Nanocellulose-based antifungal nanocomposites against the polymorphic fungus *Candida albicans*

Carla Vilela a,*, Helena Oliveira b, Adelaide Almeida b, Armando J. D. Silvestre a, Carmen S. R. Freire a

a CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

b Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

Corresponding author: C. Vilela (cvilela@ua.pt)

Graphical abstract

Highlights

- Poly([2-(methacyroyloxy)ethyl]trimethylammonium chloride)/nanocellulose composites.
- Cationic nanocomposites with UV–blocking properties and high water-uptake capacity.
BNC enhanced the thermal, viscoelastic and mechanical properties of the nanocomposites.

Bioactive nanocomposites capable of inactivating the polymorphic fungus *Candida albicans*.

*C. albicans* inactivation of 4.4– and 5.5–log CFU reduction after 24 and 48 hours.

**Abstract**

The design of functional materials capable of fighting fungal infections is of paramount importance given the intricate problem of multidrug-resistant pathogenic fungi. Herein, nanocomposites consisting of cross-linked poly(2–(methacryloyloxy)ethyl)trimethylammonium chloride) (PMETAC) and bacterial nanocellulose (BNC) were prepared, characterized and tested towards the polymorphic fungus *Candida albicans*. The BNC three-dimensional network enabled the *in-situ* polymerization of the non-toxic and bioactive quaternary-ammonium monomer, which originated transparent nanocomposites containing 10 and 40 wt.% of cross-linked PMETAC. Furthermore, the nanocomposites exhibit UV–A and UV–B blocking properties, high water-uptake capacity, thermal stability up to 200 °C, good viscoelastic (storage modulus > 1.7 GPa) and mechanical (Young’s modulus ≥ 2.4 GPa) properties and are non-cytotoxic to human keratinocytes (HaCaT cells). The fungal inactivation reached a 4.4±0.14–log CFU reduction for the nanocomposite containing only 10 wt.% of cross-linked PMETAC. Hence, these bioactive and non-cytotoxic materials can constitute potentially effective systems for the treatment of *C. albicans* infections.
Keywords: antifungal activity; bacterial nanocellulose; Candida albicans; cutaneous treatment; in-situ polymerization; poly([2-(methacryloyloxy)ethyl]trimethylammonium chloride)

1. Introduction

Fungal infections caused by Candida albicans are a major health problem since this microorganism has the potential to cause life-threatening localized and systemic infections (Dadar et al., 2018). C. albicans is a polymorphic fungus that, despite being part of the normal human microbiome as a harmless commensal, is also an opportunistic pathogen that can cause simple superficial skin and mucosal infections, or escalate into systemic infections depending on the host vulnerability, such as in the case of immunocompromised or immunologically deficient individuals (Dadar et al., 2018; Mayer, Wilson, & Hube, 2013). This major pervasive pathogenic fungus forms architecturally complex biofilms on a variety of host tissues (e.g., skin, mucosal and bed nails), teeth, dentures, catheters, and other biomedical implants (Kühbacher, Burger-Kentischer, & Rupp, 2017; Sheppard & Howell, 2016; Soll & Daniels, 2016), and the extracellular polysaccharide matrix is responsible for their resistance mechanisms (Tsui, Kong, & Jabra-Rizk, 2016). In this sense, the need to engineer antifungal materials that can avoid or reduce the growth of fungus is a research topic worth tackling (Zida, Bamba, Yacouba, Ouedraogo-Traore, & Guiguemdé, 2017).

Naturally occurring polymers, such as polysaccharides and proteins (Silva et al., 2014; Vilela, Pinto, et al., 2018), are becoming important substrates to develop multifunctional materials for antimicrobial applications (Wang & Vermerris, 2016). While some of the biopolymers are natural biocides such as chitosan (Vilela, Pinto, Coelho, et al., 2017) and lysozyme nanofibers (Silva, Vilela, Almeida, Marrucho, &
Freire, 2018), others like cellulose (the most abundant biopolymer on earth) must be combined with bioactive compounds in order to acquire such features (Li et al., 2018). In fact, cellulose is starting to make headway into antimicrobial materials particularly in its nanoscale forms, viz. bacterial nanocellulose (BNC), cellulose nanofibrils (CNFs) and cellulose nanocrystals (CNCs) (Li et al., 2018). The former nanometric form, i.e. BNC, albeit the absence of biocide properties, presents several bonus characteristics for the development of bioactive materials, namely in-situ moldability, shape retention (A. R. P. Figueiredo, Vilela, Neto, Silvestre, & Freire, 2015; Vilela, Pinto, Figueiredo, et al., 2017), three-dimensional morphology control across several length scales (Greca, Lehtonen, Tardy, Guo, & Rojas, 2018), and ability to house different (macro)molecules within its ultrafine nanofibrous network (Cielecka et al., 2018; Faria, Vilela, Mohammadkazemi, et al., 2019; Padrão et al., 2016; Subtaweesin et al., 2018). One of the easiest and versatile approach to fabricate BNC derived nanomaterials is based on the in-situ polymerization of functional monomers within the BNC porous network, as reviewed by Ray and Sain (Ray & Sain, 2016). This originates materials with properties that the exopolysaccharide does not have by itself, such as stimuli-responsiveness (Saïdi, Vilela, Oliveira, Silvestre, & Freire, 2017), ionic conductivity (Gadim et al., 2014; Vilela, Martins, et al., 2018), amphoterism (Faria, Vilela, Silvestre, et al., 2019), or antibacterial activity against for example Escherichia coli (Figueiredo, Figueiredo, et al., 2015).

