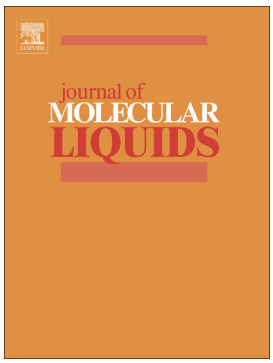

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Bacterial nanocellulose membranes loaded with vitamin B-based ionic liquids for dermal care applications

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Abbreviations:

IL	ionic liquid	B3	nicotinic acid	[Ch][B3]	cholinium nicotinate
BC	bacterial nanocellulose	B5	pantothenate	[Ch][B5]	cholinium pantothenate
Ch	cholinium	B6	pyridoxine	[Ch][B6]	cholinium pyridoxylate
VIT	vitamin B				

Abstract

The development of innovative bio-based skin care products has received an increase attention in the latter years. In the present work, the synthesis of original active principle ingredients based ionic liquids (ILs) with cholinium cation and vitamins B anions followed by their incorporation in bacterial nanocellulose (BC) membranes for topical applications is reported. Three ILs, namely cholinium nicotinate [Ch][B3], cholinium pantothenate [Ch][B5] and cholinium pyridoxylate [Ch][B6], were synthesized through a metathesis reaction and their structure characterized in detail. Thermal analysis confirmed their denomination as ILs and their high thermal resistance. The solubility of these ILs was higher than their vitamin precursors, especially in the case of [Ch][B3] whose solubility increased 30.6-fold enhancing the bioavailability of this vitamin. The incorporation of ILs in BC led to transparent and homogeneous membranes stable up to 190°C. ILs acted as plasticizers reducing BC brittleness that facilitated their application on irregular skin regions. Moreover, the re-hydration ability of BC-ILs membranes was improved 2.9 to 4.8-fold in comparison to BC, ensuring adequate hydration for ILs release, while the release of ILs in buffer solutions was more complete and faster than the release of vitamins. Finally, BC-ILs were proven not cytotoxic to skin epithelial cells and thus are suitable materials for skin care applications.

Keywords: bacterial nanocellulose, vitamin B, ionic liquids, cholinium-based ILs, skin care applications, antioxidant activity

1. Introduction

Vitamins are essential nutrients typically acquired through diet since they cannot be synthesized by human beings [1]. Among those nutrients, vitamins from complex B, namely

thiamine (B1), riboflavin (B2), niacin or nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotine (B8), folic acid (B9) and cobalamin (B12), are a class of vitamins acting as coenzymes in multiple enzymatic processes in cells. The main roles of vitamin B are in catabolic metabolism for energy production, and in anabolic metabolism for the transformation of specific bioactive molecules [2]. The use of formulations combining several vitamins from complex B to reach synergetic effects is common in food supplementation [3] and skin care [4,5].

In the last decades, vitamins from complex B have been mainly used in the pharmaceutical industry as supplements, but also by cosmeceutical companies for skin care because of their reported beneficial properties. For instance, vitamin B3 is used to prevent skin yellowing or hyperpigmentation, to increase collagen production and to reduce fine lines and wrinkles [6,7], vitamin B5 shows skin protection, *stratum corneum* hydration and anti-inflammatory properties [8], and vitamin B6 has demonstrated efficiency in the treatment of acne [9]. Additionally, B-complex vitamins formulations are recurrently used to prevent skin aging because of their antioxidant properties [10].

The design of drug-based ionic liquids (ILs) for tuned water-solubility and improved biological and/or therapeutic activities has recently been investigated for pharmaceutical and cosmeceutical applications [11–14]. ILs are organic salt-like compounds with a liquid domain below 100°C. They are promising compounds because of their high thermal stability, negligible vapor pressure and low flammability [11]. The cholinium cation, considered also part of the vitamin B group, has been largely investigated in the design of non-toxic and biocompatible ILs [15–18]. In 1962, the first cholinium-based IL, with the salicylate counter ion, showed improved water-solubility and preservation of anti-inflammatory properties in comparison to salicylic acid [19]. Accordingly, in the current work, the design of cholinium-based ILs containing anions

derived from vitamins of complex B is expected to lead to compounds with enhanced bioactive properties.

Oral administration of vitamins B is widely spread, even for skin care, but might have several side-effects. For instance, excessive ingestion of vitamin B3 can lead to liver damage [20,21], while vitamin B6 can block nociception in the brain [22]. These problems can be overcome by topical delivery, in some cases even with stronger effect applying the same dosage [22]. Three different systems can be developed for topical delivery of vitamins B, namely creams, gels and patches. Creams and gels offer the advantage of simple usage for consumers, but present the drawback of small control of the amount of the active principle applied. On the other hand, patches permit a controlled drug release and easy posology control. Typically, patches have three layers, *viz.* a drug-containing matrix, an outer membrane for easy handling and a rate controlling layer to slow down the diffusion of drugs [23–25]. An adhesive layer is also needed to guarantee the application onto the skin, but this layer tends to be replaced by matrices with inherent adhesiveness.

Bacterial nanocellulose (BC) is a nanofibrillar form of cellulose produced by several non-pathogenic bacteria of the *Gluconacetobacter*, *Sarcina* or *Agrobacterium* genus [26]. This biopolymer raised considerable interest in the pharmaceutical field, and particularly on drug delivery [27], due to its unique set of properties, such as nanofibrillar porous structure, high purity, high water-holding capacity, biocompatibility and high Young's modulus [26,28]. BC membranes have already been used to produce efficient topical drug delivery systems (TDDS) for the administration of different drugs or active molecules, namely lidocaine [29], ibuprofen [30], berberine hydrochloride [31] and also antioxidant-based ionic liquids [32], with the advantage of having a single layer structure, thus promoting a sustained release of drugs and absorption of exudates when applied in wounds.

Based on the exposed, the purpose of the current work was to synthesize novel vitamin B-based ILs, pairing the cholinium cation with anions derived from vitamins of complex B, namely cholinium nicotinate ([Ch][B3]), cholinium pantothenate ([Ch][B5]) and cholinium pyridoxylate ([Ch][B6]). The obtained ILs were characterized by nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared-attenuated total reflection spectroscopy (FTIR-ATR), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). Then, their solubility in phosphate buffer saline (PBS) aqueous solutions (pH 7.4, 0.1 M) was assessed, as well as their antioxidant properties. Finally, ILs were incorporated into BC membranes and the obtained ILs-incorporated membranes were characterized in terms of thermal and mechanical properties, and re-hydration ability to assess their suitability for skin care. The release of the ILs out of the BC membranes was investigated by dissolution tests in PBS aqueous solutions. Finally, the cytotoxicity of ILs-incorporated membranes towards skin epithelial cells was evaluated to confirm their potential for topical applications. Most of these properties were also determined for the original vitamins from complex B, for comparison purposes.

