determined by a titration method. The results show that increasing the charge at the surface of nanowhiskers tends to enhance the reinforcement effect, because of a better dispersion of nanowhiskers. However, the results also suggest the existence of an optimum surface charge, corresponding to a maximum reinforcement effect at a given volume fraction. © 2015 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2016**, *133*, 43063.

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INTRODUCTION

Hydrogels are tridimensional, natural or synthetic, permanent or transient polymeric networks able to retain a very large amount of water. They have attracted much scientific attention in the last decades.^{1–4} Many applications of hydrogels require them to have good mechanical properties both at low and high deformations, and general principles have been recently proposed in order to design such high performance hydrogels.⁵ Over the past few years, one of the major strategies used to improve and modulate the mechanical properties of hydrogels is to add nanoparticles. Most nanoparticles used in the studies on nanocomposite hydrogels are anisometric nanoparticles, either nanotubes,⁶ or nanofibers,⁷ or nanoplatelets.⁸

Adding rod-like crystalline cellulose nanoparticles, so-called nanowhiskers, in order to reinforce polymer matrices has been thoroughly studied since the pioneering work of Favier *et al.*⁹ Since then, nanocomposites with many different polymer matrices and nanowhiskers from various natural sources were used and studied, as recently reviewed.¹⁰ The fact that cellulose nanowhiskers have a very high axial Young's modulus (~ 100 GPa), a high surface area (~ 100 m² g⁻¹), and a high aspect ratio (between ~ 10 and ~ 100 , depending on the cellulose source), explains that these nanoparticles have been considered as very good candidates to improve the mechanical properties of a host polymer. The study of nanocomposites composed of a hydrogel

matrix filled with cellulose nanowhiskers is much more recent.^{11–14}

In a very recent work, we have shown that adding a small amount of tunicate cellulose nanowhiskers to an agarose hydrogel could significantly reinforce the matrix.¹⁵ More precisely, the addition of 0.13 vol % nanowhiskers was shown to increase about 12 times the elastic modulus of a 0.2 wt % agarose hydrogel. The reinforcement effect was attributed to network topology and connectivity modifications of the agarose network in the presence of nanowhiskers, the stress being transferred mostly through nanowhiskers/matrix, and not through nanowhiskers/nanowhiskers interactions. In this previous work, the only way to modulate the reinforcement effect of the agarose hydrogel was to vary the nanowhiskers volume fraction: increasing nanowhiskers volume fraction increased the elastic modulus of the hydrogel nanocomposite.

Sulfuric and hydrochloric acids are classically used for the preparation of cellulose nanowhiskers.¹⁶ Indeed, an acid treatment hydrolyzes the cellulose, leading to a degradation of the amorphous regions of the cellulose microfibrils, resulting in the production of cellulose nanocrystals. In the present work, like in our previous study,¹⁵ sulfuric acid was used as a hydrolyzing agent, the reaction with the hydroxyl groups on the surface of cellulose nanowhiskers yielding negatively charged surface sulfate groups. The present study focuses on the modulation of the

reinforcement effect of a 0.2 wt % agarose hydrogel using cellulose nanowhiskers with different surface charge densities. More precisely, the aim is to study whether the increase of the electrostatic repulsions between cellulose nanoparticles with a higher surface charge could allow to reinforce agarose hydrogels at lower nanowhiskers contents. The results could contribute to a better, and eventually optimized, formulation of green hydrogel nanocomposites having the best mechanical properties at the lowest solid contents.

MATERIALS AND METHODS

Materials

Agarose. The agarose sample used in this work has been provided by EUROGENTEC (Belgium). It has an average molar mass, estimated from intrinsic viscosity measurements, of about 100 kDa and a sulfate content below 0.1%.

Preparation of Cellulose Nanowhiskers. The cellulose source used in this work is the tunic of marine animals (*Phallusia mammilata*), provided by the Station Biologique de Roscoff (France). The proteins were extracted from washed pieces of the tunics by three successive bleaching treatments, washing with 5% potassium hydroxide at ambient temperature during 3 h and washing with chlorite at 70°C during 4 h. The tunicate nanowhiskers were prepared by acid hydrolysis of the cellulosic residue dispersed in water at a concentration of about 10%, using 96 wt % sulfuric acid, following a two-step procedure: in a first step, sulfuric acid was added drop by drop under continuous vigorous stirring of the mixture, and the temperature of the mixture was maintained at 32°C. Then, in a second step, the reaction mixture was kept at 70°C during 45 min.

