




Pure cellulose nanofibrils membranes loaded with ciprofloxacin for drug release and antibacterial activity

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Abstract The biocompatibility and very high specific area of cellulose nanofibrils (CNF) are properties of high interest for the development of active substrates for new medical device development. Enzyme pre-treated CNF (CNF-e) can be self-organized into nanostructured membranes that are suitable for active principle ingredients (API) encapsulation through adsorption phenomena. In addition, tunable surface chemistry of CNF-e, allow for covalent immobilization of API. In this work, ciprofloxacin is integrated to CNF-e membranes according to two different strategies. The first one relies only on adsorption mechanisms; ciprofloxacin is encapsulated in the bulk before the membrane formation by solvent casting. The influence of the membrane properties and preparation parameters such as grammage, thickness and drying technique, are assessed with water uptake measurements and API

release experiments. The second strategy deals with the covalent immobilization of ciprofloxacin directly onto CNF-e membrane. The two kinds of membranes are then compared in terms of antibacterial activity, in both static and dynamic conditions. Thick CNF-e membranes loaded with adsorbed ciprofloxacin that were overdried (2 h, 150 °C) prove to be more resistant in liquid medium and present a more prolonged drug release. However, these membranes rapidly lost their antibacterial activity, while CNF-e membranes with covalently immobilized ciprofloxacin remain contact active for several days. These 100% CNF active nanostructured membranes can be used as new wound dressing for topical application.

Keywords Cellulose nanofibrils · Antibacterial activity · Drug release

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Introduction

The interest for cellulose nanofibrils (CNF) has exponentially increased over the last ten years with the development of new production routes [pre-treatments, improved mechanical fibrillation (Nechyporchuk et al. 2016; Rol et al. 2018)] and announcements of its industrialization. The number of scientific paper has been multiplied by 5 between 2007 and 2017 and several books (Dufresne 2017; Lee 2018) or

reviews (Abitbol et al. 2016; Kargarzadeh et al. 2018; Klemm et al. 2011) are now available on this topic.

Such nanofibrillated cellulose can be used in several applications like paper (Aulin et al. 2010; Bardet and Bras 2014), nanocomposites (Dufresne et al. 2013), cosmetic or printed electronics (Bober et al. 2014; Hoeng et al. 2016). Most of the time, barrier, mechanical and rheological properties are the main reason behind the use of CNF, in addition to its renewable character and biodegradability. More recently, the biocompatibility and large specific surface area of CNF have motivated researchers to extend its use in the biomedical field for the development of new drug delivery and tissue engineering systems (Jorfi and Foster 2014; Laurén 2018; Lin and Dufresne 2014).

If we focus on drug delivery, CNF films and membrane have been designed as entrapping systems for hydrophobic drug in 2012 for the first time by Kolakovic et al. (2012). Since that time, very interesting interactions between some active molecules and cellulose nanofibrils were investigated. A comparison between caffeine and chlorhexidine digluconate has been performed by Lavoine et al. in terms of release profile (Lavoine et al. 2014a, b, 2016). The main outcomes indicated a prolonged controlled release of API thanks to the nanoporous network of CNF coatings. Cage-like molecules, i.e. cyclodextrins, were also used to complement the anchoring strategies of active molecules on CNF substrates. It resulted in multi-encapsulation systems (Lavoine et al. 2014c). Up to our knowledge, there is no study on the interaction of ciprofloxacin with CNF. This new second generation fluoroquinolone is very promising since it has a broad spectrum of activity against both gram positive and gram negative bacterial strains (Appelbaum and Hunter 2000). The fluoroquinolones inhibits the enzymes involved in the DNA replication process, leading to bacteriostasis and eventually cell death (Herbold et al. 2001).

In the meantime, the design of contact and long-term active structures was possible by covalent binding of molecules on CNF. For instance, penicillin, nisin or amino-silane have been grafted on CNF networks (Saini et al. 2015, 2016a, b).

The successful grafting of ciprofloxacin molecule onto CNF-e membranes through water based and thermally triggered method was demonstrated in previous study. The objective of this work is then to

compare CNF-e membranes with surface covalently bound ciprofloxacin to CNF-e membranes that have bulk adsorbed ciprofloxacin. The idea is to develop membranes with active properties for topical application. First, CNF-e membranes of different thicknesses, produced with different drying techniques, are compared in term of water uptake measurements and drug release experiments. Antimicrobial testing will be finally performed to describe the activity of CNF-e membranes with bulk adsorbed ciprofloxacin versus CNF-e membranes with covalently immobilized ciprofloxacin on its surface.

This is the first work that tries to tackle such a numerous preparation parameters and release study conditions for the design of 100% CNF-e membranes that comprise ciprofloxacin active molecule. It is believed to provide more insights on CNF-e membranes nanostructure effects on drug release.

Experimental section

Materials

The main material used is a cellulose nanofibrils suspension (CNF) that was provided by the CTP (Centre Technique du Papier, Grenoble, FR). It was isolated from a bleached birch pulp strongly refined (up to 80°SR), before an enzymatic pre-treatment and a final homogenization: 3 passes at 1500 bars in an Ariete homogenizer from GEA (Italy). The resulting 3 wt% suspension will be referred as CNF-e. Ciprofloxacin ($\geq 98\%$ CAS: 85721-33-1) and hydrogen chloride (HCl, CAS: 7647-01-0) were purchased from Sigma Aldrich. Agarose was purchased from ACROS ORGANICS (CAS: 9012-36-6) under the form of a powder which was dissolved in hot deionized water. Nutrient Agar for microbiology testing was obtained from Humeau and was composed of 3.0 g of beef extract, 5.0 g of peptone and 15.0 g of agar. Standard Nutrient broth I was composed of 15 g/l peptone, 3 g/l of beef extract, and 6 g/l of sodium chloride and 1 g/l of glucose and was purchased from Carl ROTH, as well as sodium chloride for isotonic solution preparation. Commercial gauze was obtained from adhesive bandage produced by EUROSIREL (Italy). Deionized water was used in all experiments.

Methods

Covalent immobilization of ciprofloxacin on CNF-e membranes (CNF-cip-g)

CNF-e suspension concentration was adjusted to 1 wt% and dispersed with high shear Ultra-Turrax mixer (IKA, USA). The suspension was casted in petri dishes of 90 mm in diameter and dried for at least 5 days in a conditioned room (23 °C, 50%RH) and resulted in 200 mg CNF-e membranes (about 30 g/m²). An aqueous solution of ciprofloxacin was prepared in 0.1 M HCl and CNF-e membranes were immersed for 15 min. The membranes were carefully recovered and excess ciprofloxacin solution was removed. A thermal treatment was applied for 24 h at 50 °C in a büchi oven (under vacuum) to trigger the esterification reaction. Membranes were then subjected to purification step using soxhlet extraction with high purity acetone and deionized water to remove contaminants, potential degradation products and the unbound ciprofloxacin respectively. These membranes grafted with ciprofloxacin will be designated as CNF-cip-g.

