


Retention of lysozyme activity by physical immobilization in nanocellulose aerogels and antibacterial effects

Khan M. A. Uddin · Hannes Orelma · Pezhman Mohammadi · Maryam Borghei ·
Janne Laine · Markus Linder · Orlando J. Rojas 

Received: 4 February 2017 / Accepted: 24 April 2017 / Published online: 5 May 2017
© Springer Science+Business Media Dordrecht 2017

Abstract Aerogels prepared from aqueous dispersions of anionic and cationic cellulose nanofibrils (CNFs) were investigated as solid supports for enzymes and silver nanoparticles and to elicit a sustained antibacterial effect. The imparted stabilization in dry conditions was studied with aerogels that were cast after mixing the enzymes with CNFs followed by dehydration (freeze-drying). The activity of lysozyme immobilized in the given CNF system was analyzed upon storage in liquid and air media. In contrast with aqueous solutions of free, unbound enzyme, which lost activity after the first day, the enzyme immobilized physically in unmodified and cationic CNF presented better stability (activity for a longer time). However, the enzyme activity was

reduced in the case of anionic CNF, which was prepared by TEMPO-mediated oxidation (TO-CNF). Both humidity and temperature reduced the stability of the enzyme immobilized in the respective CNF aerogel. The antibacterial activity of CNF aerogels carrying lysozyme was also tested against gram-negative and gram-positive bacteria. The results were compared with those obtained from CNF systems loaded with silver nanoparticles (AgNP) after in situ synthesis via UV reduction. Storage in cold or dry conditions preserved the activity and antibacterial performance of enzyme-loaded CNF aerogels. As expected, the lysozyme-containing aerogels showed lower inhibition than the AgNP-containing aerogel. In this latter case, the antibacterial activity depended on the concentration and size of the nanoparticles. Compared to unmodified CNF and TO-CNF, the aerogels prepared with cationic CNF, loaded with either lysozyme or AgNPs, showed remarkably better antibacterial activity. Similar experiments were conducted with horseradish peroxidase, which confirmed, to different degrees, the observations derived from the lysozyme systems. Overall, the results indicate that non-toxic and biodegradable CNF is a suitable support for bio-active materials and is effective in protecting and retaining enzymatic and antibacterial activities.

Electronic supplementary material The online version of this article (doi:[10.1007/s10570-017-1311-0](https://doi.org/10.1007/s10570-017-1311-0)) contains supplementary material, which is available to authorized users.

K. M. A. Uddin · H. Orelma · P. Mohammadi ·
M. Borghei · J. Laine · M. Linder · O. J. Rojas (✉)
Department of Bioproducts and Biosystems, School of
Chemical Engineering, Aalto University Aalto, Espoo,
Finland
e-mail: orlando.rojas@aalto.fi

H. Orelma (✉)
VTT Technical Research Centre of Finland, High
Performance Fibre Products, Espoo, Finland
e-mail: hannes.orelma@vtt.fi

Keywords Cellulose nanofibrils (CNF) · Enzyme stability · Antibacterial aerogel · Lysozyme · Silver nanoparticles

Introduction

Renewable cellulose nanofibrils (CNFs) are materials with potential in a wide range of applications due to their inherent biodegradability, biocompatibility and availability (Yano and Nakahara 2004; Syverud and Stenius 2009). They can be used in applications such as barrier coatings, food additives, transparent flexible films for packaging, composites, bioactive materials, inorganic/organic hybrids, gels, foams and in the synthesis of filaments (Nogi et al. 2009; Zhang et al. 2013). Due to their strong hydrogen bonding ability and high aspect ratio, CNFs have a strong tendency to pack into oriented structures in nanopapers, membranes, and filaments (Taniguchi and Okamura 1998; Sehaqui et al. 2010; Cai et al. 2012). Moreover, highly porous aerogels of CNF have found applications for fuel cells, liquid purification, tissue engineering, protein separation and protective clothing, among others (Piletsky et al. 2003; Ye et al. 2003; Guilminot et al. 2007; Johnson et al. 2007; Sundarrajan and Ramakrishna 2007; Sehaqui et al. 2011).

The surface of CNFs is dominated by hydroxyl groups that provide opportunities for chemical modification in organic and aqueous solvents, with the latter being most indicated, given environmental and industrial considerations. One very efficient way for modifying surface hydroxyl groups is oxidation with TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) catalyst, which results in the formation of carboxyl groups on the surface of the nanofibrillated cellulose (Saito et al. 2006, 2007). Recently, we reported that these charged groups serve as sites for the controlled nucleation or adsorption on cellulose of metallic nanoparticles, such as silver nanoparticles (AgNP) (Lokanathan et al. 2014; Uddin et al. 2014). The attachment of such AgNPs onto CNF takes place through electrostatic interactions in the colloidal system (Martins et al. 2012), which is more pronounced for highly charged CNF. This opens the opportunity for using silver metal and its different ionic forms, which are known for their strong inhibitory and bactericidal effects as well as for their broad antimicrobial spectrum (Sambhy et al. 2006; Berndt et al. 2013; Abdelgawad et al. 2014; Goli et al. 2013). In this context, silver-containing CNFs have been developed as wound dressing material that prevents infection (Wright et al. 1999) and in which the nanocellulose can act as solid support. The

antibacterial activity of silver against *S. aureus* (gram-positive bacteria) and *K. pneumonia* strains (gram-negative bacteria) has been reported (Martins et al. 2012). The effect of silver ions against bacteria is explained in a number of ways, for example, they interact with the thiol groups of enzymes and proteins that are important for bacterial respiration and the transport of important substances across the cell membrane and within the cell (Ivan and Branka 2004; Cho et al. 2005). Silver ions also bind to the bacterial cell wall, altering its function (Percival et al. 2005).

We recently introduced proteins as effective media for high-throughput production of silver nanoparticles (Mehrez et al. 2017). A further interesting approach is to prepare antibacterial materials by utilizing protein-degrading enzymes (such as lysozymes), which degrade bacteria by adherence (Hughey and Johnson 1987). Lysozyme is a hydrolytic enzyme that catalyzes the breakdown of peptidoglycan polymers, found in the bacterial cell wall, by acting on the 1–4 bond between N-acetylmuramic (NAM) acid and N-acetylglucosamine (NAG) residues. Lysozymes are used in a variety of food and pharmaceutical products to prevent bacterial-induced spoiling (Yoon et al. 2009). Unfortunately, the catalytic activity of enzymes is seriously hampered by their low thermal and chemical stabilities. One way to address this issue is by immobilizing the enzyme on a solid support. Among various methods employed, physical and chemical immobilization on solids have been most common (Cho et al. 2005; Sheldon 2007; Sheldon and Pelt 2013). Physical adsorption involves adherence onto the supporting material, for example, a porous polymer matrix (Hubbe et al. 2007). Adsorbed enzymes are shielded from aggregation, proteolysis and interaction with hydrophobic interfaces (Spahn and Minteer 2008). Adsorption makes use of the interactions generated between the support and the enzyme, which include van der Waals forces, ionic interactions and hydrogen bonding in both, organic and inorganic materials. Typically, a weak binding onto a surface implies little or no change of the enzyme's native structure. This avoids any negative effect on the active sites of the enzyme and allows for the retention of its activity (Hernandez and Fernandez-Lafuente 2011; Hwang and Gu 2013). The critical physicochemical parameters related to the material for use as support include the surface area, characteristic particle size, pore

structure and type of functional groups present. Various organic supports have been used for enzyme immobilization, the most common ones include biopolymers such as chitin (and chitosan), cellulose and alginate (Jesionowski et al. 2014). These have a common feature in that they present reactive groups that can be tailored for chemical modification, i.e., they match the conditions operative for the given enzyme and its application.