2. Materials & Methods

2.1. Materials

Cholinium bicarbonate (80% aqueous solution) was supplied by Sigma-Aldrich, nicotinic acid (99%, vitamin B3) and calcium pantothenate (pharmaceutical grade, vitamin B5) by Carlo Erba Reagents, and pyridoxine hydrochloride (99.8%, vitamin B6) by Fagron. For the preparation of the phosphate buffer saline (PBS), sodium chloride (99.5%, NaCl) was purchased from Acros Organics, and potassium chloride (99%, KCl), disodium phosphate dodecahydrate (98-102%, $Na_2HPO_4 \cdot 12H_2O$) and potassium phosphate (99%, KH_2PO_4) from Sigma-Aldrich. PBS (pH 7.4, 0.1 M) was prepared with 8.01 $g.L^{-1}$ NaCl, 0.20 $g.L^{-1}$ KCl, 2.39 $g.L^{-1}$ $Na_2HPO_4 \cdot 12H_2O$ and 0.27

$g.L^{-1}$ KH_2PO_4. All other chemicals were of analytical grade and used as received (Sigma-Aldrich). Vitamins and vitamin solutions were protected from light due to the light sensitivity of vitamin B6 [33].

The preparation of Dulbecco's Modified Eagle's Medium (DMEM) was made with 4.5 $g.L^{-1}$ glucose, L-glutamine without pyruvate (Lonza, Verviers, Belgium) containing 10% (v/v) fetal bovine serum (FBS, Biowest, Nuaillé, France) and 1% (v/v) Penicillin-Streptomycin-Fungizone (Lonza, Verviers, Belgium).

2.2. Synthesis of vitamin B-based ionic liquids

ILs comprising cholinium, as the common cation, and anions derived from vitamins of complex B ([Ch][VIT]) were synthesized by simple metathesis reactions adapted from the work of Sintra *et al.* [34]. Briefly, 1:1.05 molar ratio of cholinium bicarbonate (80 wt.% in water) and vitamins were mixed by slow addition of solid vitamins into cholinium bicarbonate solution under ambient conditions with continuous stirring. The reaction mixture was then kept at room temperature under inert atmosphere with continuous stirring for 24 h. After the synthesis, ILs were dried under vacuum at least for 72 h at 60 ºC (water content < 10.9 wt.%), collected and stored in closed vials in the dark at 4 °C up to use. Their water content was determined by coulometric Karl-Fischer titration ((Metrohm Ltd., model 831), being below 800 ppm for all ILs investigated. Three ILs have been synthesized, namely cholinium nicotinate ([Ch][B3]), cholinium pantothenate ([Ch][B5]) and cholinium pyridoxylate ([Ch][B6]). ^{1}H and ^{13}C NMR spectroscopy was used to address the ILs purity, which is > 98 wt.% for all ILs synthesized. ^{1}H and ^{13}C NMR spectra were acquired with a Bruker AMX 300 NMR equipment operating at 300.13 and 75.47 MHz, respectively. FTIR-ATR was also used to confirm the successful synthesis of ILs, as described below.

2.3. Bacterial cellulose production

BC membranes were prepared in our laboratory by incubating the bacterial strain *Gluconacetobacter sacchari* [35]. After 6 days of incubation, BC membranes were withdrawn from the culture medium, treated with a 0.5 M NaOH aqueous solution and repeatedly washed with water until neutral pH was reached. This purification procedure allows removing all culture medium and cells remaining in the BC membranes after incubation. Wet purified BC membranes were stored in distilled water at 4 °C until use.

2.4. Incorporation of vitamins and corresponding ILs in BC membranes

Wet BC membranes were cut into 2×2 cm^2 rectangular pieces (99.22 ± 0.27 wt.% water content). Membranes were pressed at room temperature until at least 50% of their water content was drained. Then, BC membranes were soaked in 1 mL of PBS aqueous solutions containing vitamins B3, B5 and B6 at a concentration of 10 $mg.mL^{-1}$, as control samples. For ILs, the concentration was adapted to incorporate the same mass of each vitamin B in the membranes, *i.e.* 18.4, 14.8 and 16.1 $mg.mL^{-1}$ for [Ch][B3], [Ch][B5] or [Ch][B6], respectively. After the complete absorption of the solutions by BC membranes, they were dried in a ventilated oven at 40 °C. Dried membranes were kept in a desiccator until use.

After, the sample holders used for the drying of membranes were rinsed with 4 mL of PBS aqueous solutions and the amount of the original vitamins or [Ch][VIT], not absorbed by the BC membranes, was determined by ultraviolet-visible (UV-Vis) spectroscopy or HPLC-DAD for vitamin B5 and [Ch][B5], as described below. By subtraction of the quantified amount of non-incorporated compounds, the mass of each vitamin and [Ch][VIT] incorporated in BC was determined.

For tensile tests, 7×7 cm² membranes were later prepared with adjusted vitamin- or [Ch][VIT]-solution volumes to keep the same vitamin/BC or [Ch][VIT]/BC ratio.

2.5. Characterization of vitamins, ILs and BC

2.5.1. Quantification of ILs and BC-ILs

UV-Vis spectroscopy, using a Thermo Scientific Evolution 600 spectrophotometer (Thermo Fisher Scientific, USA), was carried out to determine each vitamin and [Ch][VIT] concentration in aqueous PBS buffer solutions. The wavelengths of 262.5, 254.5 and 220 nm for vitamin B3/[Ch][B3], vitamin B6 and [Ch][B6] were used, respectively, using calibration curves previously established. Vitamin B5 and [Ch][B5] were quantified by HPLC-DAD, using an Ultimate 3000 chromatographer (Dionex, Germany) and a C18 column with a 5 µm pore dimension (Zorbax TMS 250×4.6 mm²). Mobile phase was acetonitrile:sodium phosphate pH 2.5 (10:90, v:v) solution, while the flow rate and injection volume were set at 1.0 $mL.min^{-1}$ and 10 µL, respectively. Quantifications and calibration curves were carried out at 198 nm.

2.5.2. Solubility assays

The solubility of vitamins and [Ch][VIT] ILs was assessed in PBS aqueous solutions at pH 7.4. An excess of each compound was added to the PBS aqueous solution and allowed to equilibrate at 25 °C under 750 rpm for 72 h. After equilibration, solutions were centrifuged during 8 min at 8000 rpm. The supernatant was recovered and centrifuged again for 8 min at 8000 rpm. Then, saturated solutions were diluted by successive 10-fold dilutions and their concentration was determined by UV-Vis spectroscopy or HPLC-DAD as described above. These experiments were performed at least in duplicate and the values expressed are an average of these results with the respective standard deviation.

2.5.3. Fourier transform infrared-attenuated total reflection (FTIR-ATR)

FTIR-ATR spectra of vitamins, [Ch][VIT] ILs and BC membranes loaded with [Ch][VIT] were obtained on a FT–IR System Spectrum BX spectrophotometer (Perkin Elmer, USA) equipped with a single horizontal Golden Gate ATR cell. Thirty-two scans were acquired in the 4000–600 cm^{-1} range with a resolution of 4 cm^{-1}. Spectra were recorded at 3 different spots of the membranes surface and averaged.

2.5.4. Thermogravimetric analysis (TGA)

Thermograms of vitamins, [Ch][VIT] ILs and BC membranes were conducted with a Setys evolution TGA analyzer (Setaram, France) equipped with a platinum cell. Samples were heated at a constant rate of 10 °C.min^{-1}, from room temperature up to 800 °C, under a nitrogen flow of 20 mL.min^{-1}. The thermal decomposition temperatures (T_{dmax}) were taken as the maximum of the derivative of TGA curves. The residue fraction at 800 °C was calculated in terms of weight percentage of the dry material.