[2-(Methacryloyloxy)ethyl]trimethylammonium chloride (METAC) is a functional monomer with a tremendous potential to be used in association with BNC. In fact, the easily polymerizable methacrylic functional group of METAC (Morales, Rivas, & González, 2016; Morales, Rivas, & Escalona, 2016) that leads to the formation of cationic water-soluble polymers and hydrogels (Nurkeeva et al., 2006) together with its
non-toxicity, mucoadhesive properties (Fefelova, Nurkeeva, Mun, & Khutoryanskiy, 2007) and antifungal activity against *C. albicans* (Stopiglia et al., 2012) are the major assets for selecting this monomer to design antifungal materials (Muñoz-Bonilla & Fernández-García, 2018). For example, Stopiglia *et al.* highlighted that METAC monomer exhibits antifungal activity against *C. albicans*, as well as other *Candida* species, with a minimum inhibitory concentration (MIC) of 50 mg mL\(^{-1}\) and a minimum fungicidal concentration (MFC) higher than 100 mg mL\(^{-1}\) (Stopiglia et al., 2012). According to these authors, METAC can be copolymerized with other methacrylic monomers to fabricate denture acrylic resins or denture temporary soft lining materials, and the presence of this fungal inhibitor will avoid denture-related stomatitis mostly caused by *C. albicans* (Stopiglia et al., 2012). In a different study, De Prijck *et al.* demonstrated that grafting PMEATC onto the surface of a voice prothesis prevented *C. albicans* biofilms formation which is a major cause of voice prosthesis deterioration in laryngectomized patients (De Prijck, De Smet, Coenye, Schacht, & Nelis, 2010).

Albeit the association between BNC and poly([2-[(methacyrloyloxy)ethyl] trimethylammonium chloride] (PMETAC) was already studied in the context of polyelectrolytic membranes with ionic conductivity for application in fuel cells (Vilela, Sousa, et al., 2017), the behaviour of PMETAC/BNC nanocomposites as antifungal materials against the polymorphic *C. albicans* has not been investigated yet. In fact, the inherent purity, biodegradability, biocompatibility and high water-holding capacity of BNC (Gama, Dourado, & Bielecki, 2017), and the antifungal activity of PMETAC against *C. albicans* (Stopiglia et al., 2012), is an interesting partnership to engineer materials capable of inactivating this opportunistic polymorphic fungus. In this perspective, the present study aims at developing nanocomposite materials consisting of cross-linked PMETAC and BNC via the *in-situ* free radical polymerization of the
bioactive quaternary ammonium monomer within the BNC porous structure. The systematic characterization of the PMETAC/BNC nanocomposites with different PMETAC and BNC contents was performed in terms of structure, morphology, optical properties, water-uptake capacity, thermal stability, mechanical and viscoelastic properties, antifungal activity against *C. albicans* and cytotoxicity against human keratinocytes (HaCaT cells).
2. Experimental

2.1. Chemicals, materials and microorganisms

[2–(Methacryloyloxy)ethyl] trimethylammonium chloride solution (METAC, 75 wt.% in H2O, Aldrich), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 97%, Aldrich) and N,N′-methylenebis(acrylamide) (MBA, 99%, Sigma-Aldrich) were used as received. Other chemicals and solvents were of laboratory grade. Bacterial nanocellulose (BNC) wet pellicles were biosynthesized in our research laboratory using the *Gluconacetobacter sacchari* bacterial strain (Trovatti, Serafim, Freire, Silvestre, & Neto, 2011). *Candida albicans* (SC5314) was provided by DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures).

The nontumorigenic immortalized human keratinocyte HaCaT cell line was obtained from Cell Lines Services (Eppelheim, Germany), phosphate buffer saline (PBS, pH 7.4), Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin and fungizone were supplied by Gibco® (Life Technologies, Carlsbad, CA, USA), and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, 98%) was purchased from Sigma-Aldrich.

2.2. PMETAC/BNC nanocomposites

Wet BNC pellicles (diameter: *ca.* 7 cm) with 40% water content were placed in stoppered glass-reactors and purged with nitrogen. Simultaneously, aqueous solutions of METAC (1:1.5 and 1:5 BNC/METAC weight fraction), AAPH (1%, w/w relative to monomer) and MBA (5%, w/w relative to monomer) were prepared and transferred to the glass-reactors containing the drained BNC pellicles. After the complete incorporation of the corresponding solution during 1 h in ice, the reaction mixtures were heated in an oil bath at 70 °C during 6 h. Afterwards, the nanocomposites were
repeatedly washed with water, oven dried at 40 °C for 24 h, and kept in a desiccator until further use. All experiments were made in triplicate and samples of cross-linked PMETAC were also prepared in the absence of BNC.

2.3. Characterization methods

Thickness: a hand-held digital micrometer (Mitutoyo Corporation, Japan) with an accuracy of 0.001 mm was used to measure the thickness of the materials. All measurements were randomly performed at different sites of the samples.

Elemental analysis: the elemental composition was determined by elemental analysis using a LECO TruSpec 630-200-200 CHNS elemental analyser (LECO Corporation, USA) with a combustion furnace and afterburner temperatures of 1075 °C and 850 °C, respectively. All samples (ca. 5 mg) were analysed in triplicate.

Attenuated total reflection-Fourier transform Infrared (ATR–FTIR): ATR-FTIR spectra were recorded with a Perkin-Elmer FT–IR System Spectrum BX spectrophotometer (Perkin-Elmer Inc., USA) equipped with a single horizontal Golden Gate ATR cell, over the range of 600–4000 cm$^{-1}$ at a resolution of 4 cm$^{-1}$ over 32 scans.

