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Research Paper

Ion-crosslinked wood-derived nanocellulose hydrogels with tunable antibacterial properties: Candidate materials for advanced wound care applications

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ABSTRACT

Development of advanced dressings with antimicrobial properties for the treatment of infected wounds is an important approach in the fight against evolution of antibiotic resistant bacterial strains. Herein, the effects of ion-crosslinked nanocellulose hydrogels on bacteria commonly found in infected wounds were investigated *in vitro*. By using divalent calcium or copper ions as crosslinking agents, different antibacterial properties against the bacterial strains *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were obtained. Calcium crosslinked hydrogels were found to retard *S. epidermidis* growth (up to 266% increase in lag time, 36% increase in doubling time) and inhibited *P. aeruginosa* biofilm formation, while copper crosslinked hydrogels prevented *S. epidermidis* growth and were bacteriostatic towards *P. aeruginosa* (49% increase in lag time, 78% increase in doubling time). The wound dressing candidates furthermore displayed barrier properties towards both *S. epidermidis* and *P. aeruginosa*, hence making them interesting for further development of advanced wound dressings with tunable antibacterial properties.

1. Introduction

Since the first modern antibiotic penicillin was discovered in 1928, hasted and unnecessary systematic administration of antibiotics has led to a more frequent occurrence of antibiotic resistant strains (Neu, 1992). In order to slow down this development, new alternative therapies are necessary (Stadler & Dersch, 2016). Proposed strategies include targeted therapy enabled by means of nanotechnology and biotechnology, as well as better preventative measures like vaccines and new diagnostics (Beyth, Houri-Haddad, Domb, Khan, & Hazan, 2015; Stadler & Dersch, 2016).

The skin is often described as a protective barrier against invading pathogens (Bowler, Duerden, & Armstrong, 2001). An interruption of the skin in the form of a wound exposes the host to local infection that may develop into a chronic wound with severe health complications, especially in diabetic or immunocompromised patients where antibiotic treatment will be necessary to resolve the infection. Targeted therapy becomes an interesting approach towards decreasing the excessive use of antibiotics when treating local infections (Freire-Moran et al., 2011). In the field of wound care, one strategy could be to develop advanced wound dressings with broad spectrum antimicrobial properties (Beyth et al., 2015; Santos et al., 2016).

All wounds contain some degree of bacterial contamination and thus any wound is at risk of becoming infected (Bowler et al., 2001). While a key function of wound dressings is to maintain a warm and moist environment for optimal healing, such an environment further increases the risk of infection (Bowler et al., 2001; Handfield-Jones et al., 1988). The consideration of antibacterial properties during wound dressing development is thus of outmost importance. At the same time, requirements on antibacterial wound dressings are stringent as they should exhibit a rapid inhibitory effect against bacterium growth while remain non-toxic and biocompatible towards host tissue (Vowden, Vowden, & Carville, 2011). Bacterial barrier properties of wound dressings are also important to reduce the risk of infection by invading pathogens and the spreading of infections between patients (Boateng, Matthews, Stevens, & Eccleston, 2008; Sood, Granick, & Tomaselli, 2014). Some commercially available antibacterial wound dressings incorporate silver or iodine in their matrix and provide a continuous and sustained release of these antibacterial ions at the wound bed (Abdelrahman & Newton, 2011). Other dressings incorporate drugs, *e.g.* polyhexamethyl biguanide, which interferes with the bacteria cell metabolism (Ovington, 2007).

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Bacterial cellulose has been extensively investigated for various biomedical applications (Petersen & Gatenholm, 2011; Picheth et al., 2017; Ul-Islam, Khan, Ullah, & Park, 2015). In recent years, another member of the nanocellulose family, wood-derived nanofibrillated cellulose (NFC), has emerged as an interesting material for the development of new biomedical applications within the fields of tissue engineering, wound healing and drug delivery (Jorfi & Foster, 2015; Lin & Dufresne, 2014). Typically, the nanofibrils are obtained through chemical and/or mechanical treatment of cellulose. Individual fibrils have a diameter of 3–5 nm and a length of several micrometers, forming 20–50 nm thick aggregates (Lavoine, Desloges, Dufresne, & Bras, 2012). This abundant biopolymer possesses typical nanomaterial characteristics like high surface area and high aspect ratio, as well as tunable physical properties that make it possible to obtain NFC in different forms, *i.e.* hydrogels, films or aerogels (Dufresne, 2013; Jorfi & Foster, 2015; Lin & Dufresne, 2014).

Hydrogels, best described as fibrous networks of crosslinked polymers that contain large amounts of water, have proven to be excellent candidates for wound care applications due to their ability to aid the natural wound healing process (Ahmed, 2015; Fernandes et al., 2013). In a previous study, Dong, Snyder, Williams, and Andzelm (2013) demonstrated the possibility to create self-standing hydrogels from negatively charged NFC by ion crosslinking. In addition, we have previously demonstrated the well-suited properties of ion-crosslinked NFC-based hydrogels for wound care applications in terms of cytocompatibility, hemocompatibility and physicochemical properties (Basu, Hong, & Ferraz, 2017; Basu, Lindh, Ålander, Strømme, & Ferraz, 2017). To continue the characterization of the wound dressing candidate materials, in the present work we investigate the effect of the ion-crosslinked wood-derived nanocellulose hydrogels on bacteria commonly found in infected wounds. To the best of our knowledge, the effect of ion-crosslinked NFC hydrogels on bacteria that typically colonize wounds has not yet been investigated.