Preparation of Nanowhiskers Mother Suspensions. Three main nanowhiskers mother suspensions in water were prepared following the same protocol. After dispersion of the cellulose nanowhiskers in deionized water, the suspensions were dialyzed until the pH of the suspension reached 7, and then they were sonicated, using a Misonic sonicator, for 10 min in order to disperse the cellulose nanoparticles. The suspensions were then treated with a mixed-bed ion-exchange resin (mixed bed resin TDM-8 from Sigma-Aldrich), and 0.02 wt % sodium azide, which acts as a bacteriostatic agent, was added to the suspension. The three resulting mother suspensions had different volume fractions: the suspension, named S_1 , had a nanowhis ker volume fraction of 0.2%, another one named S_2 , a nanowhis ker volume fraction of 0.28%, and the third one named S_3 , a nanowhis ker volume fraction of 0.37%. Two additional mother suspensions were prepared following the same protocol: S_4 having a nanowhiskers volume fraction of 0.25%, and S_5 a nanowhiskers volume fraction of 0.33%.

Preparation of Agarose Gels Filled with Cellulose Nanowhiskers. Nanowhiskers suspensions were prepared by dilution from the three main mother suspensions, S_1 , S_2 , and S_3 , leading to final suspensions at various volume fractions, ranging from 0.013% to 0.28%. Two additional suspensions with a fixed 0.13% nanowhiskers volume fraction were prepared from S_4 and S_5 . All suspensions were first sonicated during 10 min in an ice bath, and then they were agitated under mechani-

cal stirring at about 800 rpm, and heated. When the temperature reached 90°C, the required amount of agarose powder was added in order to get a suspension containing 0.2 wt % agarose. Suspensions were then cooled at room temperature in Petri dishes, allowing the agarose macromolecules to change their conformations from coils to helices, leading to the formation of a gel.¹⁷

Methods

Transmission Electron Microscopy (TEM). Transmission electron microscopy was used in order to determine the length and width distribution of the cellulose nanowhiskers in the three main mother suspensions, S_1 , S_2 , and S_3 . The aqueous nanowhiskers suspensions were placed on a carbon coated TEM copper grid. Samples, negatively stained with uranyl acetate (1%), were let to air dry before observation, using a JEOL JEM-1230 microscope (Nikon, Tokyo, Japan), equipped with a LaB6 gun filament (lanthanum hexaboride), operating at a voltage acceleration of 80 kV. The images were analyzed using SigmaScan Pro 5.0.0 software.

Atomic Force Microscopy (AFM). AFM observation was performed in order to measure the height of cellulose nanowhiskers in the three main mother suspensions, S_1 , S_2 , and S_3 . The samples were deposited on a freshly cleaved mica plane, and then dried under an Argon flux. The images were acquired in the air using a microscope AutoProbe CP Park Scientific Instrument (USA). The tips were made of silicon doped with phosphorus (Veeco Probes, USA). The resulting images were processed with the WSxM 4.0 Software (Nanotec Electronica).

Titration. Conductometric titration is commonly used to determine the amount of protonated sulfate groups at the nanowhiskers surface, resulting from the sulfuric acid hydrolysis reaction.¹⁸ 10^{-4} mol L⁻¹ sodium hydroxide was added to S_1 , S_2 , S_3 , S_4 , and S_5 nanowhiskers aqueous suspensions in order to titrate the charged sulfate groups resulting from the reaction of the sulfuric acid with the hydroxyl groups of cellulose. The number of sulfate groups at the surface of nanowhiskers per glucose unit was inferred from the overall number of sulfate groups per glucose unit (derived from the titration measurements), divided by the ratio of surface chains to total chains in a nanowhis ker, which can be calculated from the average dimensions (length L , width l , and height h) of nanowhiskers and from the crystallographic characteristics of the cellulose crystal.¹⁹

Rheometry. All rheological measurements were carried out using a controlled stress rheometer Gemini (Bohlin Instruments). Oscillatory shear tests were performed at 20°C, in parallel plate geometry (diameter: 25 mm, gap: 1.5 mm), in order to characterize the linear viscoelastic properties of all hydrogel nanocomposite samples studied in this work. The samples were transferred from the Petri dishes to the plate geometry of the rheometer, previously covered with waterproof abrasive paper of roughness of about 80 μ m in order to prevent slippage. Low viscosity silicone oil was used to prevent water evaporation during rheometrical tests.