In this investigation, we used various types of nanocelluloses as solid supports to stabilize or retain the activity of lysozyme. This effect was tested in liquid medium as well as in solids after storage in wet and dry conditions. A simple method was used to prepare light-weighted supports (aerogels) that physically immobilized the enzyme. Silver nanoparticles were also synthesized in the aerogel *in situ*, via UV reduction, and the systems were tested for antibacterial activity, which was contrasted to that of the lysozyme-containing aerogels produced with CNFs of different types and density of charges. The antibacterial activity of the enzyme and silver-containing CNF aerogels was verified.

Experimental

Materials

Cellulose nanofibrils (CNFs) were isolated from bleached birch fibers. Silver nitrate (AgNO_3) (≥ 99.0), sodium acetate, hydrogen peroxide, O-dianisidin, horseradish peroxidase (HRP), lysozyme from chicken egg white ($\geq 90\%$, lyophilized powder), *Micrococcus lysodeikticus* cells, carboxymethyl cellulose sodium salt (CMC) (Mw of 250 kDa, DS of 1.2) and chitosan (medium molecular weight) were purchased from Sigma-Aldrich, Finland. All other chemicals used in this study were laboratory grade and water used was purified with a MilliPore system.

Preparation of CNF, TO-CNF and Cationic-CNF

Unmodified CNF (referred to as CNF) was prepared by first diluting the cellulosic fibers in deionized water to a solids content of 1.5 w%. Then, the fiber suspension was sequentially passed once through a Masuko grinder, and then six times through an M110P fluidizer (Microfluidics Corp., Newton, MA, USA) equipped with a chamber pair (200 and 100 μm) and operated at

2000 bar pressure. TEMPO-oxidized CNF (hereafter referred to as TO-CNF) was prepared similarly to unmodified CNF, but the precursor cellulosic fibers were first disintegrated in alkaline TEMPO-NaBr-NaClO oxidative solution at pH 10, following the procedure reported elsewhere (Isogai and Kato 1998). Then, the TEMPO-oxidized fibers were passed once through the microfluidizer. The carboxyl content of the prepared TO-CNF was measured by conductometric titration (SCAN-CM 65.02) using a conductometric titrator 751 GPD titrino (Mertohm AG, Herisau, Switzerland). The carboxyl content of the TEMPO-oxidized fibers was 1.3 ± 0.6 mmol/g.

Cationic CNF (hereafter referred to as Cationic-CNF) was prepared following the same protocol used for unmodified CNF, except that the precursor fibers were first cationized according to a standard procedure (Hashem et al. 2003; Wang et al. 2011). Briefly, 10 g of cellulosic fibers (on the basis of dry weight) were mixed with 10.6 ml of 10 M NaOH, 14.4 ml of CHPTAC (60% w/w) aqueous solution, and 100 ml isopropyl alcohol. The reaction mixture was then heated at 60 °C for 4 h, the reaction was then quenched by adding 250 ml deionized water, and subsequently washed with deionized water by filtration until the pH value was lower than 6.

The charge density of cellulose nanofibrils was determined by polyelectrolyte titration. 40 ml of 0.8% Cationic-CNF was mixed with 2 ml PES-Na (40.5 $\mu\text{eq/ml}$) by stirring at room temperature for 30 min. The filtrate was then titrated with PDADMAC (40.5 $\mu\text{eq/ml}$, 0.1 ml aliquots) until the color changed from violet to blue in the presence of 1 ml toluidine blue (70 mg/ml). The charge density of Cationic-CNF was 144 ± 5 $\mu\text{eq/g}$, calculated according to Eq. (1):

$$\text{Charge} \left(\frac{\mu\text{mol}}{\text{g}} \right) = 40.5 \mu\text{mol/mL} * \frac{(\text{Added PESNa (in mL)}) - (\text{titrated PDADMAC in mL})}{\text{sample weight(g)}} \quad (1)$$

Preparation of aqueous dispersions for stability studies

The aqueous dispersions used in further tests included CNF, TO-CNF, and Cationic-CNF (solids content of 1 w% in MilliQ-water). The dispersions were diluted to 0.1 w% concentration with 66 mM potassium

phosphate buffer (pH 6.2) under vigorous mixing. Lysozyme enzyme solution (0.1 mg/ml) was prepared by dissolving lysozyme in the given CNF dispersion (0.1 w% concentration).

Enzyme activity assay

Lysozyme activity was determined by turbidimetric assay based on the lysis of *Micrococcus lysodeikticus* cells by monitoring the associated decrease of absorbance at 450 nm. Briefly, 2.5 ml of the *M. lysodeikticus* suspension, with an absorbance intensity (A.U.) between 0.6 and 0.7 in 66 mM potassium phosphate buffer pH 6.2 at 25 °C, was placed in a 1 cm cuvette followed by the addition of 0.1 ml of 0.1 mg/ml lysozyme solution. The suspension was mixed immediately, and the decrease in absorbance (at 450 nm wavelength) of the suspension was followed with a UV–VIS spectrophotometer (Shimadzu UV2550) over 2 min time. The enzyme activity a was calculated following Eqs. 2 and 3. A unit is equivalent to a reduction in turbidity (450 nm) of 0.001 in 1 min.

$$a \text{ (Units/ml enzyme)} = \frac{(\Delta A_{450}/\text{min}) \times (df)}{(0.001)(0.1)} \quad (2)$$

$$a \text{ (Units/mg solid)} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}} \quad (3)$$

where d_f = dilution factor, 0.001 is the ΔA_{450} per unit and 0.1 stands for the volume (in ml) of the enzyme solution.

The long-term enzyme activity was measured from solutions stored in a cupboard at room temperature over 30 days. The activity of lysozyme in CNF aerogels was measured as indicated previously, but after adding the given amount to the aerogel containing 0.01 mg of lysozyme (instead of 0.1 ml of 0.1 mg/ml lysozyme solution).