Solid-state carbon cross-polarization/magic-angle-spinning nuclear magnetic resonance ($^{13}$C CP/MAS NMR): $^{13}$C CP/MAS NMR spectra were collected on a Bruker Avance III 400 spectrometer (Bruker Corporation, USA) operating at a B0 field of 9.4 T using 9 kHz MAS with proton 90° pulse of 3 µs, time between scans of 3 s, and a contact time of 2000 µs. $^{13}$C chemical shifts were referenced to glycine (C=O at δ 176 ppm).

X-ray diffraction (XRD): XRD was performed on a Phillips X'pert MPD diffractometer (PANalytical, Netherlands) using Cu Kα radiation (λ = 1.541 Å) with a
scan rate of $0.05^\circ \text{s}^{-1}$. The XRD patterns were collected in reflection mode with the samples placed on a Si wafer (negligible background signal) for mechanical support and thus avoid sample bending.

Scanning electron microscopy (SEM): SEM images of the surface and cross-section of the samples were obtained by a HR-FESEM SU–70 Hitachi microscope (Hitachi High-Technologies Corporation, Japan) operating at 4 kV. The samples were previously coated with a carbon film.

Ultraviolet-visible spectroscopy (UV–vis): the transmittance spectra of the samples were acquired with a Shimadzu UV-1800 UV-Vis spectrophotometer (Shimadzu Corp., Japan) equipped with a quartz window plate with 10 mm diameter, bearing the holder in the vertical position. Spectra were recorded at room temperature in steps of 1 nm in the range 250–700 nm.

Water-uptake capacity ($WU$): the $WU$ was determined via immersion of dry specimens with 10×10 mm$^2$ in distilled water at room temperature (RT) during 120 h. After removing the specimens out of the water, the wet surfaces were dried in filter paper, and the wet weight ($W_w$) was measured. The $WU$ is calculated by the equation:

$$WU(\%) = \left[\frac{(W_w - W_0)}{W_0}\right]$$

where $W_0$ is the initial weight of the dry sample. At the end of the immersion tests in water (120 h), the nanocomposite specimens were oven dried at 40 °C during 24 h in a vacuum drying oven (Thermo Fisher Scientific, USA) and their dry weight measured to determine the leaching of cross-linked PMETAC. All samples were analysed in triplicate.

Thermogravimetric analysis (TGA): TGA was carried out with a SETSYS Setaram TGA analyser (SETARAM Instrumentation, France) equipped with a platinum cell. The samples were heated from RT to 800 °C at a constant rate of 10 °C min$^{-1}$ under a nitrogen atmosphere (200 mL min$^{-1}$).
Tensile tests: tensile tests were performed on a uniaxial Instron 5564 testing machine (Instron Corporation, USA) in the traction mode at a cross-head velocity of 10 mm min\(^{-1}\) using a 500 N static load cell. The specimens were rectangular strips (50×10 mm\(^2\)) previously dried at 40 °C and equilibrated at RT in a 50% relative humidity (RH) atmosphere prior to testing. All measurements were performed on five replicates.

Dynamic mechanical analysis (DMA): DMA curves of rectangular specimens with 30×5 mm\(^2\) were obtained on a Tritec 2000 DMA (Triton Technologies Ltd., UK) operating in tension mode (single strain) at 1 Hz and with 0.005 mm displacement. The temperature was sweep from −30 to 180 °C with a constant heating rate of 2 °C min\(^{-1}\). Before the measurements, the samples were kept in a conditioning cabinet at 30 °C and 50% relative humidity (RH) for 48 h.

2.4. *In vitro* antifungal activity

The antifungal activity of the nanocomposites was tested against *C. albicans* (SC5314). All fungal pre-inoculum cultures were grown overnight at 37 °C in 50 mL Yeast Glucose Chloramphenicol (YGC: 20 g L\(^{-1}\) D-glucose, 5 g L\(^{-1}\) yeast extract and 0.1 g L\(^{-1}\) chloramphenicol; Liofilchem®, Italy) broth, under shaking of 100 rpm.

For all nanocomposites, 50 mg of each material was placed in contact with 10 mL of a liquid yeast suspension, prepared by ten-fold diluting the overnight grown culture in YGC. Control yeast cell suspension and control cellulose samples were also run in each antifungal test. All samples were incubated at 37 °C. At time 0 and after 12, 24, 36 and 48 h of incubation, aliquots (500 µL) of each sample and controls were collected and the yeast cell concentration (CFU mL\(^{-1}\)) was determined by plating serial dilutions on YGC Agar (YGC: 20 g L\(^{-1}\) D-glucose, 5 g L\(^{-1}\) yeast extract, 0.1 g L\(^{-1}\)
chloramphenicol and 18 g L\(^{-1}\) agar; Liofilchem®, Italy). Three independent experiments were carried out.

Statistical analysis was performed using GraphPad Software (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA, USA). The existence of significant differences among the different samples tested was assessed by one-way ANOVA. For each situation, the significance of differences was evaluated by comparing the results obtained in the test samples with the result obtained for the corresponding control samples (difference between the concentration obtained in the control and the concentration obtained in the test sample), as well as the differences obtained between samples, for the different times of each of the three independent assays. A value of \(p<0.05\) was considered significant.