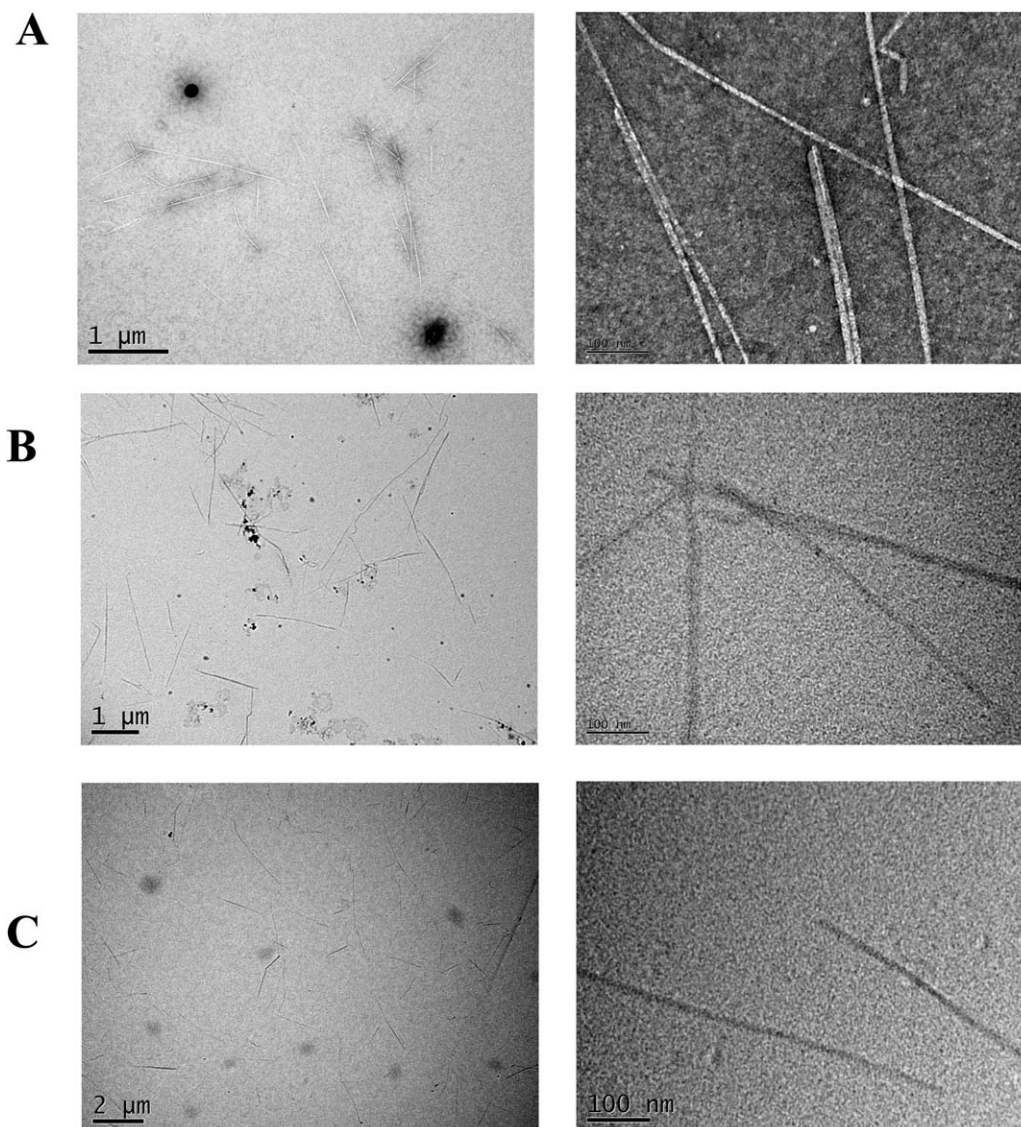


Figure 1. TEM images of nanowhiskers in S_1 (A), S_2 (B), and S_3 (C) suspensions.

