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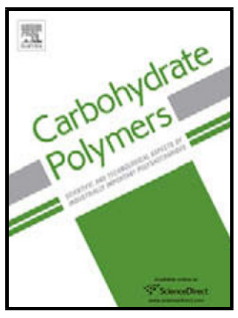

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Highlights

- Nanocellulose has shown potential usefulness in advanced wound dressing applications.
- Nanocellulose had the ability to form smooth, translucent films.
- The ability of nanocellulose to impair bacterial growth was assessed.
- Nanocellulose demonstrated impaired biofilm growth compared to the Aquacel® control.
- Cells exhibited altered morphology when grown on nanocellulose compared to Aquacel®.

An investigation of *Pseudomonas aeruginosa* biofilm growth on novel nanocellulose fiber dressings.

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ABSTRACT

Nanocellulose from wood is a novel biomaterial, which is highly fibrillated at the nanoscale. This affords the material a number of advantages, including self-assembly, biodegradability

and the ability to absorb and retain moisture, which highlights its potential usefulness in clinical wound-dressing applications. In these *in vitro* studies, the wound pathogen *Pseudomonas aeruginosa* PAO1 was used to assess the ability of two nanocellulose materials to impair bacterial growth (<48 h). The two nanocelluloses had a relatively small fraction of residual fibres (<4%) and thus a large fraction of nanofibrils (widths<20 nm). Scanning electron microscopy and confocal laser scanning microscopy imaging demonstrated impaired biofilm growth on the nanocellulose films and increased cell death when compared to a commercial control wound dressing, Aquacel®. Nanocellulose suspensions inhibited bacterial growth, whilst UV-vis spectrophotometry and laser profilometry also revealed the ability of nanocellulose to form smooth, translucent films. Atomic force microscopy studies of the surface properties of nanocellulose demonstrated that PAO1 exhibited markedly contrasting morphology when grown on the nanocellulose film surfaces compared to an Aquacel® control dressing (p<0.05). This study highlights the potential utility of these biodegradable materials, from a renewable source, for wound dressing applications in the prevention and treatment of biofilm development.

Keywords: Nanocellulose, Biofilm, *Pseudomonas aeruginosa*, Atomic Force Microscopy, Characterisation

1. Introduction

Human wound healing represents a complex sequence of inter-related and overlapping biological events. When the skin is disrupted, a series of processes are triggered to restore barrier function, prevent water loss and reduce the risk of bacterial invasion of the deeper tissues. Disruption of these processes and failure to heal is unfortunately frequent, and is estimated to occur in >60% of the population. These "chronic wounds" represent an important and unrecognised cause of morbidity and mortality and are estimated in the US alone to cost $25 billion annually (Gottrup, 2004; Gjødsbøl et al., 2006; Sen et al., 2009). In an attempt to reduce water-loss from the wound surface, decrease bacterial contamination and promote healing, specialist dressings have been employed as standard care for these wounds (Boateng et al., 2008); this market was estimated in 2011 to be, worth $12.8 billion (Transparency Market Research, 2013). Increasingly, rather than being solely "simple" inert barriers, these dressings possess impregnated biological functions, which have ranged from antimicrobials (e.g. silver/iodine) to anti-inflammatory components (e.g. oxidised cellulose). Dressings have also been used to deliver cell-based therapies (e.g. fibroblasts) with varying degrees of success (Veves, Sheehan, & Pham, 2002; Atiyeh et al., 2007; Boateng et al., 2008). The ideal dressing material would have the following properties: water loss control (to maintain a moist environment); high mechanical strength, elasticity and conformability; ability to inhibit bacterial growth and biodegradability (Sai & Babu, 2000; Kokabi, Sirousazar & Hassan, 2007). Possession of innate antibacterial properties is particularly useful for dressing materials as all wounds harbour bacteria which may directly, or indirectly inhibit wound healing and stimulate chronic inflammation within the wound bed (Gjødsbøl et al., 2006; Bjarnsholt et al., 2008; James et al., 2008).

Cellulose is the most abundant organic polymer on Earth. In attempting to deliver novel materials, researchers have developed nanocellulose structures derived from a variety of sources including, wood, annual crops and agricultural residues (Wågberg et al., 2008; Saito et al., 2009; Syverud et al., 2010; Klemm et al., 2011; Jonoobi et al., 2012; Alila et al., 2013). Nanocellulose is therefore, renewable, biodegradable and obtained from sustainable non-oil based resources (Dufresne, 2013).

In the production process, the resultant nanofibrillar structures can be produced using a variety of pre-treatments, including applying 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation, carboxymethylation, periodate oxidation; generating nanofibrillar

structures with distinct structural and chemical properties (Saito et al., 2006; Wågberg et al., 2008; Liimatainen et al., 2012). The nanocellulose structures produced with chemical pre-treatments are usually composed of nanofibrils with a narrow diameter distribution, generally less than 20 nm (Saito et al., 2006, Saito et al., 2009; Chinga-Carrasco et al., 2011) in contrast to those produced without chemical pre-treatment. Cellulose nanofibrils possess a high aspect-ratio and surface area, with resultant high tensile strength and modulus (Saito et al., 2013; Jossefson et al., 2015).

In recent years, there has been a growing interest in harnessing the potential of this abundant renewable resource as a valuable nanomaterial for biomedical applications (Lin & Dufresne, 2014; Jorfi & Foster, 2015). The use of oxidized nanofibrils for cross-linking to generate elastic cryo-gels with a desired pore size, demonstrated the potential of these materials for use in dressing materials, potentially facilitating wound fluid absorption and controlled drug release (Syverud et al., 2011b; Chinga-Carrasco & Syverud, 2014; Rees et al., 2015). Importantly, nanocellulose materials have been demonstrated to be non-cytotoxic against a series of cell-lines (Vartianen et al., 2011; Dong et al., 2012; Alexandresku et al., 2013).