2.5. In vitro cytotoxicity assays

The cytotoxicity of the nanocomposites was evaluated in human keratinocyte cell line (HaCaT cells) by using the MTT assay (Mosmann, 1983). Briefly, cells were grown in complete DMEM supplemented with 10% FBS, 2 mM L-glutamine, 10000 U mL\(^{-1}\) penicillin/streptomycin and 250 µg mL\(^{-1}\) fungizone at 37 °C in 5% CO\(_2\) humidified atmosphere. Cells were daily observed under an inverted phase-contrast Eclipse TS100 microscope (Nikon, Tokyo, Japan). The tests were performed in triplicates of pure BNC and nanocomposites PMETAC/BNC_1 and PMETAC/BNC_2. As a negative control, HaCaT cells were treated identically as described for the samples and no positive control was tested. Samples of 1×1 cm\(^2\) were prepared, rinsed with ultra-pure water, sterilized in ethanol for 1 h, rinsed several times with sterilize PBS to completely remove the ethanol, and then incubated with 1.5 mL of complete DMEM medium at 37 °C, with 5% CO\(_2\) for 24 h to prepare the extract.
In the meantime, one 96 wells plate was prepared with 5×4 wells filled up with 6000 cells/well and the cells were then incubated in complete culture medium during 24 h for adhesion. After that time, cell culture medium (in the 96 well plates) was replaced by 100 μL of each of the extracts obtained from the incubated samples and cells were then further incubated for 24 h.

At the end of the incubation time, 50 μL of MTT (at a concentration of 1 g L⁻¹) were added to each well and incubated for 4 h at 37 °C in 5% CO₂ humidified atmosphere. After that, culture medium with MTT was removed and replaced by 150 μL of dimethyl sulfoxide (DMSO) and the plate was placed in a shaker for 2 h in the dark to completely dissolve the formazan crystals. The absorbance of the samples was measured with a BioTek Synergy HT plate reader (Synergy HT Multi-Mode, BioTeK, Winooski, VT) at 570 nm with blank corrections. Data was analysed by a two-way ANOVA followed by a Holm-Šídák test to determine the significance between the two nanocomposites.

3. Results and discussion

The exopolysaccharide BNC can house all kinds of molecules from bioactive compounds (e.g. curcumin (Subtaweesin et al., 2018)) to monomers and polymers (e.g., lactoferrin (Padrão et al., 2016) and κ-carrageenan (Cielecka et al., 2018)) with the purpose of developing nanocomposite and hybrid materials with distinct properties. Therefore, the *in-situ* free radical polymerization of the cationic METAC within the swollen BNC network was used to prepare two PMETAC/BNC nanocomposites. The reaction scheme of the METAC polymerization in the presence of MBA as cross-linker and using water as solvent, is outlined in Fig. 1A. The MBA cross-linker (Shiga et al., 2018) was used with aim of retaining the bioactive quaternary ammonium-based
polymer inside the BNC porous structure, since the non-cross-linked amphiphilic PMETAC is water soluble (Fefelova et al., 2007; Nurkeeva et al., 2006), and would leach out upon contact with aqueous media (e.g., during the washing steps of the nanocomposite preparation), as indeed shown for other water soluble polymers (Figueiredo, Figueiredo, et al., 2015).

**Fig. 1.** (A) Radical polymerization of METAC in the presence of MBA as cross-linker, and (B) photographs showing the visual aspect of a dry BNC, and PMETAC/BNC_1 and PMETAC/BNC_2 nanocomposites placed in the arm.

The thickness of the prepared nanocomposites increased from 45±10 µm for the pristine BNC to 61±10 µm and 118±15 µm for PMETAC/BNC_1 and PMPC/BNC_2, respectively (Table 1). The visual examination of the nanocomposites shows no evident heterogeneities on the bottom and top surfaces, demonstrating a good dispersion of the cross-linked PMETAC inside the BNC porous network, as shown in Fig. 1B. Moreover, the two nanocomposites are more transparent than the white BNC (Fig. 1B, see section 3.2 for further details) and readily absorb moisture from the surrounding due to the hygroscopic nature of PMETAC (Goel et al., 2009). These two features are important in
the context of skin regeneration, namely for visual control of the wound healing process and absorption of exudates, respectively.

The nanocomposites present compositions of 60 or 90 wt.% for BNC (\(W_{\text{BNC}}/W_{\text{total}}\)), and 40 or 10 wt.% for PMETAC (\(W_{\text{PMETAC}}/W_{\text{total}}\)), which translates into materials containing 209±9 or 570±12 mg of PMETAC per cm\(^3\) of nanocomposite, respectively, as summarized in Table 1. This was further corroborated by elemental analysis with the following compositions: (i) C: 41.4±0.5%, H: 6.7±0.3%, O: 49.2±0.9% and N: 2.7±0.1% for PMETAC/BNC\(_1\), and (ii) C: 41.9±0.2%, H: 6.6±0.1%, O: 46.8±0.1% and N: 4.7±0.3% for PMETAC/BNC\(_2\), where, as expected, the latter nanocomposite presents a higher nitrogen content.

Table 1 Weight composition and thickness of the PMETAC/BNC nanocomposites.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Composition</th>
<th>Thickness /µm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(W_{\text{BNC}}/W_{\text{total}})</td>
<td>(W_{\text{PMETAC}}/W_{\text{total}})</td>
</tr>
<tr>
<td>BNC</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PMETAC/BNC(_1)</td>
<td>0.90±0.2</td>
<td>0.10±0.2</td>
</tr>
<tr>
<td>PMETAC/BNC(_2)</td>
<td>0.60±0.1</td>
<td>0.40±0.1</td>
</tr>
</tbody>
</table>

\(^{a}\) The composition was calculated by considering the weight of the nanocomposite (\(W_{\text{total}}\)), BNC (\(W_{\text{BNC}}\)) and PMETAC (\(W_{\text{PMETAC}}\)), and also the volume of the nanocomposite (\(V_{\text{total}}\)); all values are the mean of at least three replicates with the respective standard deviations.

The two PMETAC/BNC nanocomposites (and pure BNC and cross-linked PMETAC for comparison purposes) were characterized regarding their structure (ATR–FTIR, solid-state \(^{13}\)C CP/MAS NMR, XRD), morphology (SEM), optical properties...
(UV–vis), water-uptake capacity, thermal stability (TGA), mechanical performance (tensile tests), viscoelastic properties (DMA), antifungal activity (against \textit{C. albicans}) and cytotoxicity (towards HaCaT cells), as discussed in the following sections.