Preparation of CNF aerogels and antibacterial effects

CNF aerogels were prepared by freeze-drying aqueous dispersions of CNF, TO-CNF and Cationic-CNF. The aerogels were developed by using 25 ml of homogenized CNF dispersion (1.0%) placed into a small Petri dish (5.5 cm diameter) followed by freezing at -20 °C for 24 h. Then, the frozen sample was

lyophilized with a FreeZone freeze-dryer system (Labconco Corporation, USA) for 24 h. After lyophilization, the aerogel was approximately 1 cm thick. The same method was applied for all aerogels.

Antibacterial CNF aerogels were prepared from pure CNF with proteolytic enzyme (lysozyme) or with silver nanoparticles. Enzyme-containing aerogels were synthesized by first pre-mixing the enzyme with 25 ml of CNF dispersion (1.0%), followed by lyophilization, as described above. Silver-containing aerogels were prepared by using a molar amount of AgNO_3 which was based on the dry weight of CNF in the suspension. Silver nitrate (100 mM solution) was prepared as a stock solution by dissolving AgNO_3 (170 mg) in 10 ml water. This solution was added dropwise (additions of 100 or 500 μl) into 50 ml of CNF dispersion (1%), and mixed evenly with a magnetic stirrer to reach a loading in the aerogel equivalent to 0.02 mmol/g AgNO_3 or 0.1 mmol/g AgNO_3 . The silver nanoparticles evolved from silver cations (Ag^+) by exposing the dried aerogel to UV irradiation ($\lambda = 254$ nm, 80 W) for 30 min per each side (Arcot et al. 2015; Yan et al. 2016). The aerogel was stored in a desiccator, away from light.

Scanning electron microscopy (SEM)

The CNF aerogels were examined in cross sections by imaging under a field emission scanning electron microscope, JEOL JSM-7500FA, at 1–2 kV accelerating voltage. The samples were sputter-coated with gold–palladium before imaging to reduce charging effects. At least three different locations for each sample were analyzed.

Transmission electron microscopy (TEM)

TEM samples were prepared by placing a small piece of the aerogel in MQ water followed by sonication for 10 min in an ultra-sonication bath. A volume of 5 μL of CNF dispersion was pipetted onto carbon grids, which were contacted with the edge of a Whatman filter paper to remove excess liquid and allowed to dry in air. Brightfield TEM images were recorded using a FEI Tecnai T12 microscope (Eindhoven, Netherlands) operating at 120 kV. Size distribution histograms for AgNP in each sample were plotted based on the data collected from at least 100 particles.

Antimicrobial effect of CNF aerogels loaded with lysozyme and silver nanoparticles

The antimicrobial effect of CNF aerogels carrying lysozyme or silver nanoparticles was tested using viable cell counting against gram negative and gram positive bacteria, *Escherichia coli* and *Staphylococcus aureus*, respectively. Bacteria were cultivated at 37 °C for 14 h in 20 ml of lysogeny broth (LB), which consists of tryptone 1%, yeast extract 0.5%, NaCl 1%, pH = 7. After this, the optical density (OD) at 600 nm was adjusted to 0.5 by diluting the bacterial suspension with the same medium. The suspension was then diluted 1000 times with LB medium to give an approximate bacterial concentration of about 4×10^5 CFUs/ml. 5 mg of the aerogel samples were taken in a glass tube followed by the addition of 5 ml of the bacterial suspension. The bacteria were then cultured for 14 h under continuous shaking (230 rpm) in an incubator at 37 °C. 100 μ L of the cultured bacteria were extracted, diluted with lysogeny broth to a certain volume (to adjust the bacterial concentration of the treated solution and to ensure that the number of colonies could be counted easily), and spread on a plate containing nutrient agar. Plates containing bacteria were incubated at 37 °C for 24 h, then the number of the surviving colonies was counted. The results were compared to the number of bacterial colonies measured on a control that did not contain any sample.

Results and discussion

Lysozyme activity in aqueous media

The effect of CNF on enzyme stabilization in aqueous dispersion was studied in the case of lysozyme. The activity was measured by turbidimetric assay (wavelength of 450 nm) using a UV-spectrometer. The enzyme activity in the presence of the different types of cellulosic nanofibrils was studied over 30 days under storage in room conditions (Fig. 1a). The dispersion containing CNF and Cationic-CNF exhibited a slightly higher activity compared to that measured for the background buffer solution. A slight decrease over time in activity for the background buffer solution was observed; remarkably, in the presence of CNF and Cationic-CNF, the activity was

nearly stable over the 30 days. Similar observations were made in the case of horseradish peroxidase (HRP) in the presence of CNF and chitosan (see Supporting Information Fig S1). In contrast, the enzyme stability was clearly reduced in the presence of negatively-charged TO-CNF, which indicated an adverse effect of anionic charges against lysozyme. In the presence of the negatively charged TO-CNF, the positively-charged lysozyme undergoes strong adhesion, which causes an alteration of its structure (folding) and activity (Yabuki 2014). Similar reduction in activity was observed for HRP in the presence of TO-CNF as well as carboxymethyl cellulose (CMC) (Fig S1). Unmodified CNF, which is less charged, induced a weaker adhesion and more limited changes in the structure of the enzyme, improving the retention of its activity.

Activity of lysozyme immobilized in CNF aerogels

So far we have demonstrated that the presence of CNF and Cationic-CNF has a stabilizing effect on the enzyme, which maintains its activity over time. In this section we report on the lysozyme stability after physical immobilization in aerogels obtained by freeze drying and after storage in dry conditions. The activity was compared with that of the free lyophilized lysozyme. Figure 1b shows that the lysozyme activity remained almost unchanged when supported on CNF and Cationic-CNF aerogels. In the case of TO-CNF, the activity decreased drastically, by almost 88% compared with the lyophilized lysozyme. Thus, the type of CNF material has a significant influence on the stability of the enzyme and its activity, as observed earlier in the case of aqueous dispersions. We speculate that upon freeze drying, the strong binding between negatively-charged TO-CNF and positively-charged lysozyme irreversibly folds the enzyme and thus impairs its activity.