These studies characterized the interaction of the known wound pathogen *Pseudomonas aeruginosa* with nanocellulose materials derived from *Pinus radiata* pulp fibres. *Pseudomonas* spp. are one of the most common colonisers of non-healing wounds, being found in up to 80% of chronic venous leg ulcers (Davies et al., 2004). The ability of the materials to support *P. aeruginosa* growth in suspension and on nanocellulose films was screened to determine whether these nanoscale materials possessed any distinct, size-dependent antimicrobial properties when compared to the commercial wound dressing, Aquacel®. The surface and optical properties of nanocellulose films were also assessed to determine its suitability in wound dressing applications.

2. Materials and methods

2.1. Nanocellulose materials

Bleached *P. radiata* pulp fibers that were elemental chlorine -free (ECF) kraft market pulp were selected for use in this study. Bleached pulp fibres were pre-treated with TEMPO-mediated oxidation according to Syverud et al. (2011a). The pulp fibres were homogenized with a Rannie 15 type 12.56 X homogenizer, operated at 1000 bar pressure, where the pulp

consistency during homogenizing was 0.5%. The oxidized fibrillated materials were collected after 1 and 2 passes through the homogenizer and labelled T01 and T02 respectively. The nanocellulose materials were then diluted in deionised water to 0.4% w/v before subsequent use. The fraction of residual fibres in the T01 and T02 nanocelluloses was quantified with a FiberMaster device, as described by Chinga-Carrasco et al. (2014). The typical diameter of similar cellulose nanofibrils from the same pulp-fibre raw material has been reported to be less than 20 nm (Chinga-Carrasco et al., 2011), with the ability to form low-porosity films with density higher than 1300 kg/m^3 (Chinga-Carrasco & Syverud, 2012). The carboxyl and aldehyde content have been quantified to be 855 and 71 μmol/g cellulose, respectively (Rees et al., 2015).

2.2. Screening for the ability to impair PAO1 growth

To screen for bacterial contamination, autoclaved nanocellulose suspensions were supplemented with Mueller Hinton broth (MHB) (50%, v/v), with phosphate buffered saline (PBS; 50%, v/v) being used as the control. The suspensions were incubated at 37^{o}C and the growth (optical density) monitored over 24 hours at 600 nm (OD_{600}) in a FLUOstar Optima plate reader (BMG LABTECH). The ability of nanocellulose to impair bacterial growth was also examined by inoculating identical suspensions with an overnight culture of *P. aeruginosa* PAO1 and again monitoring growth (optical density) over 24 hours as described above.

2.3. Surface characterization of nanocellulose films

Air-dried nanocellulose films (20 g/m^2) were produced from T01 and T02 autoclaved nanocellulose suspensions (0.4% w/v). The microstructural surface of the films was characterized by laser profilometry (LP). Ten LP topography images (1 mm × 1 mm) were acquired from the top and underside surfaces of each film sample, using a lateral and z-resolution of 1 μm and 10 nm, respectively. The surface images were bandpass-filtered to enable quantification of the surface topography. ImageJ software (National Institutes of Health) and the SurfCharJ plugin were used for image processing and analysis to achieve roughness values described by the root-mean square (RMS), as previously described (Chinga-Carrasco et al., 2014). Additionally, the optical properties of the films were assessed with a UV–vis spectrophotometer (Cary 300 Conc, Varian). The wavelengths between 200 and 800 nm were included for analysis. Three replicates were measured for each series. The nanostructure of the films was characterized using atomic force microscopy (AFM). AFM

imaging was performed using a Dimension 3100 AFM (Bruker) in tapping-mode operation in air to achieve image sizes of 50 μm^2 and image resolution of 1024 x 1024 pixels.

Solubility and sorption tests of the nanocellulose films were adapted from Parr & Rueggeberg (2002). Briefly, T01 and T02 nanocellulose films (2 cm^2) were first desiccated for 24 hours and weighed, before being placed into 5 ml of de-ionised water at 37^oC for 24 h. Excess water from the sample surface was then removed with absorbent paper and the nanocellulose samples re-weighed. Samples were placed into a desiccator again for 24 hours and before being weighed again. Water sorption and solubility were then calculated as previously described Parr & Rueggeberg (2002).

2.4. Characterization of bacterial growth on air-dried nanocellulose films

The ability of T01 and T02 air-dried nanocellulose films to impair biofilm growth was compared to growth on a commercial wound dressing, Aquacel® (ConvaTec, USA). Aquacel® is a clinical hydrofiber wound dressing made from sodium carboxymethylcellulose. T01 and T02 films (2 cm^2) and Aquacel® (2 cm^2) were placed individually within a six-well plate with MHB and inoculated with an overnight culture of *P. aeruginosa* PAO1 and incubated at 37^oC for 24 h. For AFM imaging, the biofilms were gently rinsed twice to remove planktonic bacteria and allowed to air dry before imaging. A Dimension 3100 AFM (Bruker) was used to achieve AFM images, using tapping-mode operation in air and a scan speed of approximately 1 Hz. For confocal laser scanning microscopy (CLSM) imaging, the films were gently rinsed once after 24 or 48 hours growth, stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen, Paisley, UK) containing SYTO 9 dye and propidium iodide and set in Vectashield (Vector Laboratories, UK), prior to being imaged under an Olympus Fluoview FV1000 CLSM. For SEM imaging, the supernatant was removed at 24 and 48 hours and each well immersed in 2.5% glutaraldehyde and then washed thoroughly with distilled water. One ml of distilled water was added to each well and biofilms frozen (at -20^oC). Once frozen, the well plates were then freeze-dried for 24 hours. The films were imaged at 1 kV using an Hitachi S4800 SEM.