This work specifically addresses the material-bacteria interactions of ion-crosslinked NFC-hydrogels in terms of (i) antibacterial properties and (ii) the bacterial permeability through the materials, selecting *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* as model bacteria. We expect the ion-crosslinked NFC hydrogels to display bacterial barrier properties due to their tight porous structure. Moreover, we anticipate the possibility to tune the antibacterial effects of the materials by the choice of crosslinking ion. To investigate this hypothesis, both divalent calcium and copper ions are used to crosslink the negatively charged NFC.

2. Materials and methods

2.1. Chemicals and reagents

Unmodified biocide-free NFC produced from never-dried bleached sulfite softwood dissolving pulp (Trade name: Dissolving Plus, Domsjö fabriker AB, Sweden) was provided by RISE Bioeconomy (Sweden). It was prepared as described by Pääkko et al. (2007). All chemicals and reagents were obtained from Sigma-Aldrich (USA) unless otherwise stated.

2.2. Preparation of NFC hydrogels

Anionic NFC (net charge of -35 ± 7 mV, carboxyl group content of 1550 μmol/g) was obtained through 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO)-mediated oxidation (TEMPO, NaBr, NaClO, pH 10.3) of unmodified NFC as previously described by Basu, Lindh et al. (2017). The resulting anionic NFC dispersion was purified by dialysis and concentrated to 3% (w/w) by water evaporation. By addition of 100 mM (final concentration) of calcium nitrate or copper chloride to 3% (w/w) NFC dispersion in petri dishes and incubating at room temperature for 12 h, calcium- or copper-ion-crosslinked NFC hydrogels of 1 mm thickness were obtained (henceforth carrying the notations Ca-NFC hydrogel and Cu-NFC hydrogel, respectively). Removal of unbound Ca^{2+} and Cu^{2+} was done by washing the hydrogels with deionized water. The hydrogels were sterilized by UV irradiation before use.

2.3. Bacteria interaction studies

S. epidermidis Xen43 (modified with the plasmid pXen-5 containing the bacterial luminescent system LuxABCDE) (Caliper Life Sciences Inc., USA) and *P. aeruginosa* 39324 (American Type Culture Collection, USA) were used as model strains. For all experiments, overnight cultures (12 h, in mid-log phase) grown in tryptic soy broth (TSB) medium at 37 °C were used. Prior to inoculation of the experiments, bacteria concentration of the overnight cultures was determined by measuring the optical density at 600 nm (OD_{600}). Serial dilutions of each bacterium were plated in Luria-Bertani (LB) agar and cultured for 12 h at 37 °C, colony forming units (CFUs) were counted and an OD_{600} of 1 was determined to correlate to approximately 1×10^9 CFU/ml.

2.3.1. In vitro bacterial growth

S. epidermidis and *P. aeruginosa* growth in the presence of the NFC hydrogels were examined in large- and small-volume systems to obtain information concerning the bacteria's planktonic and sessile growth.

The large-volume system consisted of conical centrifuge tubes containing 10 ml of TSB and 950 mg of crushed NFC hydrogel. The tubes were inoculated with 4×10^6 CFU/ml of *S. epidermidis* and *P. aeruginosa* in their respective set-up and incubated in a heated orbital shaker (37 °C, 350 rpm) for 15 h. Every hour an aliquot of 100 μl was drawn from the tube and the bacteria concentration was determined by measurement of OD_{600} (Tecan Infinite M200 spectrofluorometer, Switzerland).

The small-volume systems were set up using 96-well plates (white for *S. epidermidis* and black for *P. aeruginosa*) with NFC hydrogel discs covering the bottom of the wells. TSB (50 μl) inoculated with 4×10^6 CFU/ml of bacteria was added onto the hydrogels. The plates were incubated in a humidified atmosphere at 37 °C for 14 h and 16 h for *S. epidermidis* and *P. aeruginosa*, respectively. Growth of the luminescent *S. epidermidis* was determined by hourly luminescence measurements using a microplate reader (Hidex Plate Chameleon, Finland). To quantify *P. aeruginosa* growth, 10 μg/ml of resazurin was added to the TSB prior to inoculation and the metabolic activity of the bacteria was measured hourly by fluorescence read outs using 590 nm emission and 550 nm excitation wavelengths (Tecan Infinite M200 spectrofluorometer, Switzerland). Bacterial growth on the Cu-NFC hydrogel could not be determined by fluorescence measurements due to interference.

In all set ups, bacterial growth in the absence of NFC hydrogels was the negative control and the commercial antibacterial dressing Aquacel® Ag+ Extra (ConvaTec, UK) was used as the positive control. Each sample and control were run in quadruplicate and each experiment was performed at least 5 times.

From the resulting growth curves, the lag phase (*i.e.* the interval of time after inoculation where the bacteria adapt to the new environment) and doubling time of the bacteria were obtained to assess bacterial growth. The length of the lag phase was determined graphically by applying a cut-off level at 2% of the highest bacteria concentration of the negative control (defined as the beginning of the exponential growth phase). The doubling time was determined by dividing the logarithm of 2 by the slope of the linear portion of the log OD_{600} versus time plot (*i.e.* the exponential phase) (Monod, 1949).

2.3.2. Sessile growth pattern and bacterial permeability of the hydrogels

NFC hydrogel discs were placed to cover the bottom of a 48-well plate onto which 150 μl of TSB inoculated with 4×10^6 CFU/ml of *S. epidermidis* or *P. aeruginosa* were added. The samples were incubated in a humidified atmosphere at 37 °C for 16 h and thereafter the culture