2.5.5. Differential scanning calorimetry (DSC)

DSC was used to determine the glass transition (T_g) and melting (T_m) temperatures of [Ch][VIT] ILs. Analyses were carried out in a power compensation Pyris Diamond differential scanning calorimeter (Perkin Elmer, USA), using hermetically sealed aluminum crucibles with a constant flow of nitrogen (50 mL.min^{-1}). Samples of about 15 mg were used in each experiment. The temperature and heat flux scales of the power compensation DSC were calibrated by measuring the temperature and the enthalpy of fusion of reference materials, namely benzoic acid, 4-methoxybenzoic acid, triphenylene, naphthalene, anthracene, 1,3,5-triphenylbenzene,

diphenylacetic acid, perylene, *o*-terphenyl and 9,10-diphenylanthracene, at the scanning rate of 2 °C.min^{-1} and under a nitrogen flow. The T_g and T_m were taken as the onset temperatures.

2.5.6. Antioxidant activity

The antioxidant activity of vitamins and [Ch][VIT] ILs was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The principle of the assay is based on the color change of the DPPH solution from purple to yellow, as the radical is quenched by the antioxidant. Change in color was monitored by visible spectroscopy at 517 nm [32]. Briefly, 3.34 mL of a DPPH solution (1 mM) in methanol was mixed with 50 mL of a vitamin solution (10 mg) or a [Ch][VIT] solution (containing 10 mg of the vitamin IL anion) in methanol. A positive control (PC) was prepared with 10 mg of ascorbic acid and a negative control with 3.34 mL of DPPH solution (1 mM) in 50 mL of methanol. Samples were kept in the dark at room temperature and the decrease in the absorbance at 517 nm was determined by UV-Vis spectroscopy [36]. DPPH radical scavenging activity, AA (%), was determined according to eq. 1:

$$AA(\%) = \frac{(A_0 - A_1)}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of the blank control and A_1 is the absorbance of the sample at 517 nm. Experiments were conducted in triplicate and the values given correspond to the average with the respective standard deviation.

2.5.7. Scanning electron microscopy (SEM)

SEM analyses of the cross-sections of BC and BC-loaded with vitamins (BC-VIT) of ILs (BC-[Ch][VIT]) were performed over fractured membranes after immersion in liquid nitrogen.

Samples were then covered with carbon and analyzed using a SU-70 high resolution microscope (Hitachi, Japan) at 4 kV and 10 mm focal distance.

2.5.8. Tensile assays

Tensile assays of dried BC, BC-VIT and BC-[Ch][VIT] membranes were performed using a 5944 universal testing machine (Instron, UK) with Bluehill 3 software in tensile mode with a 1 kN load cell. Specimens of 70×10 mm² and gauge length of 30 mm were used. The corresponding stress (MPa)–strain (%) curves were plotted to determine the Young's modulus (GPa) from the slope of the low strain region near 0.05%, maximum stress (MPa) and elongation at break (%). Experiments were conducted in quintuplicates and averaged with the respective standard deviation.

2.5.9. Re-hydration tests

Membranes (dried BC, BC-VIT and BC-[Ch][VIT]) were weighted and then soaked in individual containers with PBS aqueous solutions at room temperature, during 24 h. At different times, samples were taken out, the excess of PBS was gently removed with absorbent paper and membranes were weighted and re-immersed again. The absorbed PBS, in %, was calculated according to eq. 2:

$$PBS\ absorption = \frac{w_{wet} - w_{dry}}{w_{dry}} \times 100 \quad (2)$$

where w_{dry} and w_{wet} are the weight of dried and wet BC samples, respectively. Experiments were conducted in triplicate and averaged with respective standard deviation.

2.5.10. Dissolution assays

Dissolution assays were conducted with BC-VIT and BC-[Ch][VIT] membranes. Samples were placed in a closed flask containing 200 mL of PBS aqueous solution (pH 7.4) under magnetic stirring. The release of vitamins or [Ch][VIT] ILs was then evaluated. At determined time intervals (during 24 h), 2 mL of solution were withdrawn, and the same volume of fresh buffer was added to maintain a constant volume. The vitamins and cholinium-based ILs content in each aliquot was determined as described before. The vitamins and ILs content at each time was plotted as a cumulated concentration release (C_{cumul}), determined according to eq 3:

$$C_{cumul} = C_n + \sum_{k=0}^{n-1} \frac{C_k}{100} \quad (3)$$

where C_n is the IL concentration at time n = 0, 5, 10, 15, 20, 30, 60, 120, 240, 360 and 480 min. Weights of vitamins or [Ch][VIT] ILs leached out of the sample were calculated and the released ratio (wt.%) relative to the initial mass was calculated. Experiments were conducted in triplicate and averaged with respective standard deviation.

2.5.11. In vitro cytotoxicity assays

Cytotoxicity was ascertained on human keratinocyte cell line (HaCaT cells) obtained from Cell Line Services (Appenheim, Denmark). The cells were cultured, at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2, using Dulbecco's Modified Eagle's Medium (DMEM). BC, BC-VIT and BC-[Ch][VIT] membranes cytotoxicity was assayed through adaptation of the procedure previously described by Sadeghian *et al.* [37]. Briefly, HaCaT cells were seeded in the wells of a 96 well microplate and allowed to adhere for 24 h. Simultaneously, membranes were cut into 2×2 cm² pieces and then soaked in 10 mL of DMEM for 24 h. Although lower times will be required for skin care applications, this period was chosen as an in excess period to better guarantee the non-cytotoxic nature of the studied membranes towards human keratinocyte cells.

After this period the media was removed, and cells were washed with PBS aqueous solutions. Then, membranes were taken out of DMEM medium and the leached substances were added (direct and ½ diluted) to cells. After 24 h incubation, 25 µL of sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium (XTT) working solution were added to each well and the cells were incubated, in the dark, for 2 h. The optical density (OD) at 485 nm was then measured using a FLUOstar OPTIMA microplate reader (BMG Labtech, Germany). Experiments were conducted in quintuplicate and averaged with respective standard deviation.

The cytotoxicity results were used to calculate the percentage of inhibition after incubation with the sample using eq. 4:

$$Inhibition\ (\%) = 1 - abs_{sample}/abs_{control} \times 100 \quad (4)$$

where abs_{sample} is the absorbance in a well containing sample and $abs_{control}$ is the absorbance of untreated control cells.

3. Results and discussion

In this work, novel cholinium-based ILs paired with anions derived from vitamins B3, B5 and B6, namely, nicotinate (B3), pantothenate (B5) and pyridoxylate (B6), were synthesized and characterized by NMR, FTIR-ATR, DSC, TGA, solubility in PBS aqueous solutions and antioxidant activity. Most of these characterization studies were also conducted for the original vitamins. The obtained ILs and vitamin B precursors were then incorporated into BC membranes, characterized by NMR, FTIR-ATR, SEM, TGA, tensile tests, re-hydration capacity, dissolution and *in vitro* cytotoxicity assays, envisaging their use in skin care applications.

3.1. Synthesis and characterization of vitamin B-based ILs

The vitamin B-based ILs were synthesized by the neutralization of cholinium bicarbonate with the different vitamins B. The full names, acronyms and chemical structures of the studied ILs and their vitamin precursors are depicted in Fig. 1.