3.1. Structure and morphology

The structural characterization of the two nanocomposites and the corresponding individual components was performed by ATR–FTIR, \textsuperscript{13}C CP/MAS NMR and XRD, which allowed to confirm the effective inclusion of PMETAC into the BNC three-dimensional porous network. The ATR–FTIR spectra of both PMETAC/BNC nanocomposites (Fig. 2A) combine the absorption bands of both the cross-linked PMETAC and BNC (Fig. S1) at about 3200–3400 cm\(^{-1}\) (O–H stretching), 2900 cm\(^{-1}\) (C–H stretching), 1720 cm\(^{-1}\) (C=O stretching), 1480 cm\(^{-1}\) (N\(^+\)(CH\(_3\))\(_3\) bending), and 951 cm\(^{-1}\) (N\(^+\)(CH\(_3\))\(_3\) stretching) (Bellamy, 1975; Vilela, Sousa, et al., 2017). Moreover, the differences in the intensities of the bands allocated to the cross-linked PMETAC confirm the different contents of the bioactive quaternary ammonium-based polymer inside the BNC network (Table 1).

The solid-state \textsuperscript{13}C CP/MAS NMR spectra shown in Fig. 2B also validated the composition of the nanocomposites through the presence of the typical carbon resonances of PMETAC (Fig. S1) at \(\delta\) 19.0 ppm (CH\(_3\) of polymer backbone), 45.2 ppm (quaternary C of polymer backbone), 54.4 ppm (N\(^+\)(CH\(_3\))\(_3\) and CH\(_2\) of polymer backbone), 59.3 ppm (OCH\(_2\)), 65.0 ppm (CH\(_2\)N\(^+\)(CH\(_3\))\(_3\)) and 177.9 ppm (C=O), and of BNC (Fig. S1) at \(\delta\) 65.2 ppm (C6), 71.4–74.3 ppm (C2,3,5), 90.0 ppm (C4) and 104.8 ppm (C1) (Vilela, Sousa, et al., 2017). The different intensities of the carbon resonances assigned to the cross-linked PMETAC also corroborate the different contents of the PMETAC in the nanocomposites (Table 1).
The XRD patterns (Fig. S2) of the nanocomposites mostly preserved the semi-crystalline nature of BNC with the well-known sharp diffraction peaks at about 2\(\theta\) 14.3°, 15.9° and 22.6°, instead of the amorphous character of the cross-linked PMETAC (Qi et al., 2017).

![XRD Patterns](image)

**Fig. 2.** (A) ATR-FTIR (modes: \(\nu =\) stretching, \(\beta =\) bending) and (B) \(^{13}\)C CP/MAS NMR spectra of a) PMETAC/BNC_1 and b) PMETAC/BNC_2 nanocomposites.

Concerning the morphology, Fig. 3 displays the SEM micrographs of the surface and cross-section of the two PMETAC/BNC nanocomposites, where it is possible to observe the three-dimensional nanofibrillar network and lamellar microstructural
features of the BNC morphology. As expected, PMETAC/BNC_1 is the nanocomposite with the morphology that mostly resembles that of pure BNC because it contains 90 wt.% of BNC, i.e. only 209±9 mg of PMETAC per cm³ of nanocomposite (Table 1). On the other hand, the cross-section micrographs of PMETAC/BNC_2 with 60 wt.% of BNC show the filling of the lamellar spaces of the bacterial polysaccharide by the cross-linked cationic PMETAC with 570±12 mg of PMETAC per cm³ of nanocomposite. These micrographs demonstrate the anisotropic morphology of the nanocomposites due to the presence of BNC, along with the homogeneous distribution of the cross-linked PMETAC inside the BNC nanofibrous network.

**Fig. 3.** SEM micrographs of the surface and cross-section of the pure BNC and PMETAC/BNC nanocomposites.

### 3.2. Optical properties

The optical properties of the pristine BNC and PMETAC/BNC nanocomposites were assessed by measuring the transmittance in the range 250–700 nm. According to Fig. 4, the transmittance of pure BNC only increases in the visible range (400–700 nm) and attains a maximum of ca. 8.0% at 700 nm, which demonstrates a low transparency
in tune with its whitish colour (Fig. 1B). The inclusion of cross-linked PMETAC in the BNC porous network originated nanocomposites with higher transparency that increases over the entire visible range of wavelengths, reaching a maximum of about 33% for PMETAC/BNC_1 and 42% for PMETAC/BNC_2 at 700 nm. Furthermore, the transparency augment with the increase of PMETAC content is also observable by the visual examination of the nanocomposites, as illustrated in Fig. 1B. The rationale behind the higher transparency of the nanocomposites when compared to pure BNC might be explained by the overlapping of the optical properties of both the bioactive cross-linked PMETAC and the three-dimensional network assembly of the BNC nanofibers, as discussed elsewhere (Pinto et al., 2015).

![Graph](image)

**Fig. 4.** UV–visible transmission spectra of pure BNC and PMETAC/BNC nanocomposites.

Worth noting is also the fact that the nanocomposites, just like BNC, exhibit notable UV-absorbing properties in both UV–B (280–320 nm, short-wavelength radiation) and UV–A (320–400 nm, long-wavelength radiation) regions. The nanocomposite with the lower PMETAC content (PMETAC/BNC_1, 10 wt.%) presents a transmittance of about 1.0% at 280 nm, 2.5% at 320 nm and 7.4% at 400 nm, whereas the nanocomposite with the higher PMEATC content (PMETAC/BNC_2, 40 wt.%)

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shows a transmittance of ca. 1.9% at 280 nm, 4.6% at 320 nm and 12.5% at 400 nm. These UV-blocking properties are quite interesting for cutaneous infected wounds that worsen from exposure to UV radiation (UV-induced damage) either from sunlight or artificial light sources (Gupta, Avci, Dai, Huang, & Hamblin, 2013).