Preparation of CNF-e membranes with adsorbed ciprofloxacin (CNF-cip-ads)

A second type of CNF-e membrane was prepared by adding ciprofloxacin to a CNF-e suspension in order to allow its encapsulation through adsorption onto the nanofibrils surface. The CNF-e suspension was dispersed at 1 wt% concentration into deionized water using high shear IKA Ultra-Turrax device at 10,000 rpm for 15 s. Ciprofloxacin was dissolved in deionized water at a concentration of 2 g/L. The pH was decreased at 2.5 in order to favor the dissolution mechanism. A specific volume of this solution was then added to the 1 wt% CNF-e suspension so that the mass of ciprofloxacin accounts for 1% of the total mass of the membrane. The suspension was then magnetically stirred for at least 15 min and sonicated 30 s with a SONOREX Ultrasonic batch (Bandelin) to remove air bubbles. The suspension was then casted into 90 mm diameter petri dishes and drying was done with two different techniques. Room temperature drying was done in conditioned room at 23 °C and 50% relative humidity for at least 5 days (designated with “RT” for room temperature). Overdrying procedure was done on some of the RT dried samples in

order to further decrease the water content. The thermal treatment was performed in the oven at 150 °C for 2 h (designated as “overdried”) based on previous study (Smyth et al. 2017). Membranes of different thickness were prepared by varying the quantity of CNF-e suspension casted in the petri dish. Theoretical values of 200 mg and 400 mg of dry material were targeted, corresponding to 20 ml and 40 ml of 1 wt% CNF-e suspension. These CNF-e membranes loaded with adsorbed ciprofloxacin will be designated as CNF-cip-ads.

Physical characterization of CNF-e

CNF-e suspensions were analyzed with optical microscopy at 20× magnification on an Axio Imager A2 device equipped with an AxioCam MRm camera (Carl Zeiss, Germany). Dark field observation mode was used to obtain contrasted images. Atomic force microscopy (AFM) images were recorded on a Dimension icon® (Bruker, USA). CNF-e films were deposited on adhesive tape before stabilizing overnight at room temperature. The acquisition was performed in tapping mode using a silica coated cantilever (OTESPA® 300 kHz–42 N/m, Bruker, USA). Zones of 1 × 1 μm² were analyzed and the most representative height sensor images were chosen for analysis.

CNF-e membranes characteristics such as thickness and grammage (basis weight in gram per square meter) were evaluated. Membranes were precisely weighted on analytical scale (Mettler Toledo, Switzerland). Grammage was then calculated by dividing the weight of the CNF-e membrane by its surface. Thickness of the samples were measured by two complementary techniques; a M120 micrometer (Adamel Lhomargy, France) and scanning electron microscopy (SEM, Quanta200®) imaging of CNF-e membranes cross sections were used. Carbon tape was used to immobilize samples on supports for SEM imaging, and they were then coated with a thin layer of pure carbon thanks to an EMITECH® K450X carbon coater. The working distance was 10 mm with a 10 kV voltage and a magnitude of 1500×.

Then, the theoretical density ρ was calculated by dividing the weight of the films by the product of membranes surface and thickness. The porosity Por of the CNF-e membranes were calculated by considering

a value of 1.5 g/cm^3 for the CNF-e material mass volume ρ_{cell} with the Eq. (1).

$$Por = 1 - \frac{\rho}{\rho_{cell}} \quad (1)$$

Water uptake

The water uptake of the samples was assessed with two different methods. The first one was based on the total immersion in deionized water of 16 mm diameter discs of the CNF-e membranes which were weighted. The water uptake was measured at predetermined time intervals t , from 1 min up to 48 h. After immersion the excess water was removed with blotting paper and the sample was weighted again. The water uptake by immersion $W_i(t)$ was calculated with Eq. (2):

$$W_i(t) = \frac{m_t - m_{\text{before}}}{m_{\text{before}}} \times 100 \quad (2)$$

where m_{before} and m_t are the weight before the immersion and at the predetermined interval t respectively. The second method involved an agar gel on which the 16 mm diameter samples were deposited. The sample is expected to suck up the water in the agar gel from one side only. This is why this method better mimics the wound environment. Equation (2) was also used to calculate the water uptake from agar plate absorption $W_a(t)$. Triplicate measurements were performed for each analysis.

Release study methods

Release study of ciprofloxacin was performed with three different methods for CNF-e membranes. The first one simply exposes the samples to the release medium with a full immersion, the second one involves a specific device composed of two chambers separated by the sample and the last one uses non porous agarose gel in order to better mimic wound environment solid phase. The two first tests are continuous release system where the release medium remains the same throughout the whole experiment, while in the last test the release medium is renewed regularly. For release study, only CNF-e membranes with adsorbed ciprofloxacin (CNF-cip-ads) were characterized. Indeed, these tests are really time-consuming and devoted to detect free unbound molecules, which are supposed to be removed with

the soxhlet extractions performed on ciprofloxacin grafted membranes (CNF-cip-g). However, upon the antibacterial assays detailed later in this section, a comparison between the two types of membranes will be carried out since different modes of action are expected.

Continuous release systems: immersion release

Immersion release study was performed in a 500 ml volume of deionized water in order to achieve sink conditions (a sufficient dilution state so that the released drug do not influence the release mechanism). Experiments were done in triplicates with three different ciprofloxacin loaded CNF-e membranes (90 mm in diameter, around 200 mg) and an orbital shaker was used to maintain a slow agitation (75 rpm) while the whole system was kept at $37 \text{ }^\circ\text{C}$ in an incubator as illustrated in Fig. 1a. The CNF-e membranes were placed on a lifted mesh with big pore size inside the container in order to ensure an equivalent release from both sides of the membranes and limit CNF-e membrane deterioration. At predetermined time intervals an aliquot of 3 ml was withdrawn from the release medium and ciprofloxacin quantity was measured with UV spectroscopy at a wavelength of 271 nm (UV1800 Shimadzu), using the previously mentioned calibration curve.

Continuous release systems: release chamber

An experimental device was also developed in the laboratory (LGP2) to be able to expose the two faces of CNF-e membranes to two different media such as liquid/liquid or liquid/air. Figure 1b shows a graphic description of the device.

This system controls the flow rate of liquid which comes into contact with the sample surface in a closed loop re-circulation. It is thus possible to flow liquid in recirculation only on one side of the sample in order to stimulate the release mechanism while the other side remains in contact with air, mimicking topical applications. Aliquots of 3 ml were collected at predetermined time intervals from the flowed medium and ciprofloxacin concentration was measured with UV spectroscopy at 271 nm. At least duplicates were performed for each sample.