RESULTS AND DISCUSSION

Geometrical Characterization of Nanowhiskers

Figure 1 shows typical TEM images of nanowhiskers suspensions S_1 , S_2 , and S_3 , which were diluted in order to have a more precise measurement of the cellulosic nanocrystal dimensions.

From such TEM pictures, the length L , the width l and the aspect ratio $p = L/l$ of about 500 nanowhiskers, in S_1 , S_2 , and S_3 suspensions, were measured. The values of the average length L , the average width l and the average aspect ratio of the nanowhiskers in the three main mother suspensions are reported in Table I. It should be noted that the width l could be identified as the diameter of nanowhiskers if they were modeled as cylinders.

Table I first shows that the three nanowhiskers samples used in the present work exhibit a rather large polydispersity. However, the results also show that the differences in average length and diameter of nanowhiskers in S_1 , S_2 , and S_3 suspensions are

small, so that the geometrical characteristics of nanowhiskers in S_1 , S_2 , and S_3 suspensions will be considered as identical in the present article. It should be noted that the high aspect ratio of tunicate nanowhiskers, as compared to that of nanowhiskers from other cellulose sources,¹⁶ makes them particularly interesting as reinforcing agents. Besides, we would like to stress that the average length of the tunicate nanowhiskers is two times higher than the average mesh size of a 0.2 wt % agarose

Table I. Length L , width l , and Average Aspect Ratio $p = L/l$ of Nanowhiskers in S_1 , S_2 , and S_3 Suspensions

| | S_1 | S_2 | S_3 |
|-----------|---------------|---------------|---------------|
| L (nm) | 830 ± 600 | 940 ± 650 | 900 ± 650 |
| l (nm) | 9 ± 3 | 11 ± 3 | 9 ± 2 |
| $p = L/d$ | 90 | 85 | 100 |

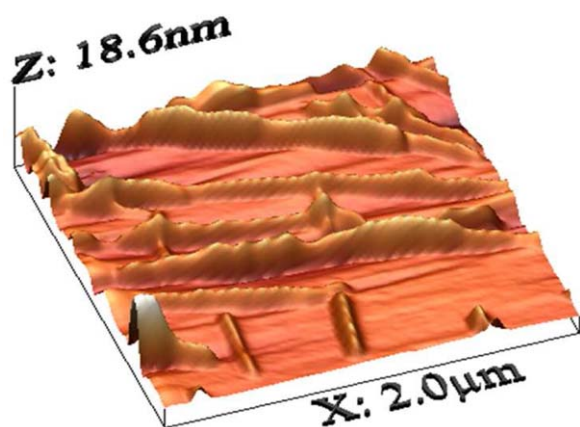


Figure 2. AFM micrograph of cellulose nanowhiskers in S_2 suspension. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrogel,²⁰ which is the matrix of the hydrogel nanocomposites studied in the present work.

AFM clichés, like that plotted in Figure 2, allowed us to determine the average height h of nanowhiskers, which was shown to be about 5 nm for nanowhiskers in S_1 , S_2 , and S_3 suspensions.

The geometrical characteristics of nanowhiskers from S_4 and S_5 suspensions have not been determined experimentally; however, they are expected to be identical to those of nanowhiskers in S_1 , S_2 , and S_3 suspensions.

Nanowhiskers Surface Charge

The surface charge of nanowhiskers in S_1 , S_2 , S_3 , S_4 , and S_5 suspensions was experimentally determined using a conductometric titration method. The number of sulfate groups per glucose unit of nanowhiskers in S_1 , S_2 , S_3 , S_4 , and S_5 suspensions was found to be 0.087, 0.028, 0.0017, 0.041, and 0.0096, respectively. The extent of the acid hydrolysis reaction being low, it was assumed that substitution occurred essentially at the surface of the nanowhiskers. The number of charged sulfate groups per glucose unit at the surface can be deduced from the ratio of surface chains to total chains in a nanowhis-ker.¹⁹

The ratio of surface chains to total chains is nearly the same, that is 0.3 for nanowhiskers in S_1 , S_2 , and S_3 suspensions, since their geometrical dimensions are about the same. Therefore, the number of charged sulfate groups per glucose unit at the surface is 0.3 for nanowhiskers in S_1 , 0.1 in S_2 , and 0.006 in S_3 suspensions. For nanowhiskers in S_4 and S_5 suspensions, assuming that they have the same geometrical characteristics, the number of charged sulfate groups per glucose unit at the surface is 0.13 and 0.032, respectively.