2.5. Cell Viability from Nanocellulose surfaces

T01 and T02 nanocellulose films, a glass slide and Aquacel®, each 2 cm^2 in size, were incubated at 37^oC in MHB after inoculation with an overnight culture of *P. aeruginosa* PAO1. After 24 hours growth, films were placed into 5 ml PBS to remove loosely adherent bacteria. This wash step was then repeated. Films were then vortexed in fresh PBS for 5

mins to remove attached bacteria and the suspension used to prepare triplicate serial dilutions and drop counts (3 x 20 µl per dilution) on dried blood agar plates and incubated overnight at 37^{o}C, before counting colonies forming units (cfu).

2.6 Statistics

The level of significant differences in this study were determined using the Mann-Whitney U test, where a *p* value of <0.05 was considered significant.

3. Results and discussion

These studies utilized growth curve assays, cell viability assays, CLSM, SEM and AFM techniques to determine whether these nanocellulose materials possessed antimicrobial properties for wound dressing applications. The nanocellulose suspensions used were first pre-treated with TEMPO-mediated oxidation, which led to region-specific oxidation of the C6-position, introducing carboxyl groups and small numbers of aldehyde groups (Saito et al., 2006). The oxidation of the pulp facilitates the fibrillation of the cellulose fibers in the homogenization process, leading to a high yield of cellulose nanofibrils (Chinga-Carrasco et al., 2011; Fukuzumi, Saito & Isogai, 2013). The effect of these highly-fibrillated nanocellulose materials on the growth of *P. aeruginosa* will be assessed in the following sections.

3.1. Growth of Pseudomonas aeruginosa *PAO1 in nanocellulose suspensions*

Optical density measurements of autoclaved T01 and T02 nanocellulose suspension supplemented with MHB revealed no bacterial growth (data not shown) and hence that no inherent bacterial contamination was present in these samples. In contrast, growth curves of nanocellulose suspensions inoculated with PAO1 showed that they appeared to support the growth of this strain over 24 hours (Fig. 1), with the greatest bacterial biomass observed in the T02 nanocellulose suspension material (366 ± 107 OD_{600} at 24h) and slightly less seen in the T01 suspension (244 ± 20 OD_{600} at 24h). However, compared to the inoculated PBS and MHB controls, growth of *P. aeruginosa* PAO1 in the presence of the nanocellulose suspensions was greatly reduced. In addition, the un-inoculated PBS- and MHB-only controls showed no change in optical density over 24 h, remaining at 0 for each time point (data not shown). Therefore, the initial growth curve assays demonstrated that the

nanocellulose materials did not appear to promote bacterial growth, (compared to both inoculated controls where growth was strong due to the added MHB); reassuringly confirming that *P. aeruginosa* PAO1 does not use the nanocellulose as a carbon source. In fact, it was apparent that the nanocellulose materials actually inhibited growth as similar growth curves to that of the inoculated PBS control would otherwise have been expected. The extent of this inhibition varied with the extent of fibrillation of the material being tested. The less fibrillated material T01 contained a larger fraction of residual fibres (3.5 ± 0.2%) due to only one homogenization pass (see also Fig. 2), imposing a slightly greater inhibitory effect on planktonic PAO1 growth than the more fibrillated material T02 which contained less residual fibres (1.3 ± 0.1%) and thus a larger fraction of nanofibrils due to two homogenization passes (see also Chinga-Carrasco et al., 2014). As the number of homogenization cycles appears to have a direct effect on planktonic bacterial growth, this may have possible implications for the future development of these materials in medical applications.