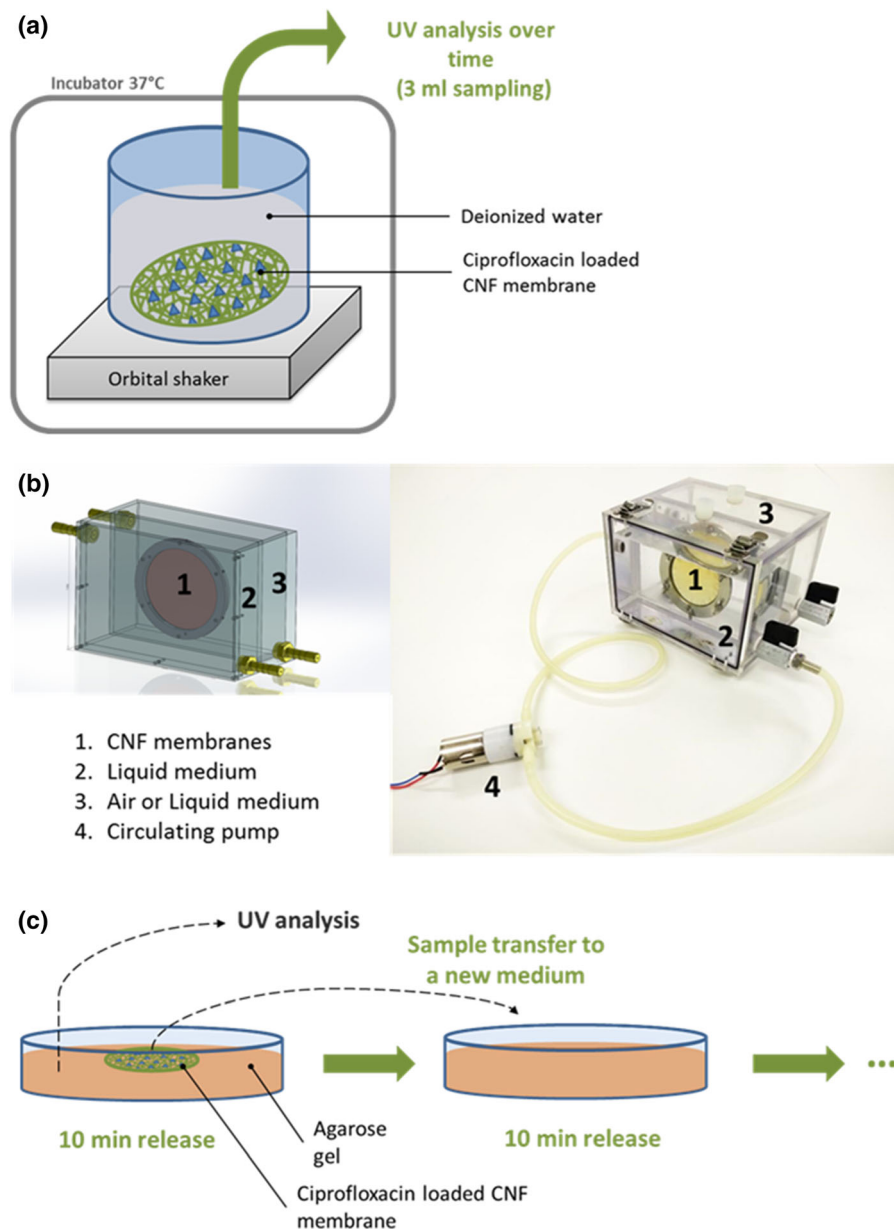


Fig. 1 Graphical description of the drug release experimental set ups, **a** continuous release, **b** release chamber and **c** intermittent release method

Intermittent release system

A third system was designed to further mimic the environment of a low exuding wound. A gel state release medium, considered as *solid* compared to previous *liquid* media, was prepared. By inspiring from nutrient agar medium used in antimicrobial testing, we chose pure agarose as a release medium. It

allowed to avoid any interactions between the releasing drug and components of nutrient agar that might affect the release dynamic while ensuring a gel state modeling low exuding wound environment. Also, release study have already been performed in agarose gel in the literature and is considered as more biorelevant for *in-vitro* measurement (Hoang Thi et al. 2010). Agarose powder was dissolved in hot

deionized water based and poured into 45 mm diameter petri dishes. After cooling down and gelification, non-porous agarose hydrogels were obtained and constituted a solid (gel state) release media. Circular disks of 10 mm in diameter were cut from ciprofloxacin loaded CNF-e membranes and were deposited on the surface of the agarose hydrogel to allow the drug to leach out of the sample from only one side as illustrated on Fig. 1c.

Every 10 min, the disk sample was recovered from the agarose hydrogel and transferred to a fresh agarose medium. This technique is used to renew the release medium and mimic the renewal of body fluids in the solid low exuding wound environment. This will be referred as a “wash” step in the results charts. After sample transfer, the agarose hydrogel was turned to liquid state with the use of micro-wave heating for at least 60 s and UV spectroscopy was used to measure the concentration of ciprofloxacin that was released from the membrane inside the agarose. Ciprofloxacin resistance to micro-wave treatment has been previously checked. Triplicate measurements were done for each sample.

Antimicrobial activity

Zone of inhibition (ZOI) testing Antimicrobial activity of the ciprofloxacin loaded CNF-e membranes was assessed through the zone of inhibition test which is inspired from the AFNOR standard NF EN 1104 test. Disks of the CNF-e membranes (10 mm in diameter) were first sterilized by a thermal treatment in an oven at 50 °C overnight. Nutrient agar was inoculated with one bacterial strain (*B. subtilis*, *E. coli* or *S. aureus*) and 10 ml of the solution was poured into 90 mm petri dishes. Once the agar solution turned to gel state, disks samples were deposited on the agar surface. After incubation during 3 days (72 h) at 37 °C, inhibition zones were detected or not onto the samples, indicating whether the ciprofloxacin has leached out of the membranes or not. The diameter or the radius (from the center of the CNF disk) of the circular inhibition zone was measured in order to quantitatively assess the antibacterial activity. A reference material was used to compare CNF-e membranes with a commercial product. A classic gauze membrane soaked into ciprofloxacin solution was chosen.

Successive ZOI were also performed. After the first 72 h of incubation over weekend, samples were transferred to another petri dish that contained also bacteria inoculated agar. Another incubation of 24 h was applied and zone of inhibition sizes were assessed. This operation was repeated every day. This complementary test evaluated the antibacterial activity of the samples over the whole week following the first 72 h incubation in order to simulate successive release like in the previous intermittent system. At least triplicates were performed for this test.

Dynamic shake flask Dynamic Shake Flask testing puts in contact the CNF-e membranes with a liquid medium that contains bacteria. Isotonic (8.5 g/l NaCl) and nutrient broth solution were prepared in deionized water. Inocula were prepared by diluting bacteria suspensions in 1/500 nutrient broth (1 ml of nutrient broth in 500 ml of isotonic solution) to reach a 5×10^5 CFU/ml concentration (CFU stands for colony forming units). Previously dry sterilized (16 h at 50 °C) and weighted (about 50 mg) CNF-e membranes were cut in small pieces of ca. 0.5×0.5 cm² and placed inside Erlenmeyer flasks in which 10 ml of inoculum was added. The flasks were then incubated for 24 h at 37 °C and under orbital stirring at 100 rpm.

After the incubation, the new bacterial concentration was measured through successive dilutions of the liquid medium in 1.5 ml eppendorfs. A volume of 100 µl was taken out of each Eppendorf and was added in petri dishes before addition of liquid agar (around 10 ml). When agar cooled down and turned to gel-like state, petri dishes were incubated overnight at 37 °C. Bacterial concentration of incubated filtrates was calculated with the following (3):

Bacterial Concentration

$$= \frac{\text{Number of bacteria counted in petri dish } n^{\circ} X}{0.1 \times \text{dilution factor of petri dish } n^{\circ} X} \quad (3)$$

The 0.1 value refers to the 100 µl added in each Eppendorf during successive dilutions. At least duplicates were performed for this assay.

Leaching assay On contrary to Dynamic Shake Flask, Leaching Assay test does not put directly into contact the samples and the bacteria. Previously dry

sterilized (16 h at 50 °C) ciprofloxacin loaded CNF-e and reference CNF-e membranes pieces (about 50 mg) were first incubated for 24 h at 37 °C and 100 rpm in a volume of 10 ml of 1/500 nutrient broth (1 ml of nutrient broth in 500 ml of isotonic solution). The liquid were then collected and filtrated with 0.45 µm pore size syringe filter in order to remove the CNF-e materials. Bacteria suspensions were prepared according to supplier information and were added to the recovered filtrates at a 10^4 CFU/ml concentration. This allowed figuring out if ciprofloxacin molecules were released during the incubation step and would inhibits the bacterial growth. After incubation for 24 h at 37 °C and 100 rpm, bacterial concentrations were measured through the same method than for Dynamic Shake Flask and Eq. (3).

The test is used to determine quantitatively the effect of potential release of ciprofloxacin from the samples. If the filtrates from reference CNF-e membranes (CNF-ref) and the filtrates from ciprofloxacin loaded CNF-e membranes present the same bacterial concentration, it means that no Ciprofloxacin was released. Duplicates were performed to further confirm the results. Both CNF-cip-ads and CNF-cip-g samples were compared with these antimicrobial test set ups.