These results show that the surface charge, ρ_s , of nanowhiskers in S_1 , S_2 , S_3 , S_4 , and S_5 suspensions can be put in descending order, $\rho_s(S_1) > \rho_s(S_4) > \rho_s(S_2) > \rho_s(S_5) > \rho_s(S_3)$, reflecting the ascending order of the initial nanowhiskers volume fraction, Φ , in the five mother suspensions, $\Phi(S_1) < \Phi(S_4) < \Phi(S_2) < \Phi(S_5) < \Phi(S_3)$. This strongly suggests that the difference in surface charge of these five nanowhiskers batches, which have been prepared following exactly the same protocol under the same acidic con-

ditions, can be attributed to differences in initial fillers concentration in the five mother suspensions. Indeed, using the same acidic conditions to treat a higher initial mass of nanowhiskers is expected to lead to a lower nanoparticle surface charge.

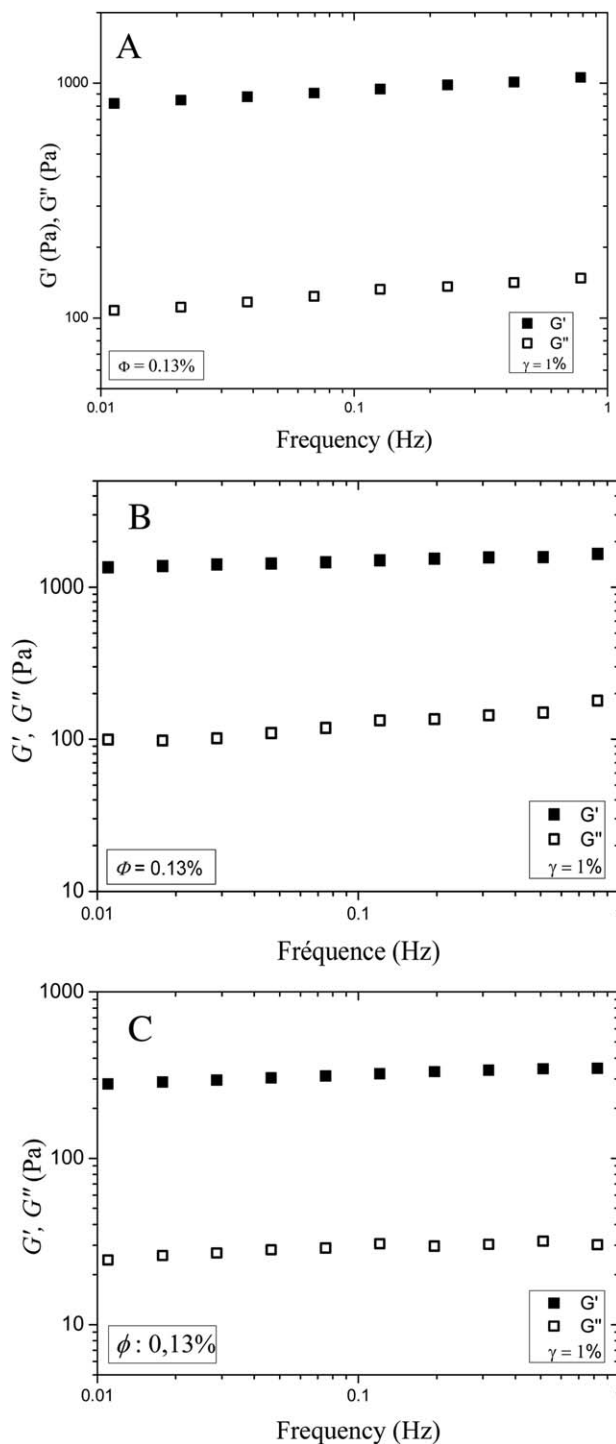


Figure 3. Storage modulus, G' , loss modulus, G'' , as a function of frequency for a 0.2 wt % agarose hydrogel filled with 0.13 vol % nanowhiskers from S_1 (A), S_2 (B), and S_3 (C) suspensions.