3.3. Water-uptake capacity

The water-uptake (WU) is an important property to assess the performance of the PMETAC/BNC nanocomposites. In fact, the WU of the biocompatible BNC is a relevant characteristic of this exopolysaccharide that allows it to absorb exudates when adhered to the skin surface (Silvestre, Freire, & Neto, 2014). According to Fig. 5, BNC absorbed water during the experiments with a WU pattern consisting of two different regions: in the first hours the absorption was fast (WU = 62.5±7% after 3 h) and then an equilibrium plateau was steadily reached (WU = 100±9% after 48 h).

The two nanocomposites absorbed water with similar profiles composed of two different regions but with distinct values. While PMETAC/BNC_1 containing 209 mg PMETAC per cm³ of nanocomposite shows a WU of 225±19% after 48 h of immersion in water, the nanocomposite with higher content of cross-linked PMETAC (40 wt.%) exhibited the higher WU value 873±56% (Fig. 5, p<0.05). As expected, these high WU values come with the increase of nanocomposite dimensions. This behaviour is in accordance with the very hygroscopic nature of tetraalkylammonium salts (Goel et al., 2009). Analogous values were reported in literature (Vilela, Sousa, et al., 2017), including for a METAC–grafted cotton fabric (~18% grafting) that reached a WU value of ca. 500% compared to the WU of ca. 150% of the pure cotton cellulose fibres present in the fabric (Goel et al., 2009). The fact that the PMETAC/BNC nanocomposites
absorb considerable amounts of water is relevant in the context of infections that originate exudates such as pus-like or clear fluids.

The samples from the WU tests were also used to determine the possible leaching of cross-linked PMETAC from the nanocomposites. After 120 h of immersion in water, the nanocomposites specimens were oven dried and the ensuing weights demonstrated a 0.5% weight-loss. Therefore, any polymer loss should be at a maximum of 0.5%, which clearly indicates that the leaching of the cross-linked PMETAC from the BNC network is minimal.

![Graph showing water-uptake as a function of time for BNC and nanocomposites PMETAC/BNC_1 and PMETAC/BNC_2](image)

**Fig. 5.** Water-uptake as a function of time (0, 1, 3, 7, 20, 48 and 120 h) for BNC and nanocomposites PMETAC/BNC_1 and PMETAC/BNC_2 when immersed in water at room temperature; the values are the mean of three replicates and the error bars correspond to the standard deviations, and the lines are for visual guidance.

3.4. Thermal stability

The thermal degradation profile of the nanocomposites was evaluated by TGA analysis in order to get a deeper insight into their thermal stability. The TGA trace of the cross-linked PMETAC indicates a weight-loss degradation profile via two consecutive stages with maximum decomposition temperatures at 279 °C ($T_{d_{max1}}$) and
407 °C ($T_{d\text{max}2}$, Table 2), due to the decomposition of quaternary ammonium groups that commonly takes place through Hofmann elimination reactions (Isik et al., 2013; Qi et al., 2017; Vilela, Sousa, et al., 2017). In turn, the trace of BNC exhibits a single-step degradation profile with a maximum decomposition temperature at 349 °C ($T_{d\text{max}}$, Table 2) associated with the pyrolysis of the cellulose skeleton (Wang, Liu, Luo, Wen, & Cen, 2007).

Regarding the nanocomposites, the TGA analysis revealed a similar profile between the two nanocomposites regardless of the amount of cross-linked PMETAC incorporated into the BNC network. The two-step degradation profile of the nanocomposites, apart from the dehydration at ca. 100 °C, is attributed firstly ($T_{d\text{max}1}$) to the degradation of pendant quaternary ammonium groups on the polymer backbone and the cellulose skeleton, and secondly ($T_{d\text{max}2}$) to the degradation of the polymer backbone (Goel et al., 2009). A final residue of around 16% was obtained at temperatures up to 800 °C for both nanocomposites (Table 2), which corresponds to a total weight-loss value of 83–84%. Worth noting is the fact that a thermal stability up to 200 °C qualifies these nanocomposites for sterilization under typical procedures at ca. 150 °C (e.g. autoclaving), which is an essential requirement for most biomedical applications.

**Table 2** TGA data of the cross-linked PMETAC, pure BNC and PMETAC/BNC nanocomposites.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_{d\text{i}}/°\text{C}$</th>
<th>$T_{d\text{max}1}/°\text{C}$</th>
<th>$T_{d\text{max}2}/°\text{C}$</th>
<th>Final residue/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMETAC</td>
<td>200±0.5</td>
<td>279±0.6</td>
<td>407±0.9</td>
<td>4.5±1.2</td>
</tr>
<tr>
<td>BNC</td>
<td>265±0.7</td>
<td>349±0.8</td>
<td>–</td>
<td>13.1±0.5</td>
</tr>
<tr>
<td>PMETAC/BNC_1</td>
<td>210±0.5</td>
<td>266±0.5</td>
<td>380±0.5</td>
<td>16.6±0.2</td>
</tr>
<tr>
<td>PMETAC/BNC_2</td>
<td>210±0.4</td>
<td>260±0.5</td>
<td>395±0.4</td>
<td>16.1±0.6</td>
</tr>
</tbody>
</table>
3.5. Mechanical and viscoelastic properties