The activity of the enzyme supported in the CNF-based aerogels was measured on day 7 and day 30 after storage under four different conditions: “Room” (storage at room conditions, 25 °C, 25% RH); “Cold” (storage in a refrigerator at 4 °C, 25% RH); “Dry” (storage at room temperature in a desiccator, 25 °C, 12% RH), and “Humid” (storage in a humid environment, 25 °C, 65% RH). The enzyme displayed roughly the same activity over 30 days when stored

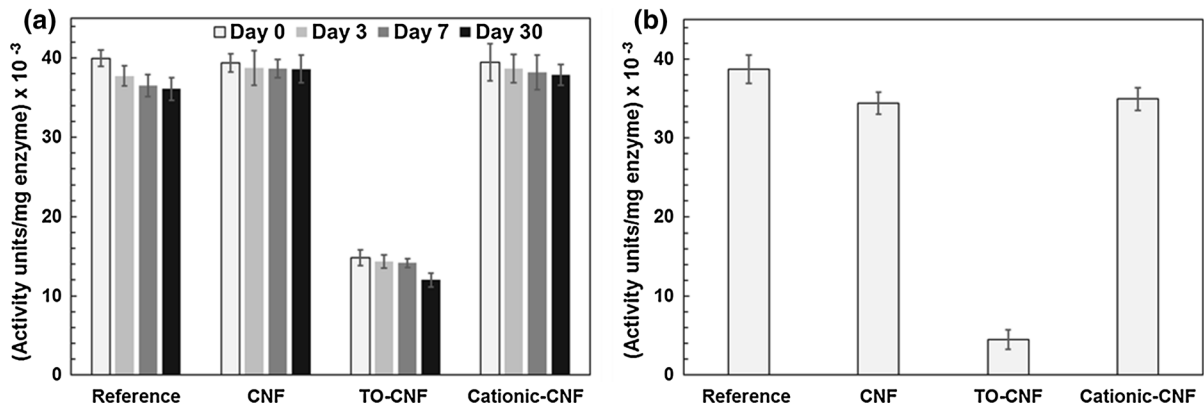


Fig. 1 **a** Lysozyme activity as a function of storage time (over 30 days in room conditions) in aqueous dispersion in the absence (reference) and in the presence of CNF, TO-CNF and Cationic-CNF, as indicated. **b** The initial enzyme activity in free

form or after immobilization in the respective nanocellulose aerogel. The experiments were performed in triplicate and the error is shown as the standard deviation

in the Cold and Dry conditions. The enzyme activity decreased very slightly when stored in Room and Humid conditions (Table 1). In Humid conditions, more water was available to hydrate the enzyme, which induced a deactivation through unfolding. Related findings about the role of water on protein stability have been reported (Wang et al. 2012).

Antibacterial aerogels

Aerogels were prepared after freeze-drying dispersions containing CNF mixed with lysozyme or with silver nitrate. The silver nitrate was converted in situ to silver nanoparticles (AgNP) by UV reduction (Fig. 2). The aerogels were then used to test their effect on bacterial growth. Compared to the aerogels free of

enzyme, which displayed a white color, the aerogels carrying immobilized lysozyme were off-white. Likewise, the color of the aerogels was altered when AgNPs were incorporated in CNF, TO-CNF and Cationic-CNF. This is expected to be the result of changes in the size and concentration of AgNPs in the aerogel (Dong et al. 2013). The surface charge of CNF has a significant effect in controlling the size of the silver nanoparticles. This is because cellulose acts as a nucleation controller and stabilizer of AgNPs in the aerogel (Lokanathan et al. 2014; Uddin et al. 2014). Thus, for a given AgNO₃ concentration, the CNF, TO-CNF and Cationic-CNF aerogels displayed different colors due to the silver plasmonic effect, which is dependent on nanoparticle size (Mogensen and Kneipp 2014) (Fig S2).

Table 1 Activity of lysozyme supported in aerogels that were stored under different conditions, as indicated

Day	Reference	CNF	TO-CNF	Cationic-CNF
0				
Room (25 °C)	38.71±1.80	34.41±1.39	4.47±1.22	34.93±1.44
7				
Room (25 °C)	37.54±2.02	34.36±2.43	3.27±1.10	34.68±2.00
Cold (4 °C)	38.85±3.76	34.64±3.55	3.57±0.85	34.91±1.11
Dry (RH 12%)	38.60±3.87	34.54±3.32	3.00±0.95	34.82±0.98
Humid (RH 65%)	37.25±1.80	34.23±2.30	3.07±0.95	34.59±1.09
30				
Room (25 °C)	37.17±1.92	33.70±1.00	3.25±0.66	34.51±1.36
Cold (4 °C)	38.54±3.30	34.20±2.37	3.12±0.55	34.78±0.40
Dry (RH 12%)	38.53±0.95	34.12±1.85	2.82±0.25	34.53±1.15
Humid (RH 65%)	37.15±1.30	34.02±1.57	2.95±0.85	34.09±1.85

The enzyme activity is given in Units/mg × 10⁻³ enzyme as measured for the given storage time: Initial (0 days), 7 and 30 days

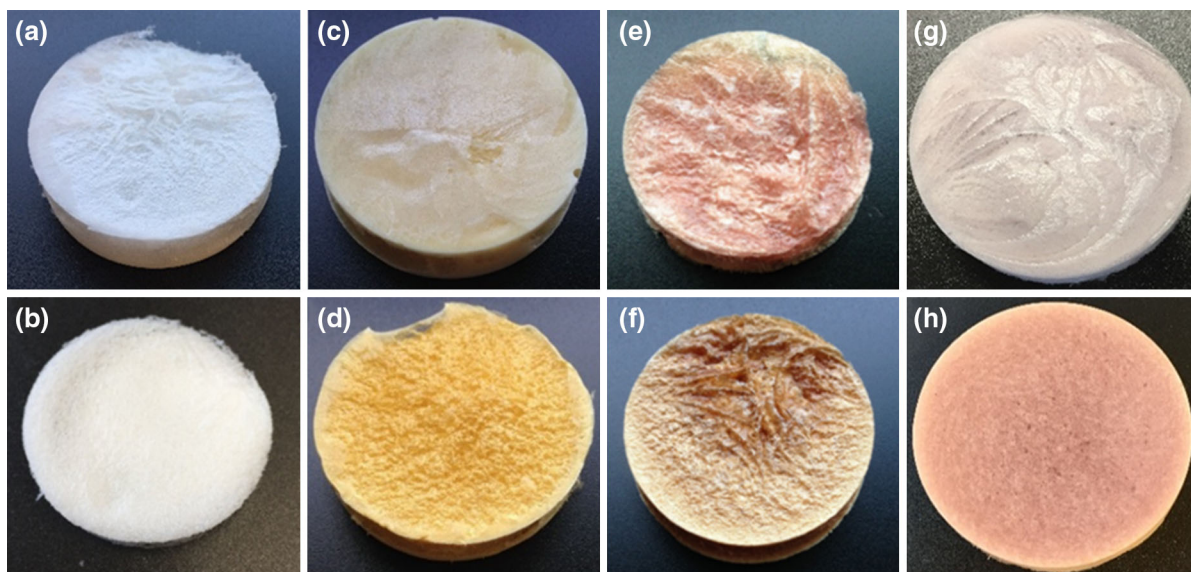


Fig. 2 Photographs of **a** neat CNF aerogel and **b** CNF aerogel carrying 0.1 g/g lysozyme. Also shown are the CNF aerogels prepared from **c** 0.02 mmol/g and **d** 0.1 mmol/g AgNO_3 precursor. TO-CNF aerogel prepared from **e** 0.02 mmol/g and

f 0.1 mmol/g AgNO_3 precursor. Cationic-CNF aerogel prepared from **g** 0.02 mmol/g and **h** 0.1 mmol/g AgNO_3 precursor. The colors developed in the aerogels containing AgNP depended on the loading of silver nitrate. (Color figure online)

The SEM cross-sections of the prepared CNF aerogels (Fig. 3) indicated an open porous structure that was formed from the ice crystals that developed upon freezing and prior the dehydration via lyophilization. Addition of lysozyme or silver decreased the porosity of the aerogels. Compared to the CNF aerogel, TO-CNF and Cationic-CNF aerogels showed less compact and uniform structures. This may be due to the higher surface charge that causes electrostatic repulsion and favors a better dispersion. Compared to CNF aerogels prepared with fast ethanol/dry ice bath (Dong et al. 2013), the aerogels reported here (prepared at $-20\text{ }^\circ\text{C}$), presented voids that were comparatively larger, due to the formation of large ice crystals. Compared to aerogels obtained from TO-CNF and CNF, those produced from Cationic-CNF showed more random and smaller voids.