Table II. Critical Strain of 0.2 wt % Agarose Hydrogels, Filled with Nanowhiskers from S_1 , S_2 , and S_3 Suspensions

| | Nanowhiskers volume fraction Φ (%) | | | | | | | |
|----------------------|---|-------|-------|-------|------|------|------|------|
| | 0 | 0.013 | 0.032 | 0.065 | 0.13 | 0.2 | 0.23 | 0.28 |
| γ_c (%) S_1 | 4 | 2 | 2 | 1.5 | 1 | 1 | - | - |
| γ_c (%) S_2 | 4 | 2.5 | 2 | 1.5 | 1.5 | 1 | 1 | 1 |
| γ_c (%) S_3 | 4 | 3.5 | 3 | 2.5 | 2 | 1.75 | - | - |

Rheology

The linear viscoelastic properties of all hydrogel nanocomposite samples were studied under small amplitude oscillatory simple shear.

Strain Sweep Oscillatory Shear

Strain sweep tests were used to determine the critical strain, γ_c (%), characterizing the extent of the linear viscoelastic response regime. The critical strain was determined from two different classical representations, which gave the same results: the first one from the storage modulus G' versus imposed strain amplitude curves, and the second one from the stress-strain curve. The values of the critical strain of 0.2 wt % agarose hydrogels, filled with nanowhiskers from S_1 , S_2 , and S_3 suspensions, at volume fractions ranging from 0.013 to 0.28%, are reported in Table II.

Table II shows that the critical strain of all hydrogel nanocomposites, prepared from S_1 , S_2 , and S_3 suspensions, are slightly below that of the pure agarose hydrogel and are nearly independent of nanowhiskers volume fraction. This result suggests that the viscoelastic properties of the hydrogel nanocomposites are governed by those of the agarose matrix, as previously observed.¹⁵ Thus, the contribution of nanowhiskers to the appearance of non-linearities in the viscoelastic behavior is weak.

However the critical strain of the agarose hydrogels filled with nanowhiskers from S_3 suspension have a slightly higher value than that of agarose hydrogels filled with nanowhiskers from S_1

or S_2 suspensions. This slight difference could be due the fact that nanowhiskers from S_3 suspension, which have a much lower surface charge, tend to aggregate via hydrogen bonding, leading to higher γ_c values than those obtained for hydrogel nanocomposites prepared from S_1 and S_2 suspensions, for which nanowhiskers are better dispersed due to electrostatic repulsions, and therefore have a stronger contribution to the appearance of nonlinearities in the viscoelastic behavior.

Frequency Sweep Oscillatory Shear

A spectromechanical characterization of all hydrogel nanocomposites was performed over a frequency range from 0.01 to 1 Hz. The storage modulus G' and the loss modulus G'' are plotted as a function of frequency, at a strain amplitude of 1%, for a 0.2 wt % agarose hydrogel filled with 0.13 vol % nanowhiskers from S_1 , S_2 , and S_3 suspensions, in Figure 3. It should be noted that the linear viscoelastic behavior of all hydrogel nanocomposite samples exhibits the same features, whatever the nanowhiskers volume fraction. The viscoelastic moduli of all samples are weakly dependent on frequency and the storage modulus is much higher than the loss modulus, highlighting the solid-like viscoelastic behavior of all hydrogel nanocomposites studied in this work.

The frequency dependence of viscoelastic moduli being weak, values of G' and G'' , and of the loss angle, $\tan \delta = G''/G'$, have been reported at an arbitrarily chosen frequency of 0.1 Hz, for all 0.2 wt % agarose hydrogels filled with nanowhiskers from S_1 , S_2 , and S_3 suspensions, in Table III.

