

In vitro toxicity assessment of hydrogel patches obtained by cation-induced crosslinking of rod-like cellulose nanocrystals.

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Abstract

With the purpose of designing active patches for photodynamic therapy of melanoma, transparent and soft hydrogel membranes (HM) have been fabricated by cation-induced gelation of rod-like cellulose nanocrystals (CNC) bearing negatively charged carboxylic groups. Na^+ , Ca^{2+} , Mg^{2+} have been used as cross-linkers of CNC. The biosafety of this material and of its precursors has been evaluated *in vitro* in cell cultures. Morphological changes, cell organelles integrity and cell survival with the tetrazolium salt reduction (MTT) assay were utilized as tests of cytotoxicity. Preliminary investigation was performed by addition of the hydrogel components to the cell culture medium and by incubations of the CNC-HM in direct and indirect contact with a confluent monolayer of A375 melanoma cells. Direct contact assays suffered from interference of physical stress. Careful evaluation of cytotoxicity was obtained considering the overall picture provided by microscopy and biochemical tests performed with the CNC-HM in indirect contact with two melanoma cell lines (A375, M14) and human fibroblasts. CNCs have been demonstrated to be a safe precursor material and CNC-HMs have a good biocompatibility provided that the excess of cations, in particular of Ca^{2+} is removed. These results indicate that CNC and can be safely used to fabricate biomedical devices such as transparent hydrogel patches, though attention must be paid to the fabrication procedure.

Key words: biocompatibility/soft/tissue; cell adhesion; cytotoxicity; dermal wound dressing;

Running Head: Cytotoxicity of cellulose nanocrystals hydrogels.

Abbreviations: HM: hydrogel membranes; CNC: cellulose nanocrystals; NCF: nanofibrillated cellulose; CNC-HM: CNC hydrogel membranes; TEMPO: 2,2,6,6-tetramethylpiperidine-1-

oxyl radical; FITC: fluorescein isothiocyanate; CNC-FL: CNC films; FBS: fetal bovine serum; MTT: (3-[4, 5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide); PBS: phosphate buffered saline; PI: propidium iodide; PET: polyethylene terephthalate; AFM: atomic force microscopy; STS: staurosporine.

Introduction

Nanocellulose is expected to find increasing applications because of its excellent mechanical performance, the optical transparency, the tunable surface chemistry together with the self-assembly behaviour, which offers a spontaneous route for fabrication of macroscopic structures.¹ The industrial production of nanocellulose is also rapidly expanding since alternative and low cost raw cellulose sources, such as food wastes, and eco-friendly purification procedures are available.^{2,3} Nanocellulose is usually obtained as nanofibrillated cellulose (NCF) and cellulose nanocrystals (CNC). The first consists of finer and branched cellulose fibrils with some amorphous hairpins, that tend to form agglomerates (diameter 20–50 nm, length of few μm),⁴ the second of rod-like or whisker shaped particles, essentially crystalline in nature, with high aspect ratio (3–5 nm wide, 50–500 nm long).⁵ The natural origin and the easy biodegradation make CNFs and CNCs particularly suited for human use. However, the nanosize could make cellulose derivatives toxic. For this reason, a careful evaluation of the safety of nanocellulose must be performed, in particular when it is used as component of biomedical devices. In this regard, one of the straightforward applications of nanocellulose is the production of skin patches. This is a growth industrial sector, since skin diseases are the fourth leading cause of disability worldwide.^{6,7} Due to the easy accessibility of the disease site, the effort is to set-up devices which combine localized delivery and extended release of drugs to the skin. Dryness and depletion of epidermal barrier properties are often associated to skin diseases or are side effects of the therapy, so that delivery