AgNP-loaded aerogels

The size of the silver nanoparticles (AgNPs) in the aerogels was analyzed by transmission electron microscopy (TEM), Fig. 4. It was observed that with higher silver nitrate loading, the proportion of large particles and aggregation increased. The number % of the finer particle fraction, obtained from a given

AgNO_3 concentration, changed significantly depending on CNF type; it increased in the order CNF < TO-CNF < Cationic-CNF, as observed in the histograms included in Fig. 4. The negative carboxylate groups and positive amino groups on the surface of TO-CNF and Cationic-CNF, respectively, provide electrostatic repulsion between nanofibrils, preventing aggregation in aqueous dispersion, which hinders the growth of silver nanoparticles. Moreover, compared to the CNF counterpart, the Cationic-CNF aerogel presented narrower particle size distribution (note the polydispersity of the particles increased with the concentration of silver nitrate, at least in the case of CNF aerogels).

Lysozyme is an enzyme with antimicrobial activity against both gram positive and negative bacteria (Barbiroli et al. 2012). Here, we investigated the antibacterial effect of CNF-lysozyme aerogels and compared their antimicrobial effect with that observed for silver-containing CNF aerogels (Yan et al. 2016). Based on 1 ml volume of bacterial suspension, CNF aerogels (1 mg carrying 0.1 mg of lysozyme) and pure lysozyme (0.1 mg) were used to evaluate the antimicrobial activity using the viable cell counting method. The activity was tested against *Escherichia coli* (gram-negative, Fig S3) and *Staphylococcus aureus* (gram-positive, Fig S4). The logarithmic reduction

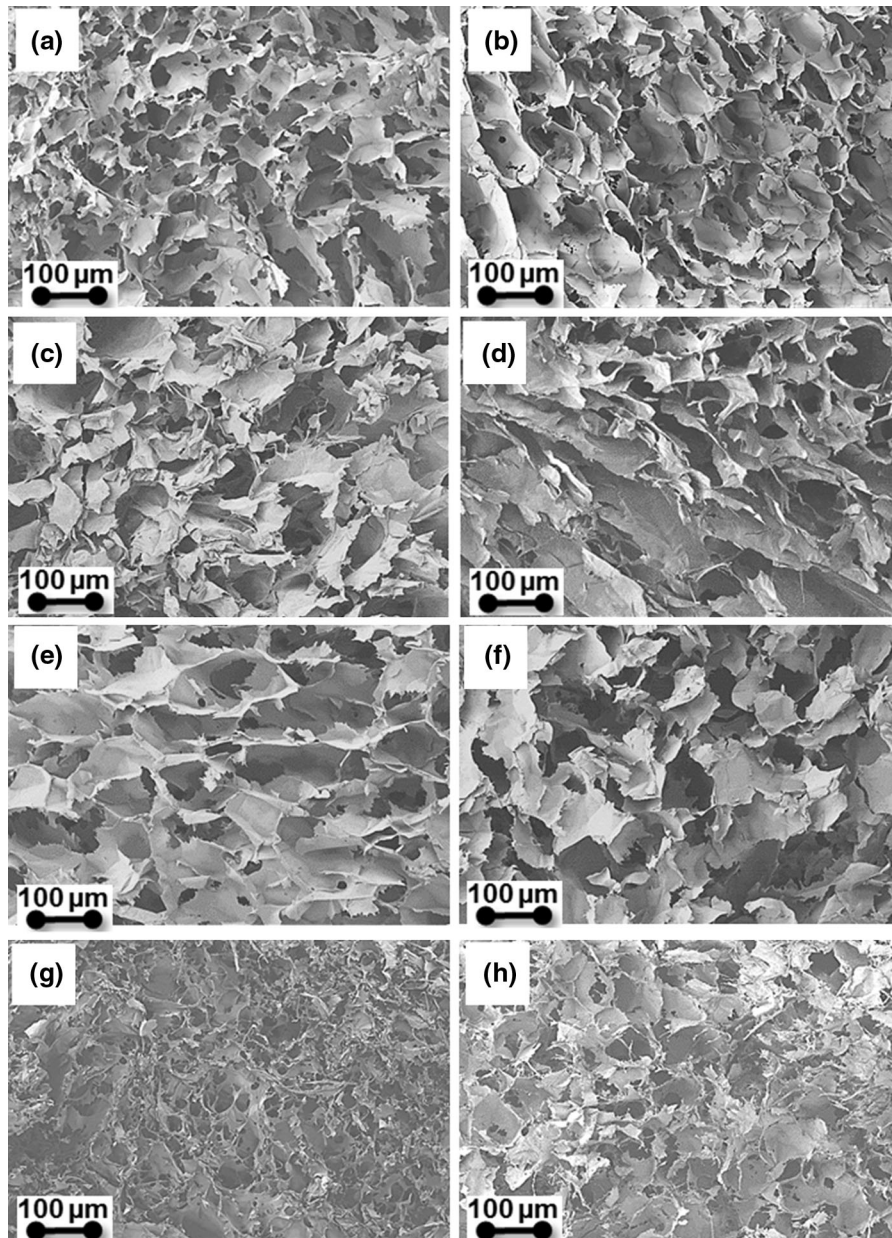


Fig. 3 SEM image of cross-sections of freeze-dried aerogel structures. **a** CNF aerogel and **b** CNF aerogel with 0.1 g/g lysozyme. **c** CNF aerogel carrying silver nanoparticles prepared from 0.02 mmol/g AgNO_3 and **d** 0.1 mmol/g AgNO_3 precursor. **e** TO-CNF aerogel carrying silver nanoparticles prepared from

0.02 mmol/g AgNO_3 and **f** 0.1 mmol/g AgNO_3 precursor. **g** Cationic-CNF aerogel carrying silver nanoparticles prepared from 0.02 mmol/g and **h** 0.1 mmol/g AgNO_3 precursor. The aerogels were stored in a desiccator and away from light

value was calculated as $\log(N_0/N)$, where N_0 = CFUs/ml of blank and N = CFUs/ml of sample (Fig. 5). For 100% inhibition of *E. coli* and *S. aureus*, the log reduction was 9.63 log and 9.39 log based on the reference. CNF and TO-CNF did not show any

significant inhibition to any of the bacteria tested. Remarkably, the Cationic-CNF showed a high inhibitory effect: the inhibition was about 92% (1.11 log) for *E. coli* and 71% (0.53 log) for *S. aureus*.