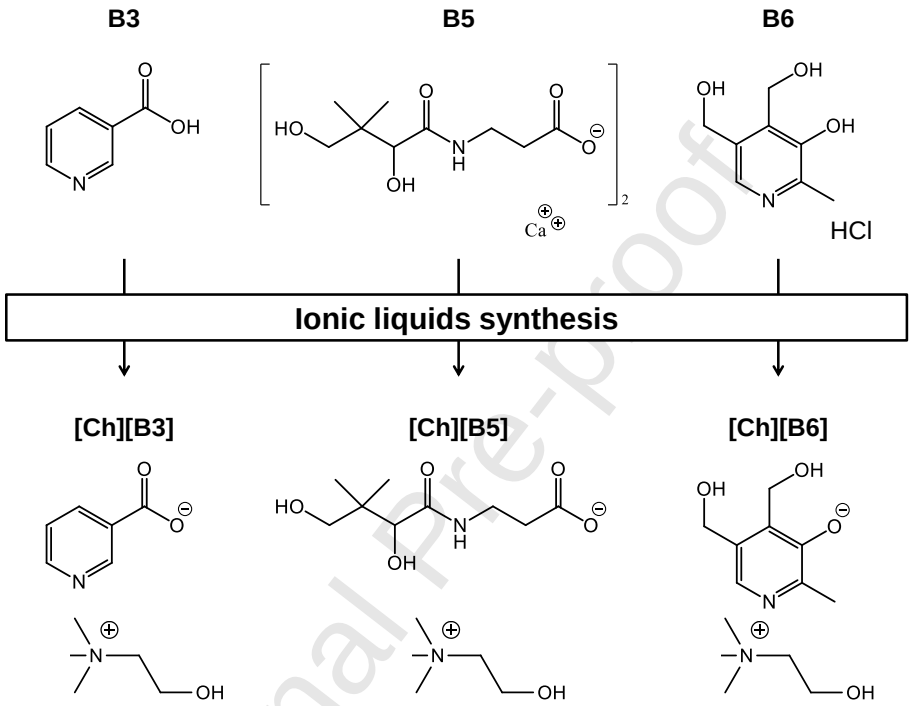

Fig. 1. Chemical structures of the vitamin B precursors (nicotinic acid (B3), calcium pantothenate (B5), pyridoxine hydrochloride (B6)) and the corresponding cholinium-based ILs (cholinium nicotinate [Ch][B3], cholinium pantothenate [Ch][B5], and cholinium pyridoxylate[Ch][B6]).

3.1.1. Structural and thermal characterization

After the synthesis of the [Ch][VIT] ILs, the cholinium:VIT molar ratios (Table S1) were confirmed based on their ^{1}H and ^{13}C NMR NMR spectra (given in Fig. S1, S2 and S3, as well as the corresponding resonance assignments), confirming the ILs successful synthesis. The FTIR-

ATR spectra of the [Ch][VIT] ILs and their vitamin precursors are displayed in Fig. S4. The spectra of all [Ch][VIT] ILs showed the stretching ν(C–O) vibration of the cholinium ion at 955 cm^{-1} [38]. Additionally, regarding [Ch][B3], the disappearance of the stretching ν(C=O) band of the carboxylic acid at 1708 cm^{-1}, typical of vitamin B3, and the appearance of a ν(C=O) stretching band of the carboxylate at 1611 cm^{-1} were observed. The ν(C=O) stretching band of carboxylate is shifted from 1556 cm^{-1} in vitamin B5 to 1585 cm^{-1} in [Ch][B5]. Finally, in the spectrum of [Ch][B6], there is the appearance of the phenolate ν($C–O^-$) vibration at 1059 cm^{-1} [38]. All these data confirm the successful synthesis of [Ch][VIT] ILs.

The thermal characterization and thermal stability of the synthesized ILs was ascertained to confirm if they are stable enough for the targeted application, and particularly to address if they support typical sterilization procedures, such as autoclaving. The glass transition (T_g), melting (T_m) and decomposition temperatures (T_{dmax}) of the prepared [Ch][VIT] ILs were determined, being reported in Table 1. All ILs show low glass transition temperatures, in the range of -25 to -75 °C. [Ch][B6] is solid at room temperature, with a melting temperature of 68 °C. On the other hand, [Ch][B3] and [Ch][B5] are liquid at room temperature and do not have any melting peak between glass transition and 100 °C, meaning that these two ILs pass from the glass to the liquid state without a solid domain identified at the studied heating speed. Overall, all synthesized compounds display a melting temperature below 100 °C, meaning that they fit within the ILs general definition.

Regarding the decomposition temperatures, [Ch][B5] (230 °C) and [Ch][B6] (200 °C) have decomposition temperatures only slightly lower than those of their precursors: B5 (232 °C) and B6 (212 °C). This is due to the fact that B5 and B6 are already in a salt form. Accordingly, the addition of an organic cation as cholinium is shown to not decrease significantly the decomposition temperatures. [Ch][B3] degrades at 224 °C, which is ca. 100 ºC below nicotinic

acid. This decrease is coherent with other studies on ILs compared with their acid precursors [15,16,39]. Nevertheless, all [Ch][VIT] ILs are thermally stable enough to support autoclaving at around 120°C to sterilize the patches for the target skin care applications.

Table 1. Thermal properties of [Ch][VIT] ILs.

	T_g (°C)	T_m (°C)	T_{dmax} (°C)
B3		232-263[a]	320[d]
B5		No defined melting[b]	231
B6		217±30[c]	212[e]
[Ch][B3]	-73	Liquid at RT	224
[Ch][B5]	-47	Liquid at RT	230
[Ch][B6]	-56	68	200

RT: room temperature, [a][40], [b][41], [c][42], [d][43], [e][44]

3.1.2. Solubility assays

The solubility results of the original vitamins and [Ch][VIT] ILs in PBS (pH 7.4) aqueous solutions are reported in Fig. 2 and Table S2. The solubility values are 5224, 608 and 751 mM for [Ch][B3], [Ch][B5] and [Ch][B6], respectively. These are higher than those of their vitamin precursors, namely 170, 160 and 683 mM for vitamins B3, B5 and B6, respectively. For most [Ch][VIT] the improvements in solubility remain in the same order of magnitude. Nevertheless, it should be remarked that (with exception of nicotinic acid) these vitamins are in a salt form, being demonstrated here that the addition of an organic cholinium cation, which is also a vitamin and can be valuable to improve biological activities, still leads to solubility improvements. The most remarkable increase (up to 30.6-fold) in solubility is observed for nicotinic acid when converted to the respective IL, namely cholinium nicotinate, since there is the conversion of an acid to a salt form. These results are in agreement with previous studies on the synthesis of cholinium-based

ILs with phenolic acids [32,34]. Although most studies on the solubility of novel ILs are carried out with water, here, aqueous PBS solutions were used due to their higher similarity to intra- and inter-cellular media in epidermis, while envisaging their use in transdermal delivery and skin care applications.