The mechanical performance of the PMETAC/BNC nanocomposites was determined from the corresponding stress-strain curves at room temperature. These properties were not evaluated for the cross-linked PMETAC owing to the lack of film-forming aptitude of this polymer. According to Fig. 6A, the Young’s modulus, tensile strength and elongation at break obtained for the nanocomposites increased with the growing content of BNC (60 and 90 wt.%), because of the exceptional mechanical properties of this bacterial polysaccharide, namely the Young’s modulus of 14.8±0.5 GPa, tensile strength of 221±4 MPa, and elongation at break of 2.4±0.2%, compatible with values reported in literature (Cacicedo et al., 2016). In fact, the Young’s modulus of the nanocomposites increased from 2.4±0.4 GPa for PMETAC/BNC_2 (60 wt.% BNC) to 9.7±0.5 GPa for PMETAC/BNC_1 (90 wt.% BNC), and the same tendency is patent on the tensile strength that increases from 37±5 MPa for PMETAC/BNC_2 to 187±8 MPa for PMETAC/BNC_1 (Fig. 6A, p<0.05). Hence, the augment from 209±9 mg of PMETAC to 570±12 mg of PMETAC per cm³ of nanocomposite points out the dependence of the nanocomposites’ mechanical performance on the amount of BNC. This behaviour is in line with previous studies dealing with other BNC-based nanocomposites (Figueiredo, Figueiredo, et al., 2015; Vilela, Gadim, Silvestre, Freire, & Figueiredo, 2016). Furthermore, the Young’s modulus and tensile strength of the PMETAC/BNC nanocomposites are superior to those described, for example, for the poly(lactic acid)/carvacrol electrospun membranes with antifungal activity against C. albicans (Scaffaro, Lopresti, D’Arrigo, Marino, & Nostro, 2018).

In the case of the elongation at break, the behaviour is different because the incorporation of 10 or 40 wt.% of cross-linked PMETAC into the BNC nanofibrous network did not have a significant effect on this parameter (Fig. 6A), given that the
means difference is not significant ($\alpha=0.05$). These results reveal that, despite the lower Young’s modulus and tensile strength values, the nanocomposites are still stiff with small values of elongation at break (PMETAC/BNC_1: $3.0\pm0.9\%$ and PMETAC/BNC_2: $2.5\pm0.8\%$) just like BNC ($2.4\pm0.2\%$). From the mechanical point of view, both nanocomposites are sufficiently pliable and workable (see Fig. 6B) for cutaneous applications dealing with the adhesion to irregular skin surfaces.

![Graph](image_url)

**Fig. 6.** (A) Young’s modulus, tensile strength and elongation at break of pure BNC and PMETAC/BNC nanocomposites; the values are the mean of five replicates and the error bars correspond to the standard deviations; the asterisk (*) denotes statistically significant differences to the pure BNC ($p < 0.05$) and (B) photograph showing the bendable nanocomposite PMETAC/BNC_2.

The viscoelastic properties the PMETAC/BNC nanocomposites were measured by DMA and the storage modulus ($E'$) and loss factor (tan $\delta$) data is plotted in Fig. 7. Once again, the lack of film-forming aptitude of the cross-linked PMETAC hindered its analysis. Concerning the pristine BNC, the variation of $E'$ with temperature has virtually no oscillations, being fairly stable up to at least 180 °C, as discussed elsewhere (Gadim et al., 2014). In the case of the nanocomposites, the story is different with...
PMETAC/BNC_1 showing better viscoelastic properties than PMETAC/BNC_2 (Fig. 7) for the entire temperature range. Nanocomposite PMETAC/BNC_1 displays a reduction in $E'$ from 9.7 GPa at $-30 \, ^\circ\text{C}$ to 5.4 GPa at 88 °C, followed by a small increase to 6.3 GPa at 125 °C, and then another decrease to 4.9 GPa at 180 °C. PMETAC/BNC_2 presents a pattern with smaller values where $E'$ decreases from 3.9 GPa at $-30 \, ^\circ\text{C}$ to 1.7 GPa at 45 °C, then increases to 3.1 GPa at 140 °C and slightly decreases to 2.9 GPa at 180 °C. Even though the $E'$ drops to values of ca. 1.7 GPa at around 45 °C, the PMETAC/BNC_2 nanocomposite still preserves a satisfactory mechanical integrity endued by BNC, as already shown for other BNC-based nanocomposites (Gadim et al., 2014, 2016; Vilela et al., 2016). Furthermore, these results agree with the data from the mechanical tests outlined in Fig. 6A.

![Graph of PMETAC/BNC_1 and PMETAC/BNC_2 nanocomposites](image)

**Fig. 7.** Storage modulus and loss factor (tan $\delta$) curves of PMETAC/BNC_1 and PMETAC/BNC_2 nanocomposites.
The loss factor (tan δ) validates the temperature dependence of the storage modulus with PMEATC/BNC_1 showing no relevant features and PMETAC/BNC_2 presenting a broad peak in the range 0 to 90 °C, as displayed in Fig. 7. The absence of any variation in the loss factor of PMETAC/BNC_1 reflects the high content of BNC (i.e., 90 wt.%), whereas the broad tan δ peak with maximum at ca. 32 °C might relate to the plasticizing effect of water on the PMETAC/BNC_2 nanocomposite due to the hygroscopic nature of PMETAC.

3.6. In vitro antifungal activity

The presence of quaternary ammonium groups in the chemical structure of the cross-linked PMETAC is expected to convert the nanocomposites into potential antifungal agents. Fig. 8 presents the antifungal activity of PMETAC/BNC nanocomposites together with that of pure BNC for comparison purposes. The experimental control was produced via inoculation of the fungus in culture media in the absence of sample. As expected, BNC has no effect on the fungal viability, showing an analogous behaviour to the control sample since BNC is deprived of antifungal activity or any other antimicrobial activity. In fact, BNC does not inhibit the growth of many microorganisms (e.g., *Escherichia coli* (Figueiredo, Figueiredo, et al., 2015; Padrão et al., 2016; Shao et al., 2016), *Staphylococcus aureus* (Padrão et al., 2016; Shao et al., 2016), *Pseudomonas aeruginosa*, *Bacillus subtilis* and *C. albicans* (Shao et al., 2016)) and can even be used as a substrate for microbial cell culture (Yin et al., 2014).