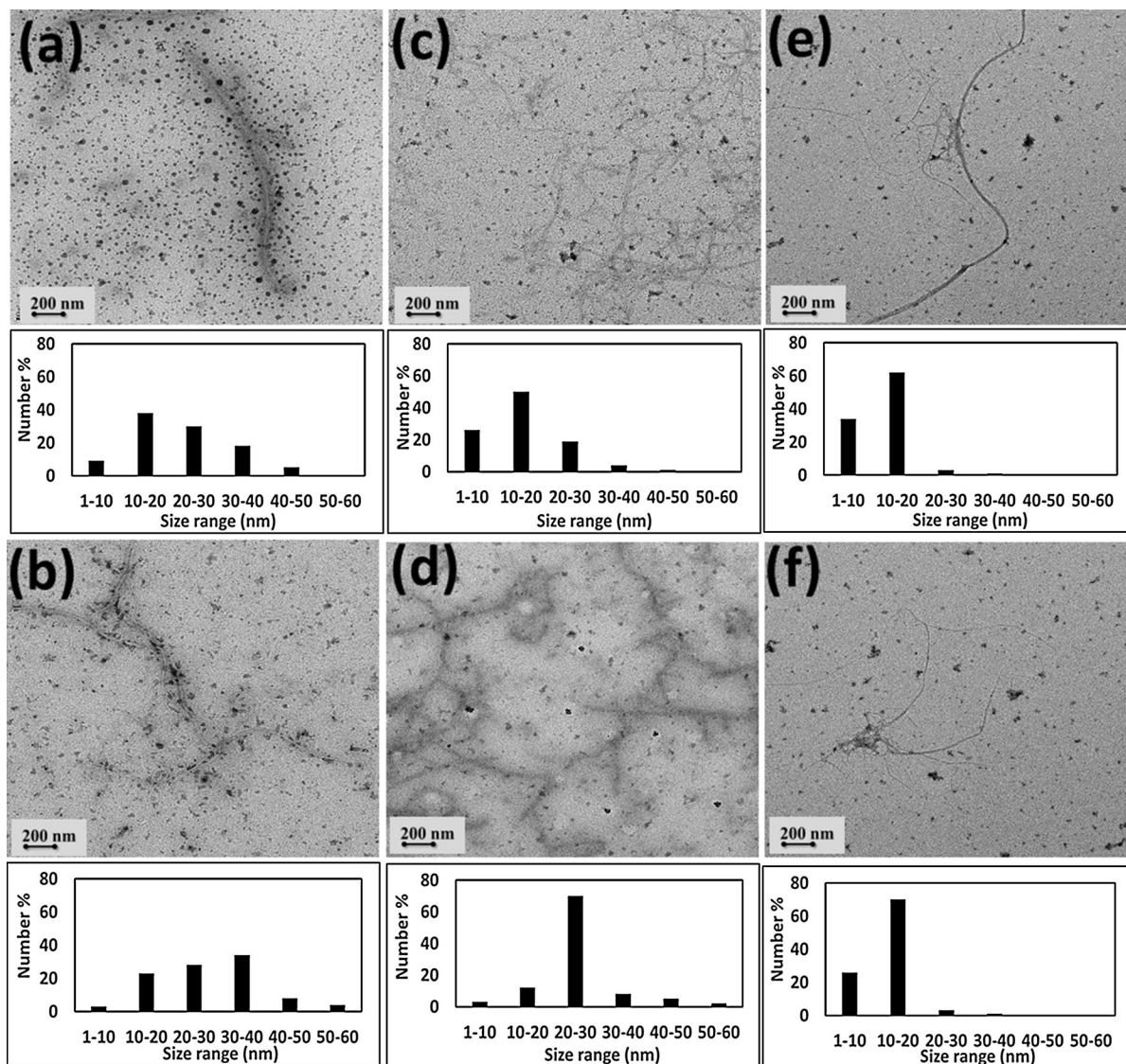


Fig. 4 TEM images and particle size histograms of Ag nanoparticles obtained from CNF aerogels that were initially loaded with AgNO₃ at a concentration of **a** 0.02 and **b** 0.1 mmol/g. Likewise, the rest of the panels correspond to aerogels comprising TO-CNF loaded with **c** 0.02 and **d** 0.1 mmol/g

AgNO₃ and Cationic-CNF containing **e** 0.02 and **f** 0.1 mmol/g AgNO₃. The number % histograms for particle size are shown in the bottom of each figure. The scale bar in all images correspond to 200 nm

The observed differences for the two types of bacteria result from the composition, thickness and density of the cell walls (those of *S. auricus* are thicker and denser). Lysozyme showed slightly higher inhibition against *S. auricus* than *E. coli*. When comparing the lysozyme inhibition against bacteria, for the different aerogels containing lysozyme, TO-CNF aerogel was shown to have much less inhibition. This is explained by the folding and strong adhesion of

lysozyme in TO-CNF, as we speculated earlier in the case of aqueous dispersions. There is a clear indication that lysozyme is active in CNF aerogels. All the aerogels containing silver nanoparticles obtained from 0.1 mmol/g AgNO₃ showed 100% inhibition (9.63 log) against *E. coli*. CNF, TO-CNF and Cationic-CNF aerogels containing silver nanoparticles obtained from 0.02 mmol/g AgNO₃ showed 99.99053, 99.99143 and 99.99999% inhibition against *E. coli*, respectively.

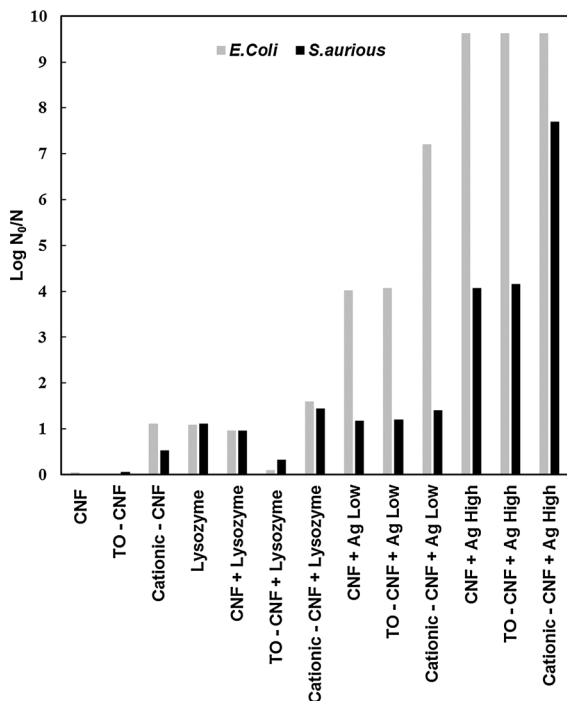


Fig. 5 Quantification of CFU reduction efficiency with different antimicrobial aerogels against both *E. coli* and *S. aureus*. The vertical axis correspond to the logarithmic reduction value (for example, a 100% reduction value for *E. coli* and *S. aureus* is 9.63 log and 9.39 log with respect to blank). A 100% reduction is reached when no CFU is detectable on the test samples. Ag Low and Ag High indicate the level of loading of the AgNPs (those obtained from 0.02 mmol/g and 0.1 mmol/g AgNO₃ precursor). Note: The error in CFU reduction efficiency is equivalent to a 0.1–0.2 log reduction