Results and discussions

Physical characterization of CNF-e membranes

The CNF-e suspension was homogeneously dispersed as depicted on Fig. 2a. The CNF-e membranes were transparent and proved to be composed of nanosized fibrils as confirmed by picture and height sensor AFM image of Fig. 2b, c. Moreover, AFM images were obtained before and after the release experiments and no differences were observed.

Different qualities of CNF membranes were prepared in order to check the influence of thickness and drying on release profiles. Indeed, a recent study has shown that such thermal treatment (150 °C for 2 h) of CNF-e membranes modify their Young's modulus in aqueous medium. The thinnest the membrane, the higher the increase of “in-liquid” mechanical properties after overdrying (Smyth et al. 2017). This study proves that thermal treatment influences the structure of CNF-e membrane and so the release mechanisms.

The difference of thickness between prepared samples will also influence the distance and specific surface area available for adsorption–desorption mechanisms that molecules undergo when leaching out of the membranes. Considering a specific surface area of 150 m²/g for the CNF-e, the surface of exchange will be of 30 m² and 60 m² for membrane of 200 mg and 400 mg respectively.

The Table 1 summarizes the characterization of CNF-e membranes in terms of grammage and thickness. Membranes produced with a 200 mg dry mass target have a grammage of 29 g/m² and 400 mg dry mass membranes, 58 g/m². The thickness was measured with micrometer. The grammage and thickness values are similar, 29 g/m² membranes have a 29 µm thickness and 58 g/m² membranes have a 58 µm thickness, which confirms the high density of the membranes as shown by the values of Table 1. CNF-e membranes were also characterized with SEM and similar thicknesses were observed (see Fig. 2d for 400 mg membranes). Also, the porosity obtained through Eq. (1) was roughly the same for both films and in accordance with data found in the literature (Henriksson et al. 2008).

Water uptake of CNF-e membranes

One of the key properties of wound-dressing for an appropriate healing is their ability to absorb exudate. Indeed, a high water uptake will allow the wound-dressing to be used on lightly and heavily exuding wound by removing the exudate from the wound and thus preventing maceration (which make the skin more prone to damage). Maceration with the exudate is known to prolong the inflammatory phase and is detrimental to healing.

Water uptake of immersed CNF-e membranes (without ciprofloxacin) was recorded over 48 h however, the absorption was very fast and data recorded in the first 10 min are presented on Fig. 3a. Membranes with lower grammage show a water uptake of 400% while membranes with higher grammage reveal a water uptake of 200%. CNF-e membranes with lower grammage were more fragile and excess water at the surface of the membranes could not be removed properly without tearing the membranes apart. Higher grammage membranes have better mechanical properties and were not affected by the removal of excess water. The maximum water uptake is quickly reached

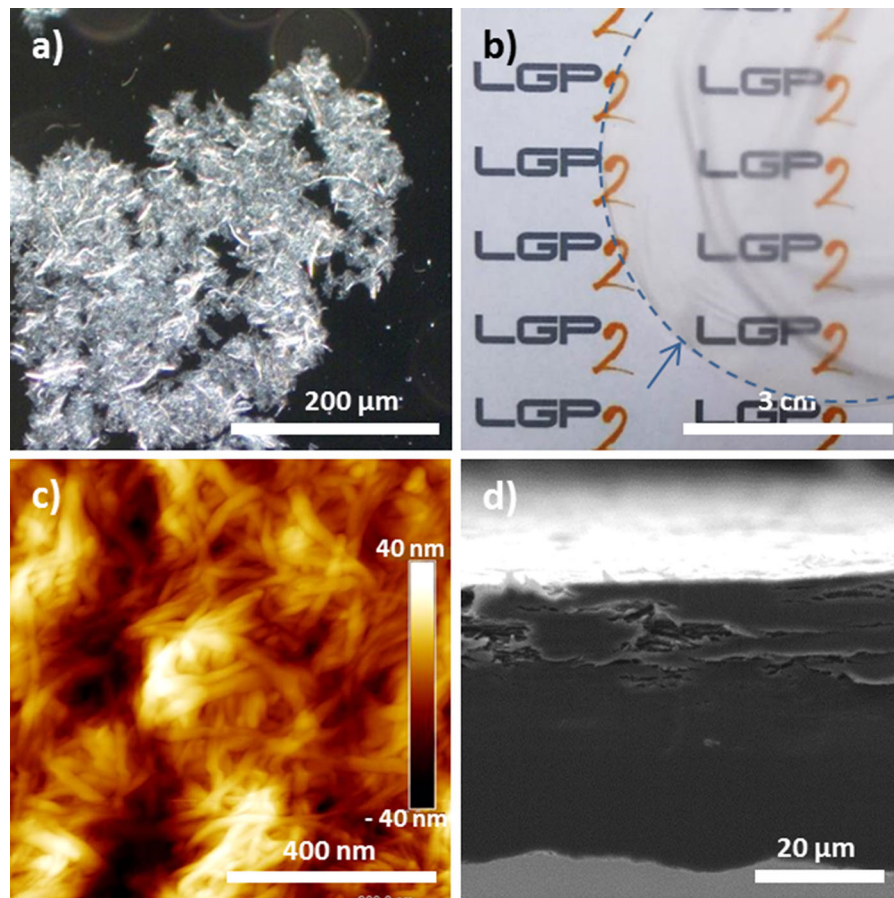


Fig. 2 Morphology of CNF-e suspension and membrane, **a** optical microscopy ($\times 20$) in dark field mode of 0.1 wt% CNF-e suspension, **b** picture (85 mm in diameter, the blue arrow

shows the edge of the film in dotted line), **c** AFM height sensor and **d** cross-section of the CNF-e membrane

Table 1 Physical characterization of CNF-e membranes

| Targeted weight of CNF films mg | Grammage | | Thickness | | Theoretical density | | Theoretical porosity (%) |
|------------------------------------|----------|---|-----------|---|---------------------|------|--------------------------|
| | g/m^2 | | μm | | g/cm^3 | | |
| 200 | 29 | 1 | 28 | 2 | 1.04 | 0.08 | 31 |
| 400 | 58 | 2 | 58 | 4 | 1.00 | 0.08 | 33 |

in about one minute for both types of CNF-e membranes. Even if thin membranes absorb more water in proportion, the absolute quantity of absorbed water is higher for thicker membranes as depicted in Fig. 3b. At least 2 h are required to clearly see a strong and stable difference. Moreover, this result also indicates that when the CNF-e quantity is doubled from 200 to 400 mg membranes, the absorbed water quantity is

really far from being doubled even after a 48 h immersion.

Overdrying of CNF-e membranes was proven to improve the mechanical properties in liquid (Smyth et al. 2017). Water uptake capacity was also assessed for overdried CNF-e membranes in order to overcome the limitation of weak samples. After 48 h of immersion of CNF-e membranes, a similar water uptake for