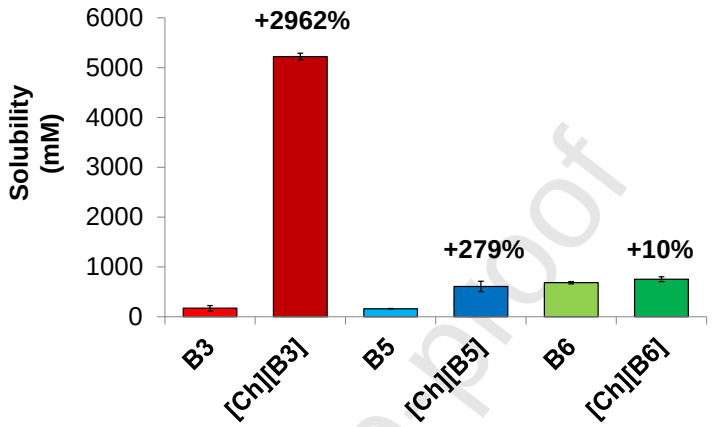

Fig. 2. Solubility of the original vitamins and [Ch][VIT] ILs in PBS (pH 7.4) aqueous solutions.

3.1.3. Antioxidant activity

The antioxidant activity of the prepared cholinium-based ILs and respective vitamin precursors was evaluated by the DPPH radical scavenging assay. Fig. 3 depicts the antioxidant activity (AA%), using ascorbic acid as positive control. The assays were carried for 3 days due to the slow kinetics of radical scavenging activity of vitamins B, as reported by Higashi-Okai *et al.* [10]. In most cases, a maximum antioxidant activity plateau was reached after 2 days for both vitamin and [Ch][VIT] samples. At the studied concentration, ascorbic acid reaches a maximum antioxidant activity of 98.0±0.6% while all vitamins reached more than 50% of antioxidant activity, namely 70.4±2.8%, 55.4±0.6% and 83.7±0.6% for B3, B5 and B6, respectively. The design of vitamin B-based ILs leads however to different effects on the antioxidant activity. While [Ch][B3] and [Ch][B6] have lower activities (63.2±0.9 and 68.4±1.4%, respectively).

[Ch][B5] shows a strong antioxidant activity increase (73.2±4.7%). This difference may be related to the conversion of the respective vitamins into the corresponding ionic liquids, in which the vitamins in [Ch][B3] and [Ch][B6] are converted to an anion, losing a proton. On the other hand, vitamin B5 is already in a salt form, following a different trend, where the replacement of sodium cation by cholinium (organic ammonium cation with a hydroxyl group) is favorable to increase the antioxidant activity of the respective IL.

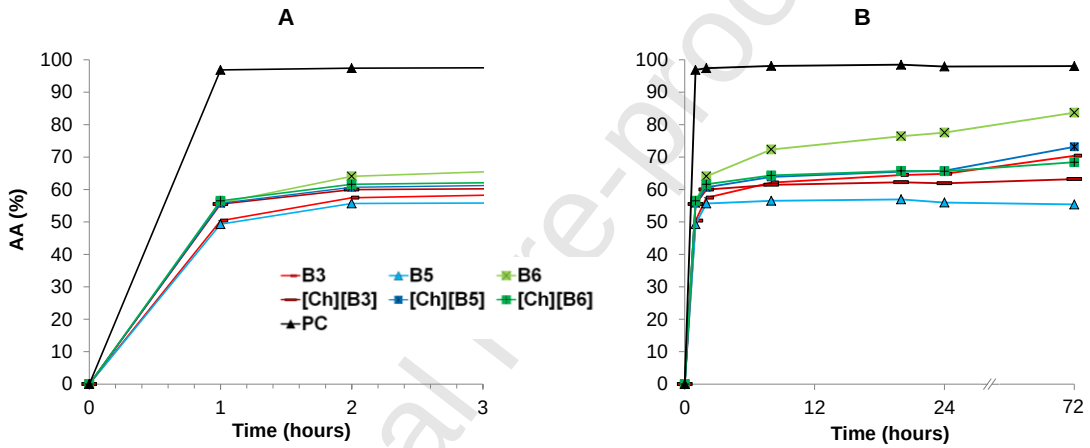

Fig. 3. Antioxidant activity of vitamins B3, B5 and B6, [Ch][B3], [Ch][B5] and [Ch][B6] ILs, and ascorbic acid as positive control (PC)for 3 hours (A) and during the3 days (B) periods.

3.2. Preparation and characterization of BC membranes enriched with vitamin B-based ILs

The obtained [Ch][VIT] ILs, and the original vitamins for comparison purposes, were incorporated into BC membranes aiming their application in skin care. The average weight of vitamins and [Ch][VIT] ILs incorporated into BC was determined by UV-Vis spectroscopy or HPLC-DAD as described before. In general, both vitamins B and ILs were incorporated in BC in high percentages, ranging from 86.9±1.7% to 98.6±0.4% of the initial amount poured on the top of BC (the respective data are given in Table S3 in the Supporting Information). Furthermore, no

significant differences in incorporation levels were observed between the original vitamins and respective ILs.

3.2.1. Structural and morphological characterization

The structural characterization of BC-[Ch][VIT] membranes was carried out by solid-state ^{13}C NMR (spectra given in Fig. S5, S6, and S7) and FTIR-ATR (Fig. S8). The solid-state ^{13}C CP/MAS NMR spectra of BC-[Ch][VIT] samples show the typical resonances of BC and of the incorporated IL, whereas the higher intensity of BC resonances agrees with the relative proportion of BC and ILs. A more detailed analysis reveals shifts of some specific carbon resonances of [Ch][VIT] after incorporation in BC, namely: the resonance of (C–**C**OO^-) carbon of [Ch][B3] that is shifted from 167.87 ppm to 173.54 ppm; the resonance of (CH_2–**C**OO^-) carbon of [Ch][B5] that is shifted from 175.17 to 165.18 ppm; and the resonance of (aromatic **C**–O^-) carbon of [Ch][B6] that is shifted from 139.17 to 134.41 ppm. These shifts suggest the establishment of interactions between ionic liquids and bacterial cellulose nanofibrils. FTIR-ATR results further confirm this behavior. The carboxylate band of [Ch][B3] moves from 1613 to 1598 cm^{-1}; and the carboxylate band of [Ch][B5] shifts from 1585 to 1564 cm^{-1}. It should be remarked that in BC-[Ch][B6], the overlapping of the phenolate band of [Ch][B6] with the (C–O–C) band of cellulose does not allow to observe any significant shift.

Both BC-VIT and BC-[Ch][VIT] dried membranes (Fig. S9 in the Supporting Information), with the exception of BC-B3, are transparent and homogeneous, similarly to BC, which is an indication of the good dispersion of the vitamins and of the corresponding ILs within the network of cellulose nanofibrils. In the case of VIT B3, the obtained BC membrane is heterogeneous, with the presence of agglomerates. On the other hand, the BC-[Ch][B3] is homogenous, avowing the formation of agglomerates. This is due to the considerable increase on the aqueous solubility

when converting vitamin B3 to the respective IL, resulting in a higher affinity to the hydrophilic cellulose matrix and dispersion in the membrane.