systems preserving skin hydration are preferred. Hydrogels meet this need, thanks to their unique properties such as high water content, softness and flexibility. Unfortunately, in spite of the large therapeutically beneficial outcomes, the high production costs of hydrogels, in particular of those of natural origin, together with the potential toxicity of residual unreacted cross-linkers or of the polymer precursors, are limiting the hydrogel dressing commercialization.^{8,9} Naturally derived cellulose nanostructures are excellent and low cost precursors of hydrogels with properties superior to those of hydrogels of artificial or petrochemical origin which at present dominate the world market.¹⁰ Compact and stable hydrogels of NCF containing negative functional groups are usually obtained with addition of divalent or trivalent cations (Ca^{2+} , Zn^{2+} , Cu^{2+} , Al^{3+} , and Fe^{3+}).¹¹ Conversely, CNC have been considered ill-suited as single component gels due to their limited ability to entangle.² Only recently, extensive sonication and ionotropic gelation have been successfully utilized to obtain soft CNC hydrogel structures of different size and shape with long-term stability and high transparency to visible light.^{12,13} In spite of the many advantages offered by nanocellulose hydrogels, toxicity concern is still an open question. In fact, thorough toxicity testing of hydrogels and all their components has not been widely executed.^{1,2,14} Hazard assessment studies have been performed mainly for NCF and few adverse biological responses have been documented.^{15,16} However, some contradictory results were obtained by investigating the toxicity of NCF films on human dermal fibroblasts exposed to extracts of the films in an indirect contact and in a direct contact cytotoxicity assay.¹⁷ The contradictory results obtained by using two different assays (direct versus indirect) raise the question of which methodologies are suitable for reliable biological evaluation of medical devices. In this regard, the International Organization for Standardization (ISO) 10993-1 and DIN EN ISO 10993-5:2009,¹⁸ suggests to test *in vitro* the cytotoxicity of each component material, including impurities, and constituents associated with processing, as well as the device both,

in direct and indirect contact with the cells. There have been only a few studies concerning the toxicity of CNCs.¹ In particular, cell cultivation experiments consisting in the monitoring of growth propagation and activity of cells in CNC hydrogel scaffolds have been reported¹⁴, while a concentration dependent cytotoxicity was observed on L929.¹⁹ In view of the use of transparent CNC hydrogels as medical devices for topical phototherapy of melanoma, herein we report the first cytotoxicity study of CNCs and hydrogel membranes (CNC-HM) produced by crosslinking CNCs with mono and divalent cations, over melanoma and fibroblasts cell cultures. We proved that CNCs are up-taken by the cells without any adverse effect. By direct and indirect toxicity test performed by an innovative protocol we enlightened the effect of mechanical stress and the role of release of the excess of gelling cations as cause of toxicity. According to a cation-induced toxicity, the use of Mg^{2+} as gelling agent provides superior hydrogel safety together with excellent physico-chemical properties.

Materials and methods

Reagents. All the chemicals were of the highest available purity and were purchased by Sigma-Aldrich. Never dried soft bleach cellulose pulp (Celeste 90) was received from SCA-Ostrand (Sweden).

Preparation of nanocellulose, films and hydrogel membranes. Carboxylic groups were introduced into never dried cellulose pulp by oxidation at alkaline pH and room temperature (RT) using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) as catalyst.²⁰ At the end of the oxidation reaction, the pH was lowered to 6.5 by washing the slurry with an equal volume of high-purity water for at least 10 times. The slurry (50 mL) containing a concentration of TEMPO oxidized cellulose in the range 5-10 g/L, was sonicated by a 13 mm diameter ultrasonic tip (Bandelin Sonopuls HD2200) for 16 min at 20 kHz and 100 W_{eff}

of output power delivered in the sample volume. At the end of this procedure a transparent suspension of rod-like nanocellulose crystallites (CNC) is obtained. The size of CNCs are rather independent of the plant source and are well known to fit in the range 3-5 nm of width and 200-500 nm of lengths (as from AFM and TEM analysis).^{12,20} By a conductimetric procedure we estimated that, the ratio between the carboxylic groups and the cellobiose units is in average 1 / 2.5 (carboxyl group concentration 9.3 mM for a 8 g/L CNC solution).¹³