The TO-CNF aerogels loaded with AgNP showed slightly higher antimicrobial activity than those based on CNF. This may be due to the smaller size of the nanoparticles in the former case. Similar to the observations made for *E. coli*, the Cationic-CNF aerogels containing AgNPs showed the highest antimicrobial activity against *S. aureus*. Overall, this is explained by a dual antimicrobial activity exerted by the cationic nature of the CNF and the AgNPs. The antimicrobial activity of silver is based on the binding of Ag⁺ with the negatively charge peptidoglycans (containing sulfhydryl groups) of the bacterial cell wall. This disturbs the cell wall permeability and cellular respiration, and leads to its disruption, namely the bacteria dissolution and death (Feng et al. 2000; An et al. 2009; Son et al. 2006). Thus, the size of the nanoparticles is important in relation to the release of

Ag⁺ as well as the binding strength with the bacterial cell wall, which inhibits its growth.

Conclusions

The charge on the surface of nanocellulose (CNF) has a significant effect on the retention of enzyme activity, when used as an aqueous dispersion or as an aerogel. As an illustration, CNF and Cationic-CNF were effective in extending lysozyme activity; however, TO-CNF had no such effect. The observation in this latter case is rationalized by the strong interaction between the oppositely charged components, which limits enzyme freedom and flexibility due to protein folding, hindering its active sites. As expected, the storage conditions influenced the retention of the activity of enzymes immobilized in the nanocellulose aerogels: compared to cold and dry conditions, storage at room and high humidity (RH 65%) atmospheres decreased the activity markedly. At an enzyme concentration of 0.1 mg/ml in aqueous dispersion, the bacterial growth was inhibited (by about 92%). The aerogels carrying physically-immobilized lysozyme showed a moderate antimicrobial effect against both gram-positive *S. aureus* and gram-negative *E. coli*. Compared to *E. coli*, lysozyme showed slightly higher antibacterial effect against *S. aureus*. The same observation was noted for CNF carrying silver nanoparticles (AgNP) that were synthesized in situ by UV reduction. In this case, the average size of the AgNPs was significantly affected by the surface charge of the nanocellulose support. The number % of small particle size (10–20 nm, which present better antibacterial activity compared to the larger fractions) was higher for the Cationic-CNF. Aerogels loaded with silver nanoparticles showed a strong antibacterial effect against *E. coli*, which depended on the nanoparticle concentration and a full inhibition of *E. coli* was observed at the highest concentration of silver nanoparticles tested. Under similar conditions of application, the Cationic-CNF aerogel carrying immobilized lysozyme or silver nanoparticles showed better antimicrobial activity compared to that of CNF and TO-CNF. Overall, the results presented in this work are relevant to the development of materials for wound dressings and enzyme supports. In this later case, for example, we will integrate nanocelluloses in units for assays that required immobilized enzymes,

immunoassays and point-of-care devices that require long shelf life in different storage conditions.

Acknowledgments The authors are grateful for funding support by the Academy of Finland (AF) through Project 295007, Nanocellulose Cyber-Physical Microsystems (ICT 2023: Advanced microsystems) as well as AF's Centres of Excellence Programme (2014–2019), under Project 264677 "Molecular Engineering of Biosynthetic Hybrid Materials Research" (HYBER). This work made use of the Aalto University Nano microscopy Center.