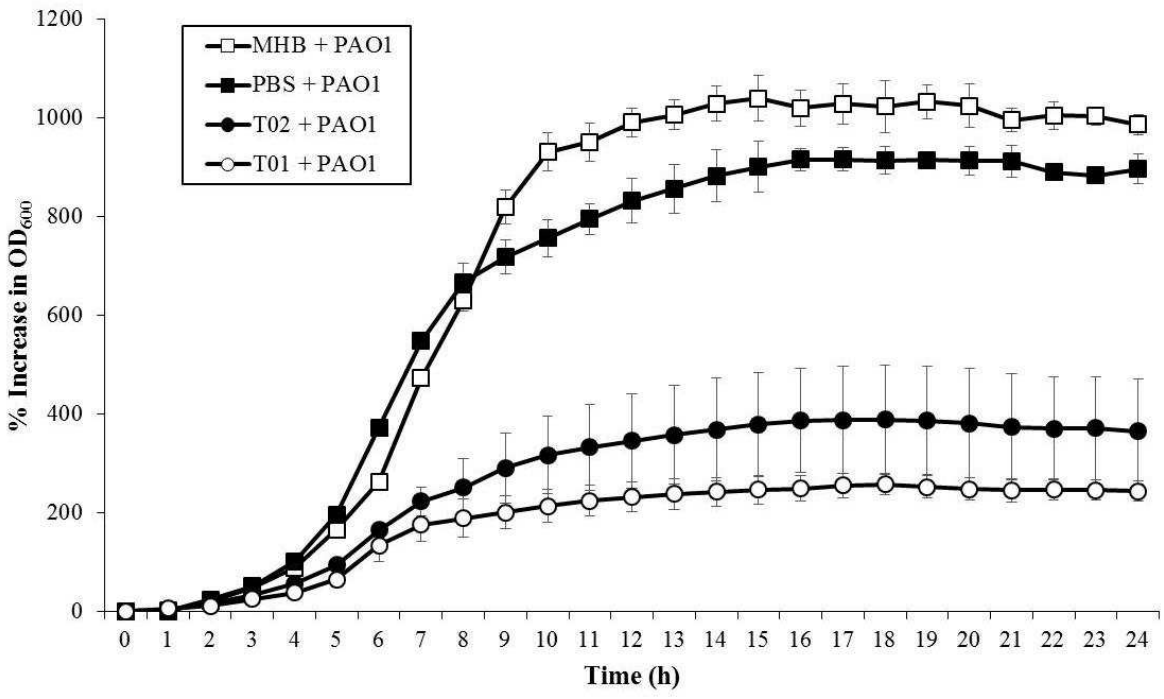

Fig. 1. Optical density (OD_{600}) of 24 hour *Pseudomonas aeruginosa* PAO1 growth in nanocellulose suspensions (T01 and T02). MHB, Mueller-Hinton Broth; PBS, phosphate buffered saline.

3.2. Characterization of the structural properties of nanocellulose films

To assess nanocellulose as a potential wound dressing material, films were prepared by air-drying and these rudimentary films were tested for relevant physical properties. The surface roughness of the films, assessed at the micrometre scale with LP (Fig. 2), revealed that the T02 film was significantly smoother than the T01 (0.53 ± 0.04 μm vs, 1.25 ± 0.1 μm respectively; $p<0.05$). This result was confirmed (but not significantly so) by the difference also observed in the surface roughness measurements obtained from the AFM images (273 ± 128 nm vs. 392 ± 146 nm, respectively; $p=0.069$) (Fig. 3). Hence, the surface roughness quantification revealed the relatively smooth surfaces for both materials, with the roughness assessed at several wavelengths indicative of a high degree of fibrillation of the material (Chinga-Carrasco & Syverud, 2014).

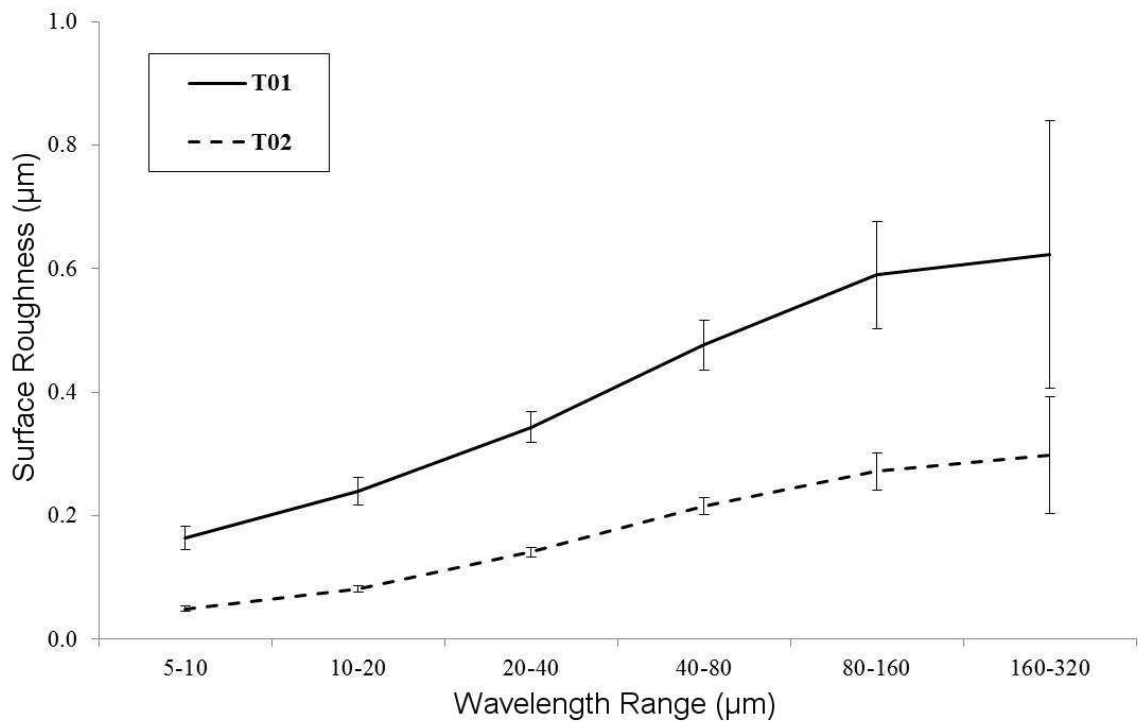

Fig. 2. Laser profilometry (LP) analysis of nanocellulose films (T01 and T02). Mean values of n=10 determinations ± standard deviations.