SEM analysis were performed on the cross-sections of BC, BC-VIT and BC-[Ch][VIT] membranes (Fig. 4). All samples present the characteristic lamellar structure of BC. Yet, BC-[Ch][VIT] membranes have less distinguishable nanofibrils, confirming improved affinity between ILs and cellulose nanofibrils and the complete filling of the spaces between the cellulose fibrils by ILs. The improvement of affinity promoted by the conversion of vitamins into the respective ILs is particularly notorious for vitamin B3, where agglomerates are easily perceptible in the cross-section of the corresponding membranes. These results are in line with the macroscopic appearance of the membranes (Fig. S9), and are in agreement with the low solubility of vitamin B3 in water and significant solubility enhancement resulting from its conversion into a cholinium-based IL (Fig. 2).

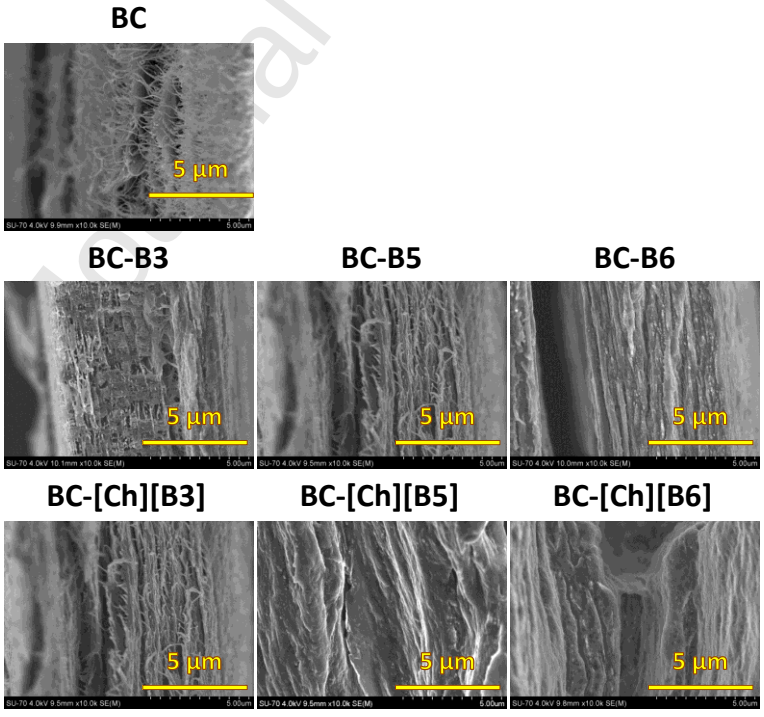

Fig. 4. SEM images of BC, BC-VIT and BC-[Ch][VIT] cross sections at ×10k magnification.

3.2.2. Thermal properties

The impact of the incorporation of vitamins and [Ch][VIT] on the thermal properties of BC membranes was investigated by TGA. Fig. S10 shows the thermographs of BC, BC-VIT and BC-[Ch][VIT] membranes, whereas Fig. S11 in the Supporting Information depicts the corresponding derivative curves. Table 2 summarizes the maximum decomposition temperatures (T_{dmax}) of each material. BC has a characteristic maximum weight loss at 339 °C [45]. BC-B3, BC-B5 and BC-B6 samples present a first weight loss at 213, 197 and 203 °C, respectively, due to the decomposition of vitamins B (cf. Table 1), and a second weight loss at 351, 355 and 327 °C due to cellulose decomposition. Regarding the BC-[Ch][VIT] samples, the first decomposition step, assigned to the degradation of the ILs, is observed at 236, 225 and 190 °C, while the decomposition of the BC-enriched fraction is observed at 288, 317, 329 and 286 °C for BC-[Ch][B3], BC-[Ch][B5] and BC-[Ch][B6], respectively. The incorporation of ILs into BC leads only to minor variations on the decomposition temperatures of the [Ch][VIT] ILs. However, a decrease on the cellulose decomposition temperature is observed in all cases, being in agreement with the literature on the decomposition temperatures of other salt-incorporated cellulose materials [46,47]. Despite these differences, BC-[Ch][VIT] samples are thermally stable at least up to 190 °C, allowing them to be submitted to thermal treatment such as autoclaving (*ca*. 120 °C) necessary in many nutraceutical and cosmetic applications, thus not compromising the envisioned skin care applications.

Table 2. Decomposition temperatures determined by DTG curves of BC, BC-VIT and BC-[Ch][VIT].

	T_{dmax} (°C)			
	T_{dmax1}	T_{dmax2}	T_{dmax3}	T_{dmax4}
BC	339			
BC-B3	213	351		
BC-B5	197	231	267	355
BC-B6	203	251	327	
BC-[Ch][B3]	236	271	317	450
BC-[Ch][B5]	225	329		
BC-[Ch][B6]	190	249	286	417

3.2.3. Mechanical properties

Mechanical properties of BC, BC-VIT and BC-[Ch][VIT] were investigated via tensile tests. Fig. 5 shows the Young's modulus, maximum stress and elongation at break calculated from stress/strain curves, given in Fig. S12 and Table S4 in the Supporting Information. The BC membrane has properties of brittle elastic materials, with 11.8±1.2 GPa of Young's modulus, 153 ± 46 MPa of maximum stress and 1.7 ± 0.6% of elongation at break [48]. The incorporation of vitamins B5 and B6 lead to small losses of both Young's modulus and maximum stress, with 12.2 ± 0.4 and 7.4 ± 1.0 GPa for Young's modulus and 117 ± 30 and 114 ± 33 MPa for maximum stress, respectively. Similarly, BC-B5 and BC-B6 samples present similar values of elongation at break when compared with BC, specifically 1.1 ± 0.3 and 2.2 ± 0.6%. On the other hand, the BC-B3 material shows a strong decrease of the Young's modulus (2.2 ± 0.4 GPa) and maximum stress (63 ± 6 MPa), and an increase in the elongation at break of 3.6 ± 0.7%. This loss of elastic properties with small compensation of plastic properties can be explained by the low affinity between B3 and the BC matrix, which is mainly due to low water solubility of the vitamin, as

discussed before, weakening the whole structure. This trend is in agreement with the morphology of BC-B3 (Fig. 4).

The incorporation of ILs into BC, in comparison with the BC-VIT membranes, leads to a significant decrease of the Young's modulus and maximum stress, with 0.7 ± 0.3, 5.1 ± 1.4 and 2.7 ± 0.4 GPa and 29 ± 17, 124 ± 19 and 104 ± 3 MPa for BC-[Ch][B3], BC-[Ch][B5] and BC-[Ch][B6], respectively. Together, the elongation at break is significantly improved with values of 7.6 ± 1.3%, 3.6 ± 0.6% and 4.5 ± 1.1% for BC-[Ch][B3], BC-[Ch][B5] and BC-[Ch][B6], respectively. These results demonstrate a clear plasticizing effect of [Ch][VIT] ILs, which is not observed with their vitamin B precursors due to the good affinity between BC and ILs demonstrated above.

Despite of the decrease of the Young's modulus for the BC-[Ch][VIT] membranes those values, as well the corresponding maximum stress and elongation at break are adequate for materials aiming topical applications, when compared with other BC-based membranes [30,49]. Furthermore, these mechanical properties have been obtained without adding any plasticizer (*e.g.* glycerol, as typically carried out [50] or polyethylene glycol [51]), with this role being fulfilled by the ILs themselves. For example, skin bio-adhesive patches composed of polyvinyl alcohol and glycerol or polyethylene glycol, as plasticizers, showed lower elongations at break, viz. 2.0% and 1.4%, respectively and 2-5 MPa maximum stress [51], than the BC-[Ch][VIT] developed in this work. These results show that the [Ch][VIT] ILs have a strong plasticizing while preserving maximum stress values suitable for topical applications.