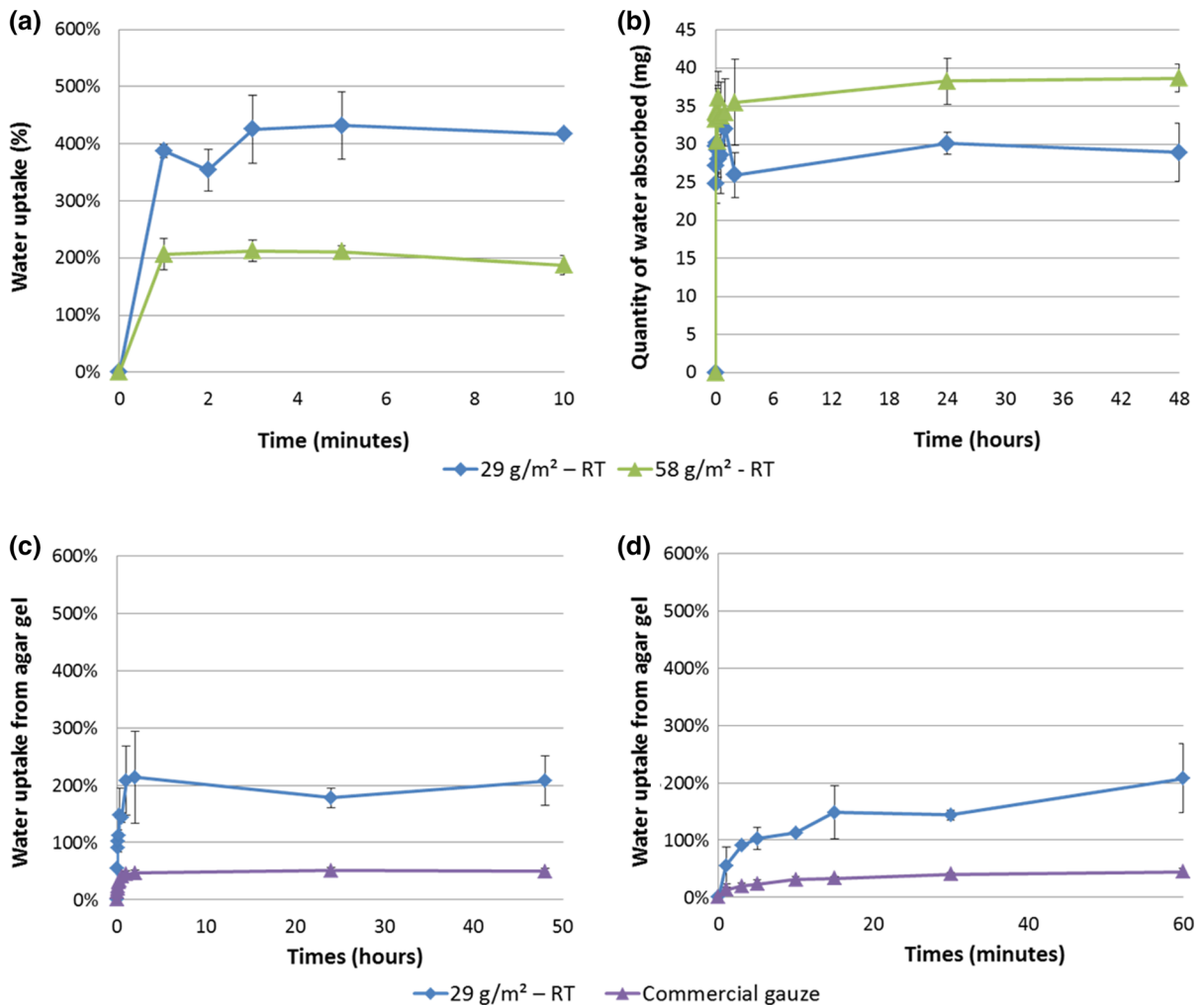


Fig. 3 Water uptake $W_i(t)$ of CNF-e membranes of two different grammage, **a** over the first 10 min and **b** absolute quantity of water absorbed over the whole 48 h of experiment.

all the samples was revealed, regardless of their grammage.

Complementary experiments were carried out with another water uptake test. Agar gel medium was used in order to better mimic the behavior associated with liquid absorption on a topical wound solid phase. A commercial wound dressing gauze was also tested and compared to the CNF-e membranes in terms of water uptake. The water uptake was measured for low grammage CNF-e membranes and wound dressing gauze over 48 h (Fig. 3c), a zoom in the first 60 min is also exposed (Fig. 3d). Maximum water uptake of CNF-e membranes is two times greater than that of the gauze, reaching 200% of the initial mass. This result is

much lower than the 400% reached by the low grammage CNF-e membranes samples with the previous test where full immersion in liquid was used. Here, samples were easier to recover because no excess water was to be removed.

These results suggest a high water uptake capacity for CNF-e membranes, reinforcing the interest of CNF for medical device development. Hydrophilicity of cellulose, together with the nanostructured network of CNF-e membranes, explain the relevant water absorption behavior. The higher capillary absorption with such CNF membrane was expected and already reported by previous research on hemostatic application (Basu et al. 2017; Sukul et al. 2017). Dimensional

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swelling that is associated with the water-uptake of CNF-e membranes justifies the high values, but it was difficult to measure because of the low thickness of the membranes and their relative weak mechanical properties in wet environment.

Innovative medical devices need to present active properties. Ability to release active principle ingredients (API) to better favor healing procedure and prevent infection thus appear as a promising opportunity for CNF-e based medical devices.

Release study

Based on recent literature previously cited, CNF-e membrane nanostructured network seems to be a good candidate for API encapsulation and subsequent release. The release profiles of ciprofloxacin molecule from CNF-e membranes were then investigated for the first time. The influences of the CNF-e membranes production method as well as the type of CNF were studied. The medium chosen for release experiment is deionized water. Actually, other medium such as phosphate buffer saline (PBS) are available and often use in release study experiments since they mimic the human body fluids (Smyth 2017). However, some preliminary experiments of release of ciprofloxacin in PBS medium showed strong interaction of the drug with the ions of the buffer. With deionized water as the release medium, the influence of the above mention parameters on the release profiles is expected to be highlighted, without any interfering phenomena due to the presence of ions.

The effect of CNF-e membranes drying procedures was first investigated with 29 g/m^2 and 58 g/m^2 membranes that were dried (1) at room temperature over several days or (2) at room temperature over several days followed by an overdrying treatment at $150 \text{ }^\circ\text{C}$ for 2 h. The release of ciprofloxacin molecule was then measured with immersion protocol over at least 48 h in triplicates for each membrane. The release profiles of four different samples, dried according to the two afore mentioned drying procedures, are shown on Fig. 4. The four samples reached the same maximum release rate around 70% of the theoretical quantity of ciprofloxacin as depicted on Fig. 4a. This first result indicates that the overdrying ($150 \text{ }^\circ\text{C}$ for 2 h) do not trigger any chemical immobilization of ciprofloxacin onto the CNF-e membranes. It also confirms the stability of ciprofloxacin

molecules inside CNF-e membranes, when exposed to this thermal treatment. A zoom into the first 30 min of the release expose on Fig. 4b, proves that for 29 g/m^2 membranes, overdried samples kinetic of release is similar than that of room temperature dried ones. Only 3 min is necessary for both samples to reach 70% of drug released.

However, for thicker membranes of 58 g/m^2 , the tendency is not the same and overdried samples seem to behave slightly different even if we consider the high deviation obtained for the data point at 10 min on Fig. 10b. The overdrying is limiting the burst effect and creates a more prolonged release. This result suggests that a more controlled release of drug can be achieved by increasing the thickness of CNF-e membranes.

More precisely, only 5 min are required for 29 g/m^2 membranes to reach the maximum release rate of 70%. In 5 min, 58 g/m^2 membranes are barely at 40% for the room temperature dried membranes and 20% for overdried membranes. Thicker membranes will also need at least 30 min to reach the maximum release rate, which is six time longer. The analysis of additional quantity of drug released in between two measurement points also helps the discussion: between 5 and 10 min, the thinner membranes release 1–2% of ciprofloxacin (1–2 mg) whereas thick membranes will still release about 10% of ciprofloxacin (40 mg). The same result can be extracted from additional quantity between 10 and 15 min or 15 and 30 min. In thicker CNF-e membranes, the time required to swell the nanofibrils network across the section is higher. So the drug diffusion is longer, which explains the shift observed on Fig. 4b.