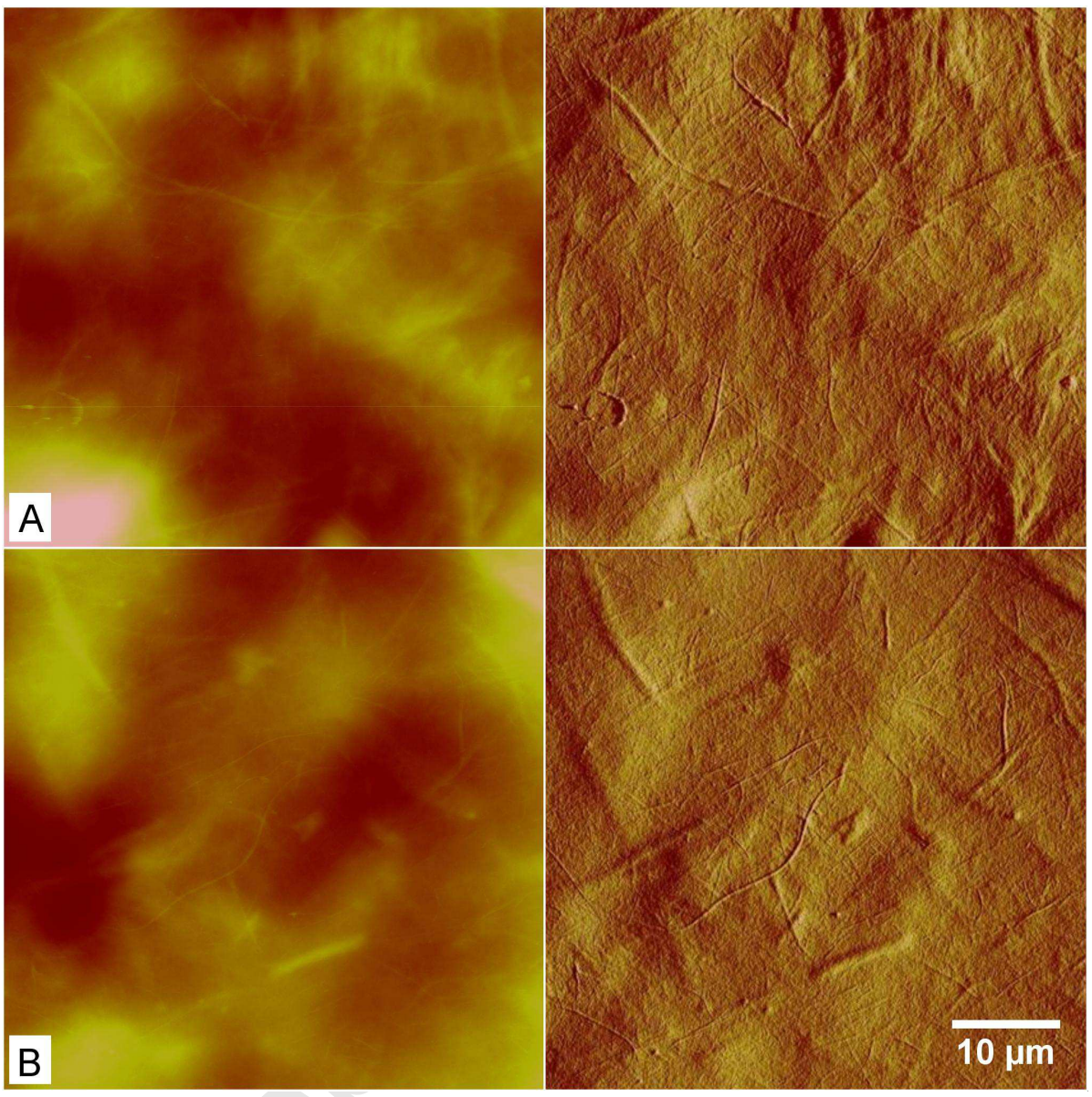

Fig. 3. Atomic force microscopy (AFM) imaging of nanocellulose films. AFM topographical (left) and amplitude (right) imaging of (A) T01 (Z-height = 2 µm) and (B) T02 (Z-height = 1.5 µm) respectively. (Scale bar is 10 µm).

Interestingly, both the T02 and T01 nanocellulose films revealed very high water sorption values (2232 ± 113 % vs. 2153 ± 22 % respectively) most likely due to their high surface area to volume ratio and highly absorbent nature. Solubility values of the T01 and T02 nanocellulose films were low (15.1 ± 3.2 % vs. 15.6 ± 1.8 % respectively). No significant differences were found between T01 and T02 films for either water sorption and solubility (p>0.05).

In addition, T01 and T02 nanocellulose films, characterized by UV vis spectrophotometry, were found to possess a high degree of light transmittance (91% at a wavelength of 650 nm; Fig. 4) confirming the high degree of fibrillation in the materials. T01 and T02 nanocellulose materials thus formed films with a high degree of light transmittance, due to the dense packing of the nanofibers, and fissures between the fibers (Nogi et al., 2009). High translucency of the films can be a great advantage for wound dressing applications, as the wound bed could potentially be monitored by visual inspection without the need for the removal of the dressing from the wound.