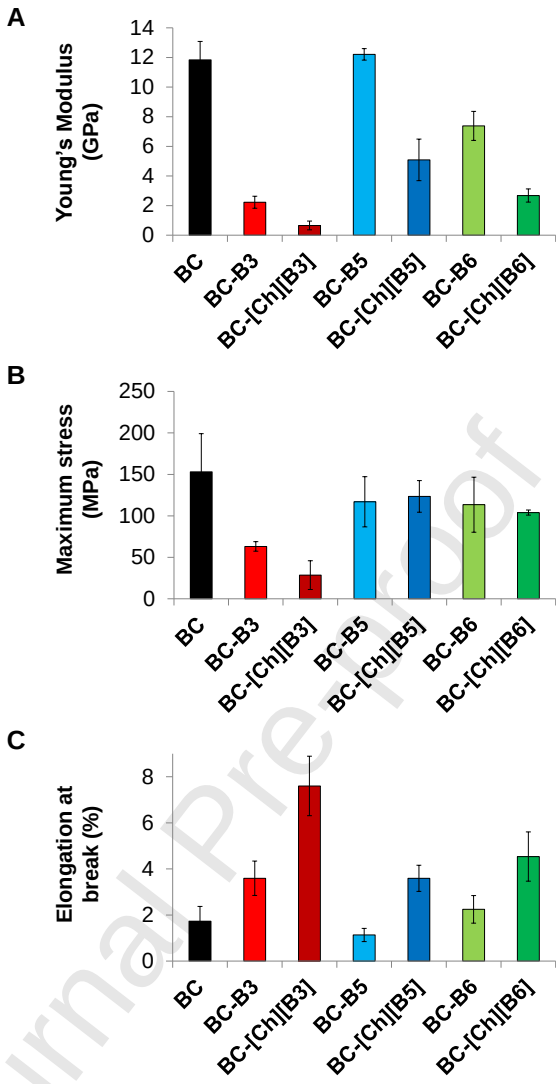

Fig. 5. Young's modulus (A), maximum stress (B) and elongation at break (C) of BC, BC-VIT and BC-[Ch][VIT] membranes.

3.2.4. Re-hydration and drug release capacity

In order to evaluate the ability of membranes to re-hydrate when applied on the skin, re-hydration tests in PBS (pH 7.4) aqueous solutions were performed during 24 h. The respective results are given in Fig. 6. BC membranes are able to absorb 326±11% of their dry weight in PBS

aqueous solutions. BC-B5 presents higher absorption capacity 1224±171% while BC-B3 and BC-B6 exhibit a lower absorption capacity, with 210±11% and 211±4%, respectively.

Remarkably, all BC-[Ch][VIT] membranes exhibit higher re-hydration values than BC and BC-VIT membranes, with values of 1576±125%, 1697±80% and 960±69% for BC-[Ch][B3], BC-[Ch][B5] and BC-[Ch][B6], respectively. This re-hydration improvement is in accordance with the increased solubility of [Ch][VIT] ILs in PBS aqueous solutions. FTIR-ATR and NMR spectroscopy observations suggest interactions between ILs and BC, while the SEM images show homogeneous distribution of ILs in the BC network. Therefore, the affinity between ILs and BC leads to less interlayer hydrogen bond formation during the drying of membranes, avoiding the BC structure to collapse and allowing also an easier re-hydration [52]. This improvement in absorption capacity of the membranes afforded by ILs is highly relevant toward the envisioned skin care applications. In fact, the rehydration values observed for the BC-[Ch][VIT] ILs membranes are higher or similar to those obtained for BC membranes loaded with other drugs, such as lidocaine [29], diclofenac sodium [50] and caffeine [49], and in most cases plasticized with glycerol (values ranging from 80-1400%). Other biobased materials developed for topical drug delivery as, polyvinyl alcohol/chitosan/BC patches loaded with sodium ibuprofen [53] and polyvinyl alcohol or polycaprolactone nanofibers incorporated with timolol maleate [54] also show considerably lower % rehydration capacities (up to around 200%).

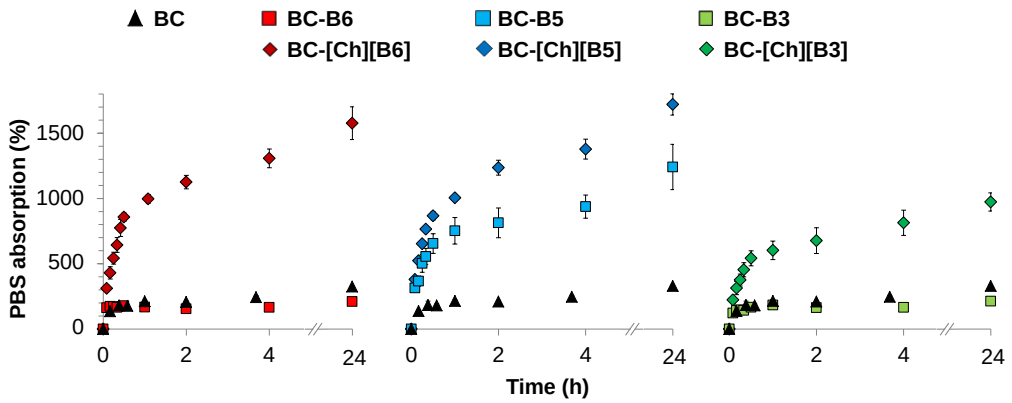

Fig. 6. PBS (pH 7.4) aqueous solutions absorption by BC, BC-VIT and BC-[Ch][VIT] dried membranes.

The kinetics of release of vitamins and [Ch][VIT] from the corresponding BC membranes in PBS aqueous solutions was assessed by dissolution assays (Fig. 7). These results are relevant because the buffer solution can serve as model for cytoplasmic medium and intercellular medium and thus predict the behavior of our compounds *in vivo*. All samples show a strong burst effect with at least 66% of the incorporated compound being released in the first 5 min. The use of ILs leads to a faster and more complete release of the target compounds when compared to the original vitamins. The Weibull model [55] was used to fit the 1st order kinetics of the release profiles and the respective parameters and linear regression coefficients are listed in Table S5 (Supporting Information). All BC-VIT samples release profiles are well described by the model, while for BC-[Ch][VIT] profiles only BC-[Ch][B5] follows this model (regression coefficients given in Table S5 in the Supporting Information). Samples described well by the Weibull model behave as if BC had no barrier properties in the release of the incorporated compounds. The other samples present an almost instantaneous dissolution in PBS.

If proper amounts are loaded into the membranes, the high percentage release of vitamin B-based ILs from BC is very interesting for topical skin care applications, particularly for short-term masks to be used in the cosmetic or pharmaceutical field or for moisturizing the skin. Depending on the target application, it should be remarked that the fast release of [Ch][VIT] ILs can eventually be delayed by the addition of compounds with tuned barrier properties, such as poly(ethylene-*co*-vinyl acetate), poly(ester urethanes) or polyacrylates [23].