Another release device has been designed to expose one side of the CNF-e membrane to a liquid release medium in continuous closed loop re-circulation while the other side is in contact with the air. This better mimic the external application for heavily exuding wounds. As mentioned before and confirmed with above described experiments, overdried samples exhibit a higher resistance to liquid medium exposition and prolonged release abilities. They were thus chosen to carry on the study with the release chamber device. Figure 5 shows that the closed loop re-circulation of liquid medium in the device provided an extended release for 29 g/cm^2 membranes. Indeed, about 90% of the theoretical amount of ciprofloxacin was released in 24 h in these dynamic conditions

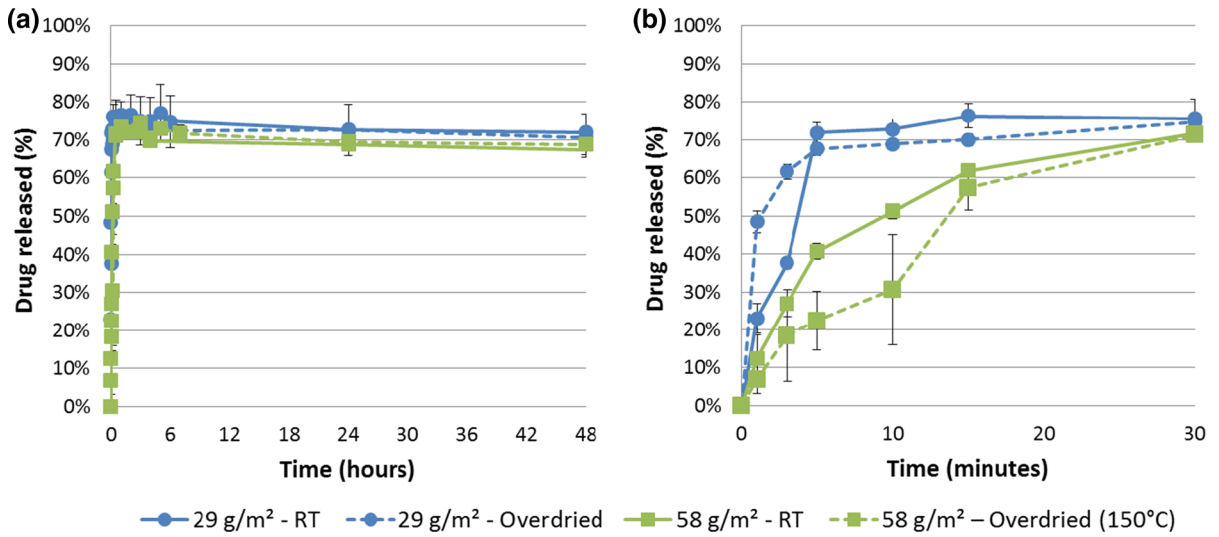


Fig. 4 Influence of drying procedures and CNF-e quantity on drug release profile of 29 g/m² and 58 g/m² ciprofloxacin loaded CNF-e membranes over a **48 h** and **b** zoomed in the first

30 min of continuous release immersion experiment (the legend is common to both the charts)

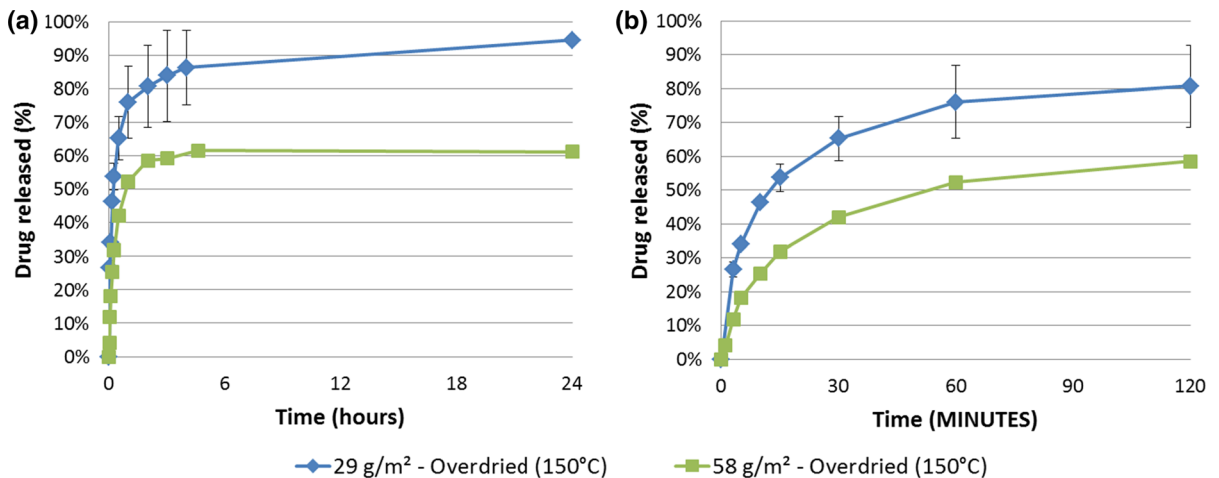


Fig. 5 Release study for overdried ciprofloxacin loaded membranes in the release chamber with closed loop liquid medium recirculation (the legend is common to both the charts)

while only 70% was released with the mild agitation in the immersion protocol used in the previous studies. This tendency is not observed for the thicker membrane and suggests that the ciprofloxacin entrapped in the membrane side that is exposed to the air is more complicated to retrieve with the re-circulating liquid medium. This could also be a reservoir layer for longer time release.

A third set up of experiment was used to further characterize the overdried samples. This one was designed to mimic low exuding wound environment.

Samples were in contact with agarose gel that was renewed every 10 min (the “wash” step). Figure 6 shows the comparison of the drug concentration evolution in agarose media for 29 g/m² and 58 g/m² overdried membranes, over the number of washing steps. Similar evolutions are observed with a strong decrease from the first washes toward stabilization after about 10 washes. The lowest minimum inhibitory concentrations of ciprofloxacin for bacteria commonly found on wound infection sites (*Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Streptococcus*

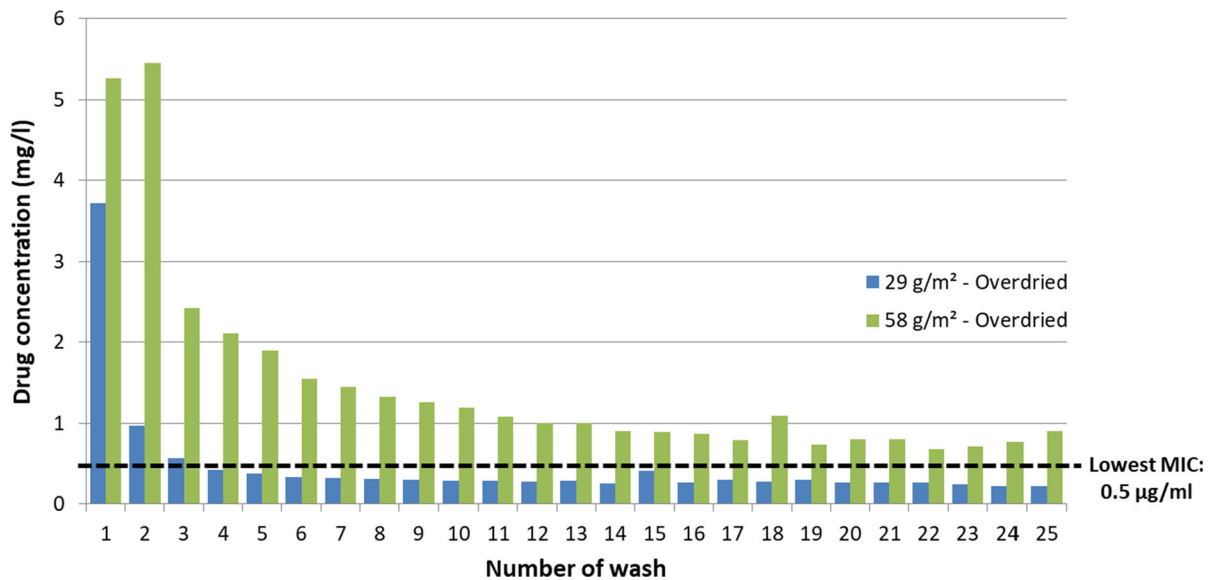


Fig. 6 In solid release study, agarose gel was used to release ciprofloxacin from 58 g/m² overdried membrane

pneumoniae) is 0.5 µg/ml (Dow et al. 1999; Hooper and Wolfson 1991; Markham 1999). The thin membranes can be considered as non-active since the drug concentration that was detected rapidly goes under this value after 3 washes. On the contrary, after 25 washes, thick membranes still release significant amount of ciprofloxacin.