Preparation of fluorescein isothiocyanate –labelled CNC. Fluorescence labelling of the CNCs was performed via covalent coupling of FITC which reacts with the free hydroxyl groups forming a thiocarbamate bond.²¹ 0.75 mL of 2mM fluorescein isothiocyanate (FITC) were added to 5 mL of a 0.1M NaOH aqueous suspension of CNC (3 mg/mL) and then stirred for 6 days in the dark at RT. The suspension was dialyzed against 400 mL NaOH (0.05M) for 7 days by changing the dialysis solution 4 times and then dialyzed against deionized water until pH 7.0 was reached. The reaction efficiency was verified by acquiring the fluorescence spectra of the labelled CNCs carefully purified from unreacted FITC. At pH 7 fluorescein covalently bound to CNCs shows the absorbance maximum at 491 nm and the maximum emission at 518 nm. The quantum yield of labelled CNCs was estimated according to a procedure outlined by Horiba Scientific²² based on the comparative method.^{23,24} Assuming the quantum yield of FITC equal to 0.75²⁵ we obtained a quantum yield for FITC on CNC around 0.20±0.02. On the basis of a calibration curve obtained with FITC solutions in PBS at pH 7 and considering the measured quantum yield, we estimated that the grafted FITC amount was 1.76 nmol/ g

Preparation of CNC films To obtain CNC films (CNC-FL), the CNC suspension was casted on a Petri dish and left to dry at 60°C for 24 hours in an oven. The thickness of the film was in the range 10-12 µm.

Preparation of CNC-HM. The CNC-HMs were obtained by cation-induced gelation. 25 mL of 8 g/L CNC solution were transferred into an 85 mm diameter Petri dish over which 10 mL of 1M salt solution (CaCl_2 , MgCl_2 , NaCl) were dropped under gentle agitation. Cation induced gelation of CNC is a reaction-diffusion controlled process¹³ and a compact hydrogel membrane is obtained almost instantaneously under gentle mixing of the two reactants. The resulting 85 mm diameter membrane was shaped in smaller CNC-HMs disks using a cylindrical 9 mm diameter die and placed in 20 mL of 1M salt solution (CaCl_2 , MgCl_2 , NaCl) for 24 hours. The CNC-HMs were rinsed in pure water to remove the excess of salt solution from the surface. If not otherwise specified the membrane was used as obtained at the end of this procedure (rinsed-CNC-HMs). In some experiments the CNC-HM were soaked in water or ethanol for 5 days (referred to as water-CNC-HMs and ethanol-CNC-HMs respectively) or soaked in water for 2 hours and then let at 60°C for one hour (referred to as 60°C-CNC-HMs).

Cell cultures Primary dermal fibroblast (Normal, Human, Adult ATCC PCS201012™), malignant melanoma cells (Human, adult, female, A375), were provided by the American Type Culture Collection. The malignant melanoma cell strain (human, adult, male), UCLA-SO-M14, hereafter called M14 was a kind gift of dr. A. Molinari (Istituto Superiore di Sanità, Rome). Cells were grown in monolayer in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (50 U/mL) - streptomycin (50 µg/mL), and 1% non-essential aminoacids in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C.

Cytotoxicity tests MTT assay. Cell viability was assessed by (3-[4, 5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) MTT assay (Sigma Aldrich). This test measures the activity of the enzymes, above all mitochondrial enzymes, which reduce MTT to formazan, imparting a blue/purple color to the dye. After removing cell medium, untreated and treated cells with were washed with

phosphate buffered saline (PBS) solution and incubated with 0.5 mg/mL MTT solution, for 2 h at 37 °C. After removing the MTT solution, the samples were lysed by 100 µL DMSO, and analysed by a microplate reader (Bio-Rad) at 570 nm. Untreated cells, that is cells from wells which did not contain the tested compounds or HMs, were used as a negative control for each MTT measurements. As positive control, 1 µM staurosporine (STS) from Sigma Aldrich for 24 h was used. Cell viability (%) was calculated as follows: (absorbance mean value of the treated sample/absorbance mean value of the control sample) × 100.

Phase contrast microscopy. A375 cells untreated and treated cells with CNC-HMs were observed by phase contrast microscope (Zeiss, Axiovert200).

Confocal laser scanning microscopy. Cells (4×10^5) were seeded in completed RPMI 1640 medium for 24 h prior to the experiment. After carefully removing the growth medium, cells were washed with PBS and incubated with FITC-labelled CNCs: A) 0.13 ± 0.03 mg/mL; B) 0.4 ± 0.1 mg/mL; C) 0.53 ± 0.13 mg/mL for 24h of treatment. At the end of incubation, slides were washed in PBS and fixed in 3.7% paraformaldehyde in PBS for 30 min at RT. After washing with PBS, cells were permeabilized in Triton-X-100 (0.5% in PBS) and then incubated with propidium iodide (PI) nuclear staining ($1 \mu\text{g/mL}$) for 30 minutes, after that, mounted on glass microscope slides.