Table III. Storage Modulus, G' , Loss Modulus, G'' , and Loss Angle, $\tan \delta$, at 0.1 Hz, of 0.2 wt % Agarose Hydrogels Filled with Nanowhiskers from S_1 , S_2 , and S_3 Suspensions

| | Nanowhiskers Volume Fraction Φ (%) | | | | | | | | |
|-------|---|-------|-------|-------|------|------|------|------|------|
| | 0 | 0.013 | 0.032 | 0.065 | 0.13 | 0.2 | 0.23 | 0.28 | |
| S_1 | G' (Pa) | 80 | 200 | 320 | 590 | 940 | 830 | - | - |
| | G'' (Pa) | 7 | 15 | 40 | 60 | 125 | 150 | - | - |
| | $\tan \delta$ | 0.09 | 0.07 | 0.12 | 0.1 | 0.13 | 0.18 | - | - |
| S_2 | G' (Pa) | 80 | 190 | 325 | 830 | 1500 | 2800 | 2300 | 2270 |
| | G'' (Pa) | 7 | 15 | 30 | 85 | 135 | 260 | 250 | 245 |
| | $\tan \delta$ | 0.09 | 0.08 | 0.09 | 0.1 | 0.09 | 0.09 | 0.11 | 0.11 |
| S_3 | G' (Pa) | 95 | 160 | 220 | 260 | 400 | 490 | - | - |
| | G'' (Pa) | 7 | 14 | 21 | 24 | 35 | 44 | - | - |
| | $\tan \delta$ | 0.1 | 0.09 | 0.09 | 0.09 | 0.09 | 0.09 | - | - |

To focus on the contribution of nanowhiskers to the reinforcement effect, the reduced storage modulus $(G' - G'_{\text{agarose}})/G'_{\text{agarose}}$ as a function of nanowhiskers volume fraction was plotted in Figure 4.

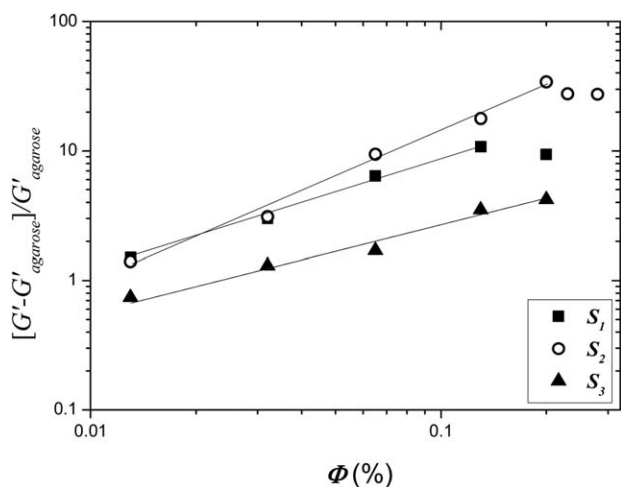


Figure 4. Reduced storage modulus $(G' - G'_{\text{agarose}}) / G'_{\text{agarose}}$ as a function of nanowhiskers volume fraction for samples prepared from S_1 , S_2 , and S_3 mother suspensions.

First of all, Figure 4 shows an increase of the storage modulus of the hydrogels with increasing volume fraction of nanowhiskers from S_1 , S_2 , or S_3 suspensions, at least for volume fractions up to 0.13% for nanowhiskers from S_1 suspension and 0.2% for nanowhiskers from S_2 suspension. The increase of elasticity provided by the cellulose nanowhiskers has been observed and discussed in our previous work.¹⁵ The reinforcement effect was attributed to changes in the agarose network topology and connectivity induced by the presence of nanowhiskers, the stress being transferred through nanowhiskers/agarose interactions.

However, Figure 4 shows that, at a given nanowhiskers volume fraction, the reinforcement effect brought by nanowhiskers from suspension S_3 is much less marked than that brought by nanowhiskers from S_1 and S_2 suspensions. This result strongly suggests that the reinforcement effect is much less efficient when nanowhiskers surface is weakly charged. This electrostatically driven effect could be attributed to the fact that nanowhiskers from S_3 suspension, which have a much lower surface charge, tend to aggregate via hydrogen bonding, leading to a more heterogeneous structure of hydrogel nanocomposites. Indeed, the reinforcement effect is all the more marked as the nanowhiskers dispersion state is better, allowing a better stress transfer.

Besides, Figure 4 also shows some significant differences between the reinforcement brought by nanowhiskers from suspension S_1 and that from suspension S_2 , which both have a relatively high surface charge. The differences appear at volume fractions higher than 0.03%. First, the reinforcement effect is more marked for nanowhiskers from suspension S_2 , even though their surface charge is lower, which seems to contradict our previous interpretation. Moreover, the maximum reinforcement effect appears at a higher volume fraction (0.2%) for nanowhiskers from suspension S_2 than that (0.13%) for nanowhiskers from suspension S_1 . These results show that increasing the nanowhiskers surface charge does not result systematically in an enhancement of the reinforcing effect, suggesting the existence of a maximum surface charge for reinforcement.