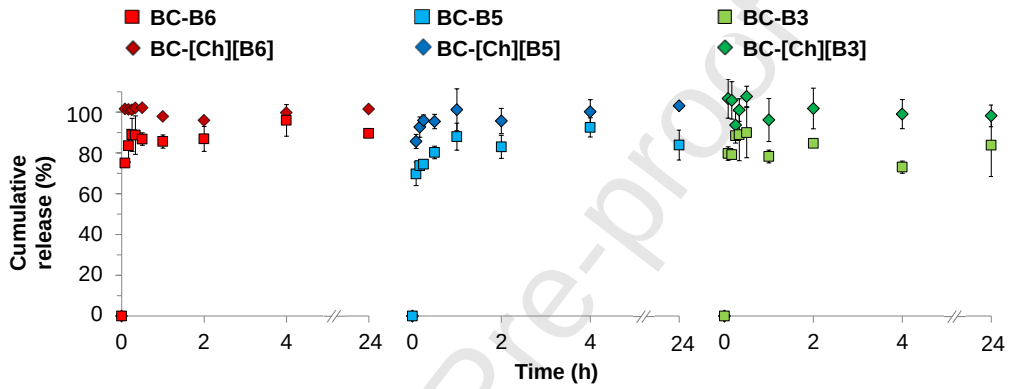

Fig. 7. Cumulative release of vitamins and [Ch][VIT] ILs in PBS (pH 7.4) aqueous solutions from BC-VIT and BC-[Ch][VIT] membranes.

3.2.5. In vitro cytotoxicity assays

In order to ensure the safety of the prepared membranes and the possibility for cosmeceutical and pharmaceutical applications, the cytotoxicity of BC, BC-VIT and BC-[Ch][VIT] membranes was finally assessed against HaCaT cells (Fig. 8). As expected, the BC membrane are non-cytotoxic, with only 5.9±3.9% of inhibition for the exposure time, being in agreement with its well-known non-cytotoxicity towards several cell lines [56] (including HaCaT cells [57]). The BC-VIT and BC-[Ch][VIT] membranes are also non-cytotoxic, with inhibition of HaCaT cells ranging from -7.7±1.1% to 3.7±0.5%. To be considered as non-cytotoxic materials,

membranes have to exhibit an inhibition rate below 20% [58], which is the case of all membranes prepared and investigated.

Although ILs have been described as expensive solvents for several years, which may compromise the envisioned application, mainly because of their restricted use and thus production in low amounts in the academia sector, in the last decade this scenario has changed. When produced at large-scale their price is competitive, and sometimes even lower, than other solvents [59]. They are currently used in industrial applications, at large-scale, meaning that their cost is no more a drawback, as appraised in a recent published book presenting case studies of commercial applications of ILs written by the inventor or the company using the technology involving ILs [60]. Accordingly, if produced at large scale, the cost of vitamin B-based ILs is not a drawback, particularly considering that a simple metathesis reaction is used to produce them and that the starting materials are not expensive compounds. Furthermore, the improvement of the properties offered by these ILs when compared with the original vitamins, namely (i) their high thermal resistance, (ii) higher solubility thus improving their bioavailability, (iii) ability to act as plasticizers reducing BC brittleness while facilitating their application on irregular skin regions, (iv) higher re-hydration ability of the respective membranes ensuring adequate hydration for ILs release, and (v) non-cytotoxicity behavior towards skin epithelial cells, make them suitable materials to be investigated in skin care and other related applications. However, for this application to become a reality, more biological tests need to be performed and the respective ILs need to be produced at a larger scale to reduce their cost.

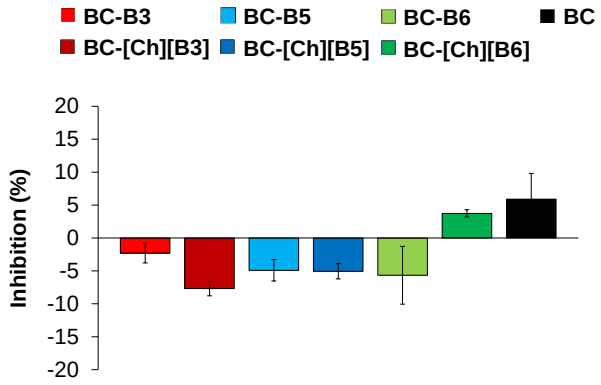

Fig. 8. Inhibition of HaCaT cells in contact with BC, BC-VIT and BC-[Ch][VIT] membranes.

4. Conclusions

Vitamin B-based ionic liquids were successfully synthesized and incorporated into bacterial cellulose membranes envisioning their use in skin care applications. These ILs exhibit high thermal stability (at least 120 °C), display melting temperatures below 100 ºC (where [Ch][B3] and [Ch][B5] are liquid at room temperature) and show increased solubility in aqueous PBS buffer solutions, particularly for vitamin B3 whose solubility increased 30.6-fold when converting it to an IL. The antioxidant properties of [Ch][VIT] revealed different behaviors, compared to their precursors namely an improvement of the radical scavenging activity for [Ch][B5 and a loss of activity for [Ch][B3] and [Ch][B6]. The incorporation of [Ch][VIT] into BC led to transparent and homogeneous materials with high thermal stability and improved elongation at break due to the plasticizing effect of ILs which is particularly relevant to avoid the use of plasticizers. Additionally, the ability of BC-[Ch][VIT] membranes to absorb buffer aqueous solutions has been improved about 3 times in comparison to BC, and the release of ILs in PBS has been more complete and faster than the release of the pristine vitamins. Finally, BC-VIT and BC-[Ch][VIT] were shown to be non-cytotoxic for human dermal cells (HaCaT). Based on the overall results, BC-[Ch][VIT] membranes appear to be a promising material for topical

delivery of vitamins B in skin care applications. Further investigations should focus on additional biological assays to further address their cytotoxicity, the kinetic of release of ILs through the epidermis, and on the anti-aging properties of ILs, such as collagen production enhancement, moisturizing of the *stratum corneum* skin layer and wound healing.

Supplementary information

Tables S1-4: cholinium:vitamin B ratio in ILs evaluated by ^{1}H NMR, solubility of vitamins and ILs in PBS, average weight of each compound incorporated in BC membranes, Young's modulus, maximum stress and elongation at break data, and parameters related with the regression by the Weibull model, respectively.

Figures S1-12: liquid-state ^{1}H and ^{13}C NMR spectra of ILs and peak attributions, FTIR-ATR spectra of ILs and their precursors, solid-state ^{13}C NMR spectra of BC-IL membranes and peak attributions, FTIR-ATR spectra of BC-ILs, photographs of membranes, TGA and DTG curves of membranes and stress/strain curves of BC-IL and BC-VIT membranes after tensile tests, respectively.

Acknowledgments

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Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Carmen Freire

Author Statement

Original active principle ingredients based ionic liquids (ILs) with cholinium cation and vitamins B anions were developed and incorporated in bacterial nanocellulose (BC) membranes for potential topical applications

Graphical abstract

Highlights

• Simple synthesis of original cholinium-vitamin B ionic liquids.

• Successful design of non-cytotoxic IL-incorporated bacterial nanocellulose membranes.

• Improvement of vitamins solubility in buffer, especially for nicotinic acid IL.

• Fast and complete release of ILs in buffer solutions and improved re-hydration capacity of membranes.