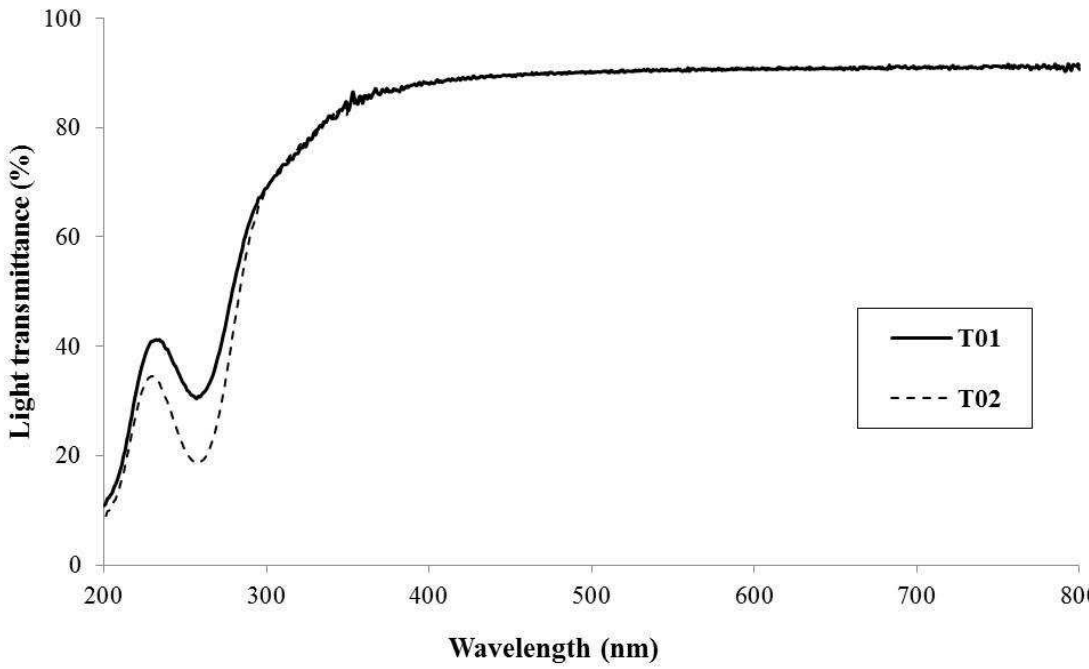

Fig. 4. Light transmittance using UV Vis spectrophotometry of air-dried T01 and T02 nanocellulose films (n=3).

The TEMPO-mediated oxidation modified the surface of cellulose nanofibrils, introducing both carboxyl (855 μmol/g) and aldehyde groups (71 μmol/g) (Rees et al., 2014). The occurrence of aldehyde groups was confirmed by the ‘‘shoulders’’ at bands between 200 and 300 nm (Fukuzumi et al., 2009). The T02 sample had a more pronounced decrease of light transmittance at the 200-300 nm band, probably indicative of a larger number of exposed aldehyde groups due to the high nanofibrillation of this material (see also Chinga-Carrasco, 2013).

These carboxyl and aldehyde groups could be used to add functionality to the material (Chinga-Carrasco and Syverud, 2014). This ability to modify nanocellulose materials to covalently bond biomodulatory agents (e.g. growth factors or quaternary ammonium compounds), suggests that it may be possible to combine the physical properties of nanocellulose films (i.e. high surface area to volume ratio and small pore size) with antimicrobials (Andresen et al., 2007). Such modification of nanocellulose materials with antimicrobials is however, not necessarily beneficial; surface-modified nanocellulose with quaternary ammonium compounds having previously been shown to be cytotoxic (Alexandresku et al., 2013).

3.3. Bacterial biofilm growth on dried nanocellulose films

Within chronic wounds, bacteria exist as biofilms, tightly adhering to material and host-tissue surfaces, rather than as “free-floating” (planktonic) bacteria (Bjarnsholt et al., 2008; Hill et al., 2010). It was, therefore, important, to determine the ability of the nanocellulose materials to support biofilm growth. In these experiments, we studied the physical interaction of the bacteria and subsequent biofilm formation with the film surface using a variety of imaging techniques and quantified biofilm cell viability on the film surfaces in comparison to the commercially-available dressing Aquacel®. Whilst, CLSM and SEM have been employed to examine bacterial and *Candida* growth on wound dressings (Newman et al., 2006; Tran et al., 2009; Anghel et al., 2013), AFM has attracted relatively little attention (Oh et al., 2009; Wright et al., 2010).

CLSM imaging with LIVE/DEAD® staining demonstrated less bacterial growth on the T01 and T02 nanocellulose materials and more cell death when compared to Aquacel®, for both 24 and 48 hours growth (Fig. 5). Decreased PAO1 biofilm growth was thus evident on the nanocellulose materials. The observed biofilm clusters corresponded with SEM imaging, which revealed that PAO1 adherence and growth resulted in distinct clusters of bacterial cells on the nanocellulose materials when compared to Aquacel®. In addition, the biofilm clusters on both films appeared to be considerably more developed at 48 hours than 24 hours (Fig. 6).