To investigate the possible existence of an optimum surface charge at a given nanowhiskers volume fraction, the storage modulus G' , at 0.1 Hz, of 0.13 vol % nanowhiskers suspensions, prepared from the additional S_4 and S_5 mother suspensions, were measured: $G' = 1570$ Pa for nanowhiskers suspension prepared from S_4 , and $G' = 1000$ Pa for nanowhiskers suspension prepared from S_5 . The storage modulus G' , at 0.1 Hz, of all 0.13 vol % nanowhiskers suspensions, prepared from S_1 , S_2 , S_3 , S_4 , and S_5 suspensions, was then plotted as a function of the number of sulfate groups per glucose unit at the surface of nanowhiskers, in Figure 5. This figure highlights the electrostatically driven modulation of the reinforcement effect, and supports the existence of an optimum value of the nanowhiskers charge, corresponding to a maximum reinforcement effect at a given volume fraction. Data in Figure 5 suggest that the optimum number of sulfate groups per glucose unit at the surface of nanowhiskers is close to 0.15, for 0.13 vol % nanowhiskers suspensions.

To explain the existence of an optimum surface charge, we propose the following interpretation: when nanowhiskers surface charge is too high (nanowhiskers from S_1), hydrogen bonds between nanowhiskers and agarose molecules are less numerous, leading to a less effective nanowhiskers/matrix stress transfer, and therefore to a less pronounced reinforcement effect. Moreover, when nanowhiskers surface charge is too high, the topological constraints exerted by nanowhiskers on the agarose network are more marked because nanowhiskers electrostatically repel each other strongly. This phenomenon could explain that, when nanowhiskers surface charge is too high, a lower nanowhiskers volume fraction is needed for the maximum reinforcement effect to appear: 0.13% for nanowhiskers from S_1 compared to 0.2% for nanowhiskers from S_2 . The decrease of the reinforcing effect of nanowhiskers above this volume fraction could be due to the densification of the junction zones of agarose, resulting in a decrease of the agarose network connectivity, and therefore in a decrease of elastic modulus.

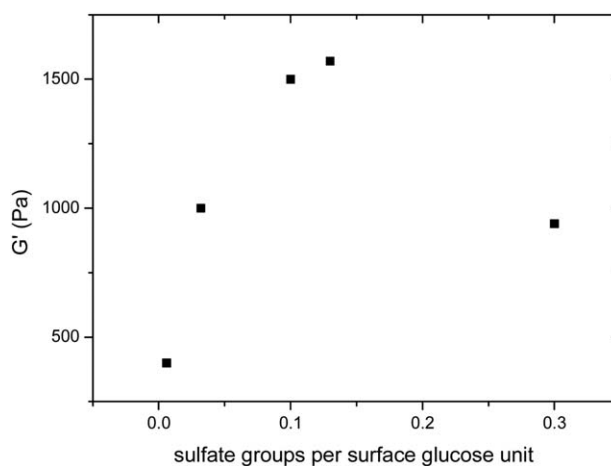


Figure 5. Storage modulus G' , at 0.1 Hz, of 0.13 vol % nanowhiskers suspensions, prepared from S_1 , S_2 , S_3 , S_4 , and S_5 suspensions, as a function of the number of sulfate groups per glucose unit at the surface of nanowhiskers.

CONCLUSION

The linear viscoelastic properties of 0.2 wt % agarose hydrogels filled with tunicate cellulose nanowhiskers having different surface charge densities were studied at various nanoparticle volume fractions, ranging from 0.013% to 0.28%. The results show that the reinforcement effect due to the addition of nanowhiskers can be modulated, not only by varying the nanoparticle volume fraction, but also by varying the nanowhiskers surface charge.

The study shows that increasing the surface charge of nanowhiskers increases the reinforcement effect because of greater electrostatic repulsions between nanowhiskers, which improves the nanoparticle dispersion state and the nanowhiskers/matrix stress transfer. However, the results also strongly suggest the existence of an optimum surface charge, corresponding to a maximum electrostatically driven reinforcement effect at a given volume fraction.

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