CNF-e membranes with increased thickness (and associated grammage) that are overdried are recommended for the development of active medical devices for topical applications since they exhibit better resistance when exposed to liquid medium and revealed a more controlled release in immersion conditions together with active behavior in agarose release system. These samples will be used in priority for the antimicrobial testing and referred as CNF-cip-ads.

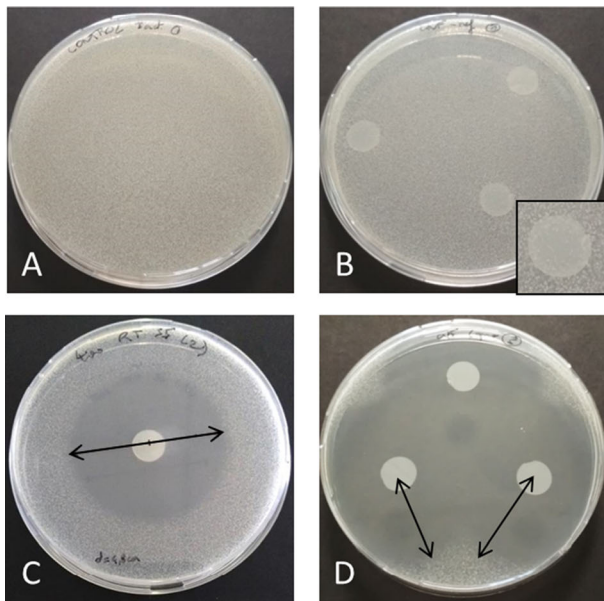
Antimicrobial activity

Antibacterial activity testing was carried out in order to evaluate the capacity of ciprofloxacin loaded CNF-e membranes (CNF-cip-ads, *overdried* and CNF-cip-g) to be active against bacterial strain. In parallel, reference CNF-e membranes of 29 g/m² without ciprofloxacin are also characterized and referred to as CNF-ref. Widely used *B. subtilis* strain was chosen to perform zone of inhibition (ZOI) testing as depicted on Fig. 7 where a clear ZOI is observed on CNF-cip-

ads (C) and CNF-cip-g (D) samples while CNF-ref disks do not exhibit any activity (B) even close to the disk sample (inset). Both samples proved to be strongly active against *B. subtilis* since ZOI radius of 2.4 and 2.6 cm were measured confirming the antibacterial activity of such CNF-e membranes.

All the samples were then exposed to a new inoculated medium during 24 h of incubation for 3 more cycles. Figure 8 shows the evolution of ZOI radius over this prolonged incubation. CNF-cip-ads samples were tested against *B. subtilis* only while CNF-cip-g samples were tested against *E. coli* and *S. epidermidis*. In both cases, CNF-ref membranes do not show any antibacterial activity. The activity of CNF-cip-ads membranes described by the ZOIs radius is strongly decreasing with the number of cycle from 2.4 cm until it reaches zero. This membrane is detected to be inactive within only 3 cycles. CNF-cip-g membranes show a smaller ZOI radius of 1.0 and 1.2 cm but a different phenomenon occurs after cycle 1. The detected ZOI are limited to the edge of the sample disk indicating a contact active antibacterial behavior. This suggests that the covalently bound ciprofloxacin acts locally at the surface of the CNF-cip-g membrane, which confirms a prolonged activity against both gram positive and gram negative bacterial strain.

The test of ZOI detection is only qualitative and performed in static conditions. However, most of the

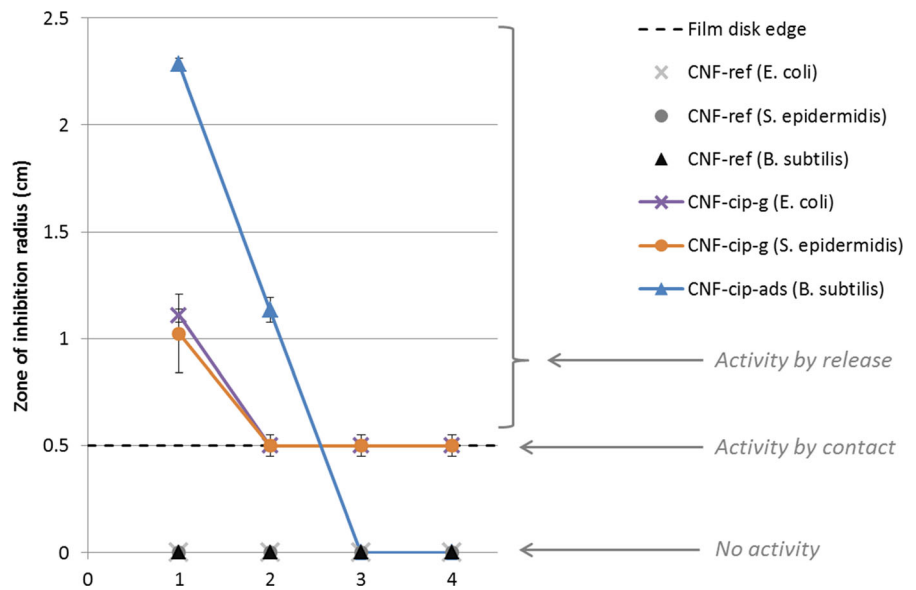


| | | ZOI radius (cm) |
|---|-------------|-----------------|
| B | CNF-ref | 0 |
| C | CNF-cip-ads | 2.4 |
| D | CNF-cip-g | 2.6 |

Fig. 7 Zone of Inhibition testing of CNF-cip-ads (overdried) and CNF-cip-g against *B. subtilis* strains and values of ZOI radius. Picture A proves the correct growth of the bacterial

strain. Pictures B, C and D refer to CNF-ref, CNF-cip-ads and CNF-cip-g respectively

Fig. 8 Successive ZOI experiments results, the radius of ZOIs is plotted against the number of cycle of exposition, only 29 g/m² membranes are compared. CNF-cip-ads samples are tested against *B. subtilis*; CNF-cip-g samples are tested against *S. epidermidis* and *E. coli*



release experiments that were discussed in the previous section were performed in dynamic conditions, especially those that were set up to mimic heavily exuding wounds (continuous release in immersion with orbital shaking or in the release chamber with closed loop re-circulation). Complementary antibacterial testing is necessary to assess the activity of the

ciprofloxacin loaded CNF-e membranes in similar conditions.