In the case of the studied nanocomposites, both inhibited the growth of *C. albicans* with 48 h of contact time, but the concentration of cross-linked PMETAC is the major factor affecting the growth inhibition. PMETAC/BNC_1 reached a 3.4±0.07–log reduction after 24 h and a maximum of 4.4±0.14–log reduction after 48 h, whereas the
highest growth inhibition was observed for the nanocomposite PMETAC/BNC_2 containing the highest content of cross-linked PMETAC with about 4.6±0.13–log reduction after 24 h and 5.5±0.19–log reduction after 48 h of incubation (Fig. 8). Clearly, these nanocomposites can be termed antifungal since they exhibit a minimum 3–log CFU reduction that is indicative of a killing efficiency higher than 99.9%. Shiga et al. verified that PMETAC has a MIC of 0.37 mg mL\(^{-1}\) (Shiga et al., 2018), and the nanocomposite with the lower PMETAC content (PMETAC/BNC_1) presents fungicidal activity at a concentration of 0.5 mg mL\(^{-1}\). When compared with other systems, PMETAC/BNC nanocomposites present a comparable growth inhibition of fungal cells with, for example, the non-cytotoxic tissue conditioners based on poly(acryloyloxyethyltrimethyl ammonium chloride)–grafted chitosan (Lee et al., 2018).

![Fig. 8. Antifungal activity of BNC and nanocomposites PMETAC/BNC_1 and PMETAC/BNC_2 after 0, 24 and 48 h. Values represent the mean of three independent experiments; error bars represent the standard deviations; the asterisk (*) denotes statistically significant differences to the control and pure BNC (p < 0.05).](image-url)
3.7. *In vitro cytotoxicity assay*

The cytotoxicity of the PMETAC/BNC nanocomposites was assessed in HaCaT cells through the indirect MTT assay (Mosmann, 1983). The metabolic activity of the HaCaT cells was normal for the negative control, viz. 100% cell viability (Fig. 9). BNC was non-cytotoxic to HaCaT cells for the exposure time, as already reported in literature for the same cells (Saïdi et al., 2017) and other cell lines (Figueiredo et al., 2013). This was expected given the recognised biocompatibility of BNC as discussed elsewhere (Silvestre et al., 2014; Sulaeva, Henniges, Rosenau, & Potthast, 2015).

![Cell viability graph](image)

**Fig. 9.** Cell viability of HaCaT cells after exposure to negative control, BNC, PMETAC/BNC_1 and PMETAC/BNC_2 on the HaCaT cells for exposure time of 24 h. Values represent the mean of six replicates; error bars represent the standard deviation; the asterisk (*) denotes statistically significant differences to the control and pure BNC ($p<0.05$).

The HaCaT cells cultured with different extracts of PMETAC/BNC nanocomposites showed a PMETAC content-dependent cell viability (Fig. 9, $p<0.05$). In fact, the cell viability of PMETAC/BNC_1 that contains 10 wt.% of cross-linked PMETAC was reduced to 81.4±2.1%, whereas for PMETAC/BNC_2 with 40 wt.% of...
cross-linked PMETAC the cell viability decreased to 72.4±3.1%. Nevertheless, both nanocomposites are considered non-cytotoxic under these conditions since the cell viability was not reduced by more than 30% (ISO 10993-5:2009(E), 2009). These results agree with the non-cytotoxicity of PMETAC reported for BJ (CRL2522) (Mishra, Ramasamy, Ahmad, Eshak, & Majeed, 2014) and HeLa (Wang et al., 2018) cell lines.

The beauty of these PMETAC/BNC nanocomposites lies in the combination between the antifungal activity against *C. albicans* and their customizable properties depending on the amount of the individual components, namely the UV-blocking properties, water-uptake capacity, thermal stability, mechanical performance and non-cytotoxicity. Under these premises, we anticipate the application of these bioactive nanocomposites as potentially effective systems for the treatment of *C. albicans* cutaneous infections or even for application in medical devices requiring biocides to reduce fungal adhesion and biofilm formation such as catheters and prostheses.

### 4. Conclusions

The exploitation of the bioactive cationic poly([2–(methacryloyloxy)ethyl] trimethylammonium chloride) and the hydrophilic bacterial nanocellulose generated nanocomposites capable of inactivating the polymorphic fungus *C. albicans*. This one-pot strategy originated optically transparent PMETAC/BNC nanocomposites containing 209±9 and 570±12 mg of PMETAC per cm³ of nanocomposite. Besides, these cationic nanocomposites exhibit UV–A and UV–B blocking properties, high water-uptake capacity, thermal stability up to 200 °C, along with good viscoelastic properties (storage modulus > 1.7 GPa) and mechanical performance (Young’s modulus ≥ 2.4 GPa). The *in vitro* cytotoxicity assay showed that the nanocomposites are non-cytotoxic toward
HaCaT cells. Furthermore, their antifungal activity was demonstrated by the fungal inactivation of *C. albicans* that registered a 4.4±0.14–log reduction for the nanocomposite containing only 10 wt.% of PMETAC after 48 h. Hence, these customizable nanocomposites might show potential as bioactive systems to address the intricate issue of fungal infections, where antifungal materials play a key role in reducing/avoiding these types of ailments.

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