References

- Abdelgawad AM, Hudson SM, Rojas OJ (2014) Antimicrobial wound dressing microfiber mats from multicomponent (chitosan/silver-NPs/polyvinyl alcohol) systems. *Carbohydr Polym* 100:166–178
- An J, Zhang H, Zhang J, Zhao Y, Yuan X (2009) Preparation and antibacterial activity of electrospun chitosan/poly (ethylene oxide) membranes containing silver nanoparticles. *Colloid Polym Sci* 287:1425–1434
- Arcot LR, Uddin KMA, Chen X, Xiang W, Xianming K, Johansson LS, Ras RHA, Rojas OJ (2015) Paper-based plasmon-enhanced protein sensing by controlled nucleation of silver nanoparticles on cellulose. *Cellulose* 6:4027–4034
- Barbiroli A, Bonomi F, Capretti G, Iametti S, Manzoni M, Piergiovanni L, Rollini M (2012) Antimicrobial activity of lysozyme and lactoferrin incorporated in cellulose-based food packaging. *Food Control* 26:387–392
- Berndt S, Wesarg F, Wiegand C, Kralisch D, Muller FA (2013) Antimicrobial porous hybrids consisting of bacterial nanocellulose and silver nanoparticles. *Cellulose* 20:771–783
- Cai J, Liu S, Feng J, Kimura S, Wada M, Kuga S, Zhang L (2012) Cellulose–silica nanocomposite aerogels by in situ formation of silica in cellulose gel. *Angew Chem* 124(9):2118–2121
- Cho KH, Park JE, Osaka T, Park SG (2005) The study of antimicrobial activity and preservative effects of nanosilver ingredient. *Electrochim Acta* 51:956–960
- Dong H, Snyder JF, Tran DT, Leadore JL (2013) Hydrogel, aerogel and film of cellulose nanofibrils functionalized with silver nanoparticles. *Carbohydr Polym* 95:760–767
- Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JOA (2000) Mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J Biomed Mater Res* 52:662–668
- Goli K, Gera N, Liu X, Rao B, Rojas OJ, Genzer J (2013) Generation and properties of antibacterial coatings based on electrostatic attachment of silver nanoparticles to protein-coated polypropylene fiber. *ACS Appl Mater Interfaces* 5:5298–5306
- Guilminot E, Fischer F, Chatenet M, Rigacci A, Berthon-Fabry S, Achard P, Chainet E (2007) Use of cellulose-based carbon aerogels as catalyst support for PEM fuel cell electrodes: electrochemical characterization. *J Power Sources* 166:104–111
- Hashem M, Hauser PJ, Smith B (2003) Reaction efficiency for cellulose cationization using 3-chloro-2-hydroxypropyl trimethyl ammonium chloride. *Text Res J* 73:1017–1023
- Hernandez K, Fernandez-Lafuente R (2011) Control of protein immobilization: coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance. *Enzyme Microb Technol* 48:107–122
- Hubbe MA, Hubbe Rojas OJ, Lucia LA, Jung TM (2007) Consequences of the nanoporosity of cellulosic fibers on their streaming potential and their interactions with cationic polyelectrolytes. *Cellulose* 14:655–671
- Hughey VL, Johnson EA (1987) Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl Microbiol Biotechnol* 53(9):2165–2170
- Hwang ET, Gu MB (2013) Enzyme stabilization by nano/microsized hybrid materials. *Eng Life Sci* 1:49–61
- Isogai A, Kato Y (1998) Preparation of polyuronic acid from cellulose by TEMPO-mediated oxidation. *Cellulose* 5:153–164
- Ivan S, Branka SS (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for gram negative bacteria. *J Colloid Interface Sci* 275:177–182
- Jesionowski T, Zdarta J, Krajewska B (2014) Enzyme immobilization by adsorption: a review. *Adsorption* 20:801–821
- Johnson J, Ghosh A, Lannutti J (2007) Microstructure-property relationships in a tissue-engineering scaffold. *J Appl Polym Sci* 104:2919–2927
- Lokanathan AR, Uddin KMA, Rojas OJ, Laine J (2014) Cellulose nanocrystal-mediated synthesis of silver nanoparticles: role of sulfate groups in nucleation phenomena. *Biomacromol* 15:373–379
- Martins NCT, Freire CSR, Pinto RJB, Fernandes SCM, Neto CP, Silvestre AJD, Causio J, Baldi G, Sadocco P, Trindade T (2012) Electrostatic assembly of Ag nanoparticles onto nanofibrillated cellulose for antibacterial paper products. *Cellulose* 19:1425–1436
- Mehrez EE-N, Eisa W, Abdelgawad AM, Rojas OJ (2017) Clean and high-throughput production of silver nanoparticles mediated by soy protein via solid state synthesis. *J Clean Prod* 144:501–510
- Mogensen KB, Kneipp K (2014) Size-dependent shifts of plasmon resonance in silver nanoparticle films using controlled dissolution: monitoring the onset of surface screening effects. *J Phys Chem C* 118:28075–28083
- Nogi M, Iwamoto S, Nakagaito AN, Yano H (2009) Optically transparent nanofiber paper. *Adv Mater* 21:1595–1598
- Percival SL, Bowler PG, Russell D (2005) Bacterial resistance to silver in wound care. *J Hosp Infect* 60:1–7
- Piletsky S, Piletska E, Bossi A, Turner N, Turner A (2003) Surface functionalization of porous polypropylene membranes with polyaniline for protein immobilization. *Biotechnol Bioeng* 82:86–92
- Saito T, Nishiyama Y, Putaux JL, Vignon M, Isogai A (2006) Homogeneous suspensions of individualized microfibrils from TEMPO-catalyzed oxidation of native cellulose. *Biomacromol* 7:1687–1691

- Saito T, Kimura S, Nishiyama Y, Isogai A (2007) Cellulose nanofibers prepared by TEMPO-mediated oxidation of native cellulose. *Biomacromol* 8:2485–2491
- Sambhy V, MacBride MM, Peterson BR, Sen A (2006) Silver bromide nanoparticle/polymer composites: dual action tunable antimicrobial materials. *J Am Chem Soc* 128:9798–9808
- Sehaqui H, Liu A, Zhou Q, Berglund LA (2010) Fast preparation procedure for large, flat cellulose and cellulose/inorganic nanopaper structures. *Biomacromol* 11(9):2195–2198
- Sehaqui H, Zhou Q, Ikkala O, Berglund LA (2011) Strong and tough cellulose nanopaper with high specific surface area and porosity. *Biomacromol* 12:3638–3644
- Sheldon RA (2007) Enzyme immobilization: the quest for optimum performance. *Adv Synth Catal* 49:1289–1307
- Sheldon RA, Pelt SV (2013) Enzyme immobilisation in biocatalysis: why, what and how? *Chem Soc Rev* 42:6223–6225
- Son WK, Youk JH, Park WH (2006) Antimicrobial cellulose acetate nanofibers containing silver nanoparticles. *Carbohydr Polym* 65:430–434
- Spahn C, Minter SD (2008) Enzyme immobilization in biotechnology. *Recent Pat Eng* 2:195–200
- Sundarrajan S, Ramakrishna S (2007) Fabrication of nanocomposite membranes from nanofibers and nanoparticles for protection against chemical warfare stimulants. *J Mater Sci* 42:8400–8407
- Syverud K, Stenius P (2009) Strength and barrier properties of MFC films. *Cellulose* 16:75–85
- Taniguchi T, Okamura K (1998) New films produced from microfibrillated natural fibres. *Polym Int* 47(3):291–294
- Uddin KMA, Lokanathan AR, Liljeström A, Chen X, Rojas OJ, Laine J (2014) Silver nanoparticle synthesis mediated by carboxylated cellulose nanocrystals. *Green Mater* 2:183–192
- Wang Z, Hauser PJ, Laine J, Rojas OJ (2011) Multilayers of low charge density polyelectrolytes on thin films of carboxymethylated and cationic cellulose. *J Adhesion Sci Technol* 25:643–660
- Wang J, Yiu B, Obermeyer J, Filipe CDM, Brennan JD, Pelton R (2012) Effects of temperature and relative humidity on the stability of paper-immobilized antibodies. *Biomacromol* 13:559–564
- Wright JB, Lam K, Hansen D, Burrell RE (1999) Efficacy of topical silver against fungal burn wound pathogens. *Am J Infect Control* 27(4):344–350
- Yabuki S (2014) Supporting materials that improve the stability of enzyme membranes. *Anal Sci* 30:213–217
- Yan J, Abdelgawad AM, El-Naggar ME, Rojas OJ (2016) Antibacterial activity of silver nanoparticles synthesized In-situ by solution spraying onto cellulose. *Carbohydr Polym* 147:500–508
- Yano H, Nakahara S (2004) Bio-composites produced from plant microfiber bundles with a nanometer unit web-like network. *J Mater Sci* 39:1635–1638
- Ye SH, Watanabe J, Iwasaki Y, Ishihara K (2003) Antifouling blood purification membrane composed of cellulose acetate and phospholipid polymer. *Biomaterials* 24:4143–4152
- Yoon J, Park JM, Jung SK, Kim KY, Kim YH, Min J (2009) Characterization of antimicrobial activity of the lysosomes isolated from *Saccharomyces cerevisiae*. *Curr Microbiol* 59:48–52
- Zhang Y, Nypelö T, Salas C, Arboleda J, Hoeger IC, Rojas OJ (2013) Cellulose nanofibrils: from strong materials to bioactive surfaces. *J Renew Mater* 1(3):195–211