In the dynamic shake flask protocol, samples are put in contact with liquid medium that contains bacterial strains for several hours. The quantitative evolution of the logarithm of bacterial concentration over incubation time reveals the activity of the CNF-e

substrates, as displayed on Fig. 9. CNF-ref samples give a very similar result when compared to the positive control (“No sample”). From time 0 to 3 and 24 h of incubation, a growth of bacteria is suggested by the increase in bacterial concentration observed for both strains from 5.5 log to more than 7.5 log. On the contrary, ciprofloxacin loaded CNF-e membranes present strong decreases in bacterial concentration. Within only 3 h, the CNF-cip-g samples reduce the bacterial concentration to zero while the CNF-cip-ads membranes only give a 2 log and 1 log reduction for *E. coli* and *S. aureus* respectively. After 24 h of incubation of the samples in the bacteria containing liquid medium, both ciprofloxacin loaded CNF-e membranes reduce the bacterial concentrations to zero. The CNF-cip-g antibacterial activity is stronger than CNF-cip-ads samples in Dynamic Shake Flask test conditions. The 24 h release applied prior to the test must have depleted the ciprofloxacin quantity of CNF-cip-ads whereas the covalently bound ciprofloxacin in CNF-cip-g samples was not affected.

In Dynamic Shake Flask test, samples are in direct contact with the bacteria. The bacterial growth inhibition can thus be explained both by the release of active molecule and contact active inhibition phenomena. In order to be able to draw precise conclusions, a last complementary test was used. As for the Dynamic Shake Flask, it measures quantitatively the evolution of bacterial concentration of an inoculated medium, but this liquid does not contain the sample itself. The sample was exposed to the liquid and then

removed before the test, eliminating the possibility of contact active inhibition phenomena. In the Leaching Assay, samples are put in contact with a liquid medium that does not contain bacteria, in immersed conditions for 24 h (incubation 1). After this, the solid samples are recovered and then the liquid is inoculated with bacteria and incubated for 24 h (incubation 2). If some active substances leached out from the samples during the incubation 1, the bacterial growth during incubation 2 will be affected. If the sample did not release any active substances, bacteria concentration is supposed to remain stable or to slowly increase with bacterial growth.

The logarithms of bacterial concentrations after the incubation 2 are compared in Fig. 10 for each sample, including a positive control that was not put in contact with any CNF-e substrates. The positive control shows a bacterial growth up to 6.1 and 6.6 log for *S. aureus* and *E. coli* respectively, compared to the initial bacterial concentration of 4.1 log. CNF-ref samples have a very similar response since bacterial growth is confirmed for both strains. For *E. coli* the bacterial growth is significantly higher than the positive control, suggesting that the CNF-e promote the bacterial growth. This has been already observed and indicates good nutrient conditions for bacterial growth (Saini et al. 2015). The CNF-cip-ads sample obviously released ciprofloxacin molecules during the immersion since these conditions are exactly the same than for immersion release experiments discussed previously. After 24 h of incubation with bacteria, the

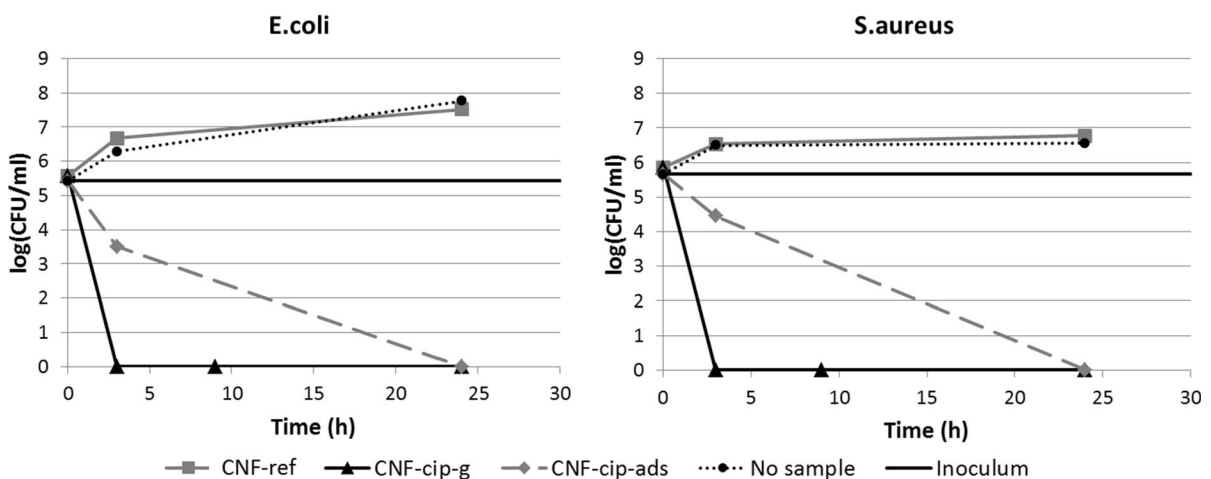


Fig. 9 Dynamic shake flask test applied on CNF ref, CNF-cip-ads and CNF-cip-g samples against two bacterial strains, *E. coli* and *S. aureus*

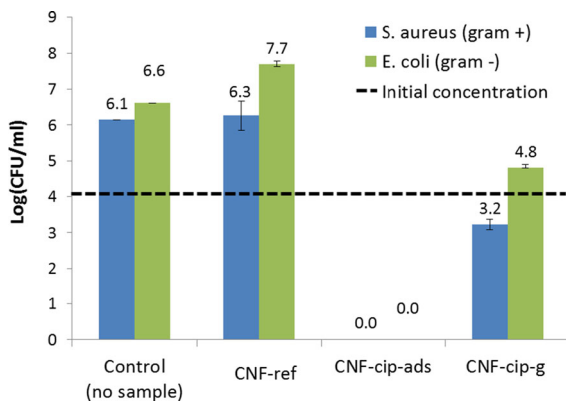


Fig. 10 Leaching assay that assess the release of active substances from the samples, CNF-ref, CNF-cip-ads and CNF-cip-g. All samples were subjected to a 24 h release in immersed conditions before the test

liquid medium does not exhibit any remaining bacterial activity. The CNF-cip-g sample shows a bacteriostatic effect since a log variation inferior to 1 compared to the initial concentration is detected. This result confirms again that ciprofloxacin is actually covalently bound to the CNF-e surface and did not leach out from the membrane during the incubation 1. Moreover, the initial concentration for Leaching Assay is 1 log inferior to that of Dynamic Shale Flask. A ten times less concentrated medium is much more sensitive to the presence of active compounds, which also confirms the insignificant effect of CNF-cip-g in these conditions.

This last result closes the comparison between CNF-cip-ads and CNF-cip-g in terms of antibacterial activity. Both samples demonstrated strong antibacterial activity against gram positive (*B. subtilis*, *S. aureus*, *S. epidermidis*) and gram negative strains (*E. coli*), CNF-cip-ads by release mechanisms and CNF-cip-g by contact active phenomena. CNF-cip-g is then preferable when a quick and persistent long term activity as shown on Figs. 8 and 9.

Conclusion

Overdried thick CNF-e membranes proved to be able to absorb more water and better resist the exposition to liquid medium. The multiple release study experiments allowed the ciprofloxacin loaded CNF-e membranes to be exposed to different conditions that mimicked both heavily and low exuding wounds

environment. Thick overdried membranes demonstrated the most prolonged release in immersion and release chamber protocols. Compared to thin overdried membranes, they were also more active in the intermittent release protocol with above-MIC ciprofloxacin concentrations. The antibacterial experiments were used to compare CNF-e membranes with bulk adsorbed ciprofloxacin (CNF-cip-ads) versus CNF-e membranes with surface grafted ciprofloxacin (CNF-cip-g). Both samples offered a similar response to classic ZOI testing, in static conditions. However, upon successive ZOI measurements, CNF-cip-ads rapidly lost its activity while CNF-cip-g proved to have a stable contact activity thanks to the covalently bound ciprofloxacin. Both CNF-e membranes are then good candidate for the development of medical device for topical applications but CNF-cip-g membranes seems to present better long term persistent contact antibacterial activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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