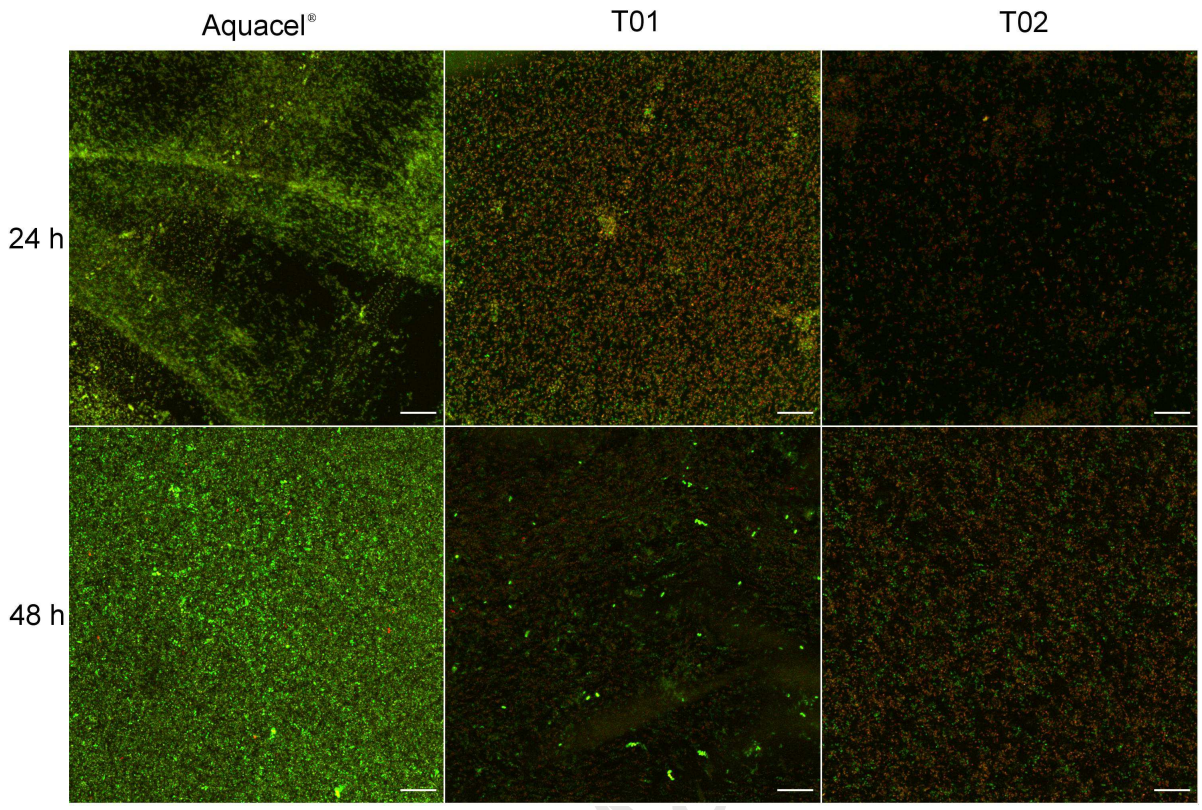

Fig. 5. Confocal laser scanning microscopy (CLSM) imaging of 24 and 48 hour *Pseudomonas aeruginosa* PAO1 growth on T01 and T02 nanocellulose films and Aquacel® using LIVE/DEAD® staining, showing live (green) and non-vital cells (red). (Scale bar = 20 µm).

Aquacel® itself demonstrated greater bacterial coverage seen throughout the material for both 24 and 48 hours compared to either of the nancellulose materials (Fig. 6). A previous study revealed that the bacterial population in contact with a Hydrofiber® (Aquacel®) dressing could maintain viability for at least 21 hours (Newman et al., 2006). Comparing the nanocellulose materials to this non-antimicrobial, commercially used, wound dressing, showed distinct differences, with the nanocellulose appearing to possess inherent anti-microbial properties.

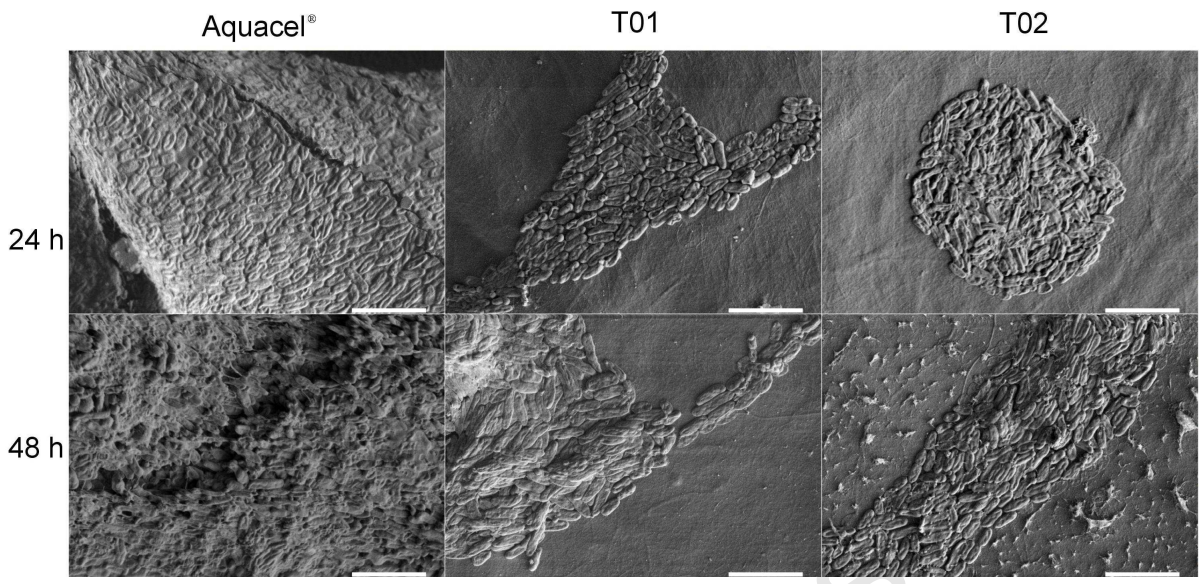

Fig. 6. Scanning electron microscopy imaging of 24 and 48 hour *Pseudomonas aeruginosa* PAO1 growth on T01 and T02 nanocellulose film surfaces and within the lower, more fibrous Aquacel® dressing layer. (Scale bar = 5 µm).

Under conditions of hydration, Aquacel® forms a cohesive gelled surface where the material under the surface remains more fibrous and less gel-like, giving two distinctly different environments for bacterial growth. This smooth amorphous gel surface can make it difficult to discern bacterial cells or fibres using SEM imaging (Walker et al., 2003). In contrast, bacterial cells growing under this gelled surface (in the more fibrous dressing material) can be more readily visualised with SEM imaging, but only through cracks in the Aquacel® surface, as also demonstrated in this study.

In contrast to SEM, AFM is able to more fully map the surface topography of a material, giving a 3-dimensional image of higher magnification. Therefore, AFM was employed to give an enhanced view of the surface structure of the smooth Aquacel® gel sample and the T01 and T02 materials (**Fig. 7**). Interestingly, the PAO1 cells exhibited a very different morphology in the biofilm on the Aquacel® surface, in that the cells were significantly smaller in length (1.02 ± 0.18 vs. 1.61 ± 0.37 µm; $p<0.05$) and had a less wrinkled appearance compared to those on the nanocellulose materials. The gelled surface of Aquacel® may offer an altered micro-environment for bacterial growth, not present in the novel nanocellulose materials, which could have resulted in these observed differences. Furthermore, the cells on the Aquacel® appeared less ‘stressed’ compared to those growing on the T01 and T02 materials, perhaps due to nutritional stress on the latter, which is known

to induce cell filamentation (elongation). In contrast, SEM imaging of the bacterial cells below the gelled surface (in the more fibrous Aquacel® dressing material) did not exhibit this altered morphology when compared to the nanocellulose films.

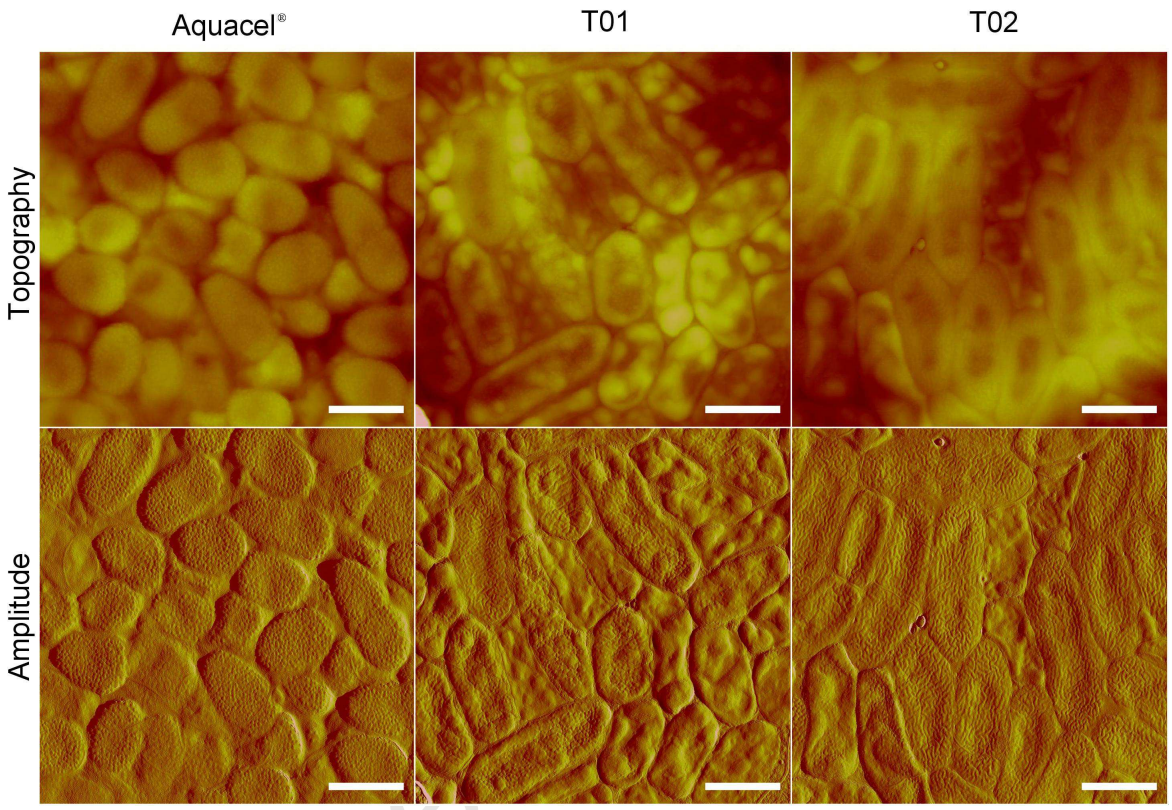

Fig. 7. Atomic force microscopy imaging of 24 hour *Pseudomonas aeruginosa* PAO1 growth on the T01 and T02 nanocellulose film surfaces and Aquacel® dressing surface. (Scale bar = 1 µm).

Cell viability studies showed no significant difference in cell numbers (CFU/mL) between either the T01 (1.7×10^8), T02 (1.2×10^8) or Aquacel® (2.5×10^8) surfaces, whilst the glass slide control showed significantly less bacterial attachment (2×10^6; $p<0.05$). The similar cell viability between the materials was possibly due to the previously described gelling properties of Aquacel® (Walker et al., 2003). Even though CLSM confirmed increased and indeed confluent biofilm growth on the Aquacel®, this was not reflected in the viability counts. Aquacel® has been shown to encapsulate populations of pathogenic bacteria under its gelled surface, providing an ideal environment to immobilise them (Walker et al.,

2003) and the vortexing used in this study was clearly insufficient to release all the bacterial cells from the dressing material for the viability assay.

Although in suspension, T01 showed better bacterial growth impairment than T02, interestingly, no difference in biofilm growth was evident between the films with CLSM, AFM and SEM. The two experiments assessed different modes of bacterial growth; planktonic versus biofilm, and on different media (liquid and solid) thereby accounting for the differences seen. When the nanocellulose is in film form, other properties become important for bacterial attachment and growth, such as surface roughness, which is irrelevant in suspension.

4. Conclusions

The results of this study highlight the potential usefulness of nanocellulose, from a renewable source. They demonstrate that whilst these bio-degradable materials were not utilised as a carbon-source to support bacterial growth of *P. aeruginosa*, they instead are able to effectively inhibit bacterial growth. The ability to control the physical material properties e.g. tensile strength, surface roughness and translucency, as well as the ability to covalently link therapeutic compounds to their surface, has many applications in wound healing and surgery.

Conflict of interest

There are no conflicts of interest to report.

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