The analysis of the intracellular distribution of FITC-CNCs and PI for nuclear counterstain was carried out using a Leica TCS SP2 spectral confocal microscopy equipped with argon-helium neon lasers. FITC-labelled CNCs was excited at a wavelength of 488 nm and PI at 594 nm; emission lines were collected after passage through a DD488/543 filter in a spectral window ranging from 515-700 nm. Signals from different fluorescent probes were taken in sequential scan mode, which allows the elimination of channel cross talk and colocalization was detected in an overlay model. Acquisition parameters: 63.0/1.4 NA objective, image size: 1024×1024 , pinhole size 1 Airy; step size 0.5 µm. Images were processed by using LCS (Leica Microsystems) and Photoshop (Adobe System Inc. Mountain) software programs.

CNC-HM contact toxicity tests with melanoma cells. To determine the 2D cell culture response to the direct contact between the CNC-HM and melanoma cells, the cells were initially placed (4×10^5) in 35 mm tissue culture dish and allowed to grow in culture medium as a confluent monolayer. After for 24 h of growing, the CNC-HM was laid down on the monolayer cells and cell proliferation and morphology were monitored for 24h.

CNC-HM indirect contact toxicity test. The indirect toxicity tests were performed by protecting the cell layer from possible mechanical stress damage induced from CNC-HM contact, using a cell culture insert with a microporous (8 μ m), permeable polyethylene terephthalate (PET) membrane (FalconTM Cell culture insert, Corning inc. North Carolina). The insert housing was laid over 0.5 cm from the confluent cell monolayer avoiding the direct contact but favouring the molecules diffusion. Then, the CNC-HM was laid over the PET membrane and the well plate was filled with the culture broth up to the CNC hydrogel membrane. The sketch of the experimental set-up is shown in Figure 1. This set-up allows diffusion of CNCs molecules through the PET membrane, however it avoids the mechanical stress due to contact between cells and CNC-HM.

Statistical analysis. The experiments were performed at least in duplicate and all the data are reported as the sample mean $\pm$ the standard deviation (SD). The distribution of each measurement was examined for the assumption of normality with the Shapiro–Wilk test. One-way Analysis of Variance (ANOVA)²⁶ was applied to detect the treatment effect, with Bonferroni post hoc analysis being applied to reveal differences between the control cells and all the treatments. The alpha level was set at $p < 0.05$.

Results

CNC-FL and CNC-HM.. Transparent CNC-FL with thickness in the range of 10-12 μ m have been obtained by casting the aqueous dispersion of CNC in a Petri dish followed by drying

at 60°C.²⁷ The dried CNC-FL were compact and flexible, and immersed again in aqueous solution become soft and pliable but kept their structure, without releasing visible amounts of the CNC components, as shown in Figure 2 (panel b and c).

Disk-shaped, about 4-5 mm thick, transparent CNC-HM obtained by addition of Na⁺, Ca²⁺ and Mg²⁺ chloride salts to CNC suspensions are shown in Figure 2 (panel a). Detailed investigation of the structural and mechanical properties of these hydrogels previously reported revealed the absence of any ordered domain, with the mesh size of the order of tens of nm and a modest scaling of changing the crosslinker cation, while the stiffness is almost linearly dependent on the charge/radius ratio of these species.¹² After gelification, the CNC-HMs were processed to remove the excess of salt and increase the stability in water solutions. Four processing strategies were evaluated: a) short washings (5 min) in water, b) incubation in water (50 mL) for 5 days, changing the 50 mL water once a day; c) conversion to alcoholgel by repetitive soakings in ethanol, followed by 5 days soakings in aqueous solution; d) 2h washing in water followed by 1h drying at 60°C. Table 1 reports a summary of the different CNC-HMs.

The morphological appearance and the stability of the CNC-HMs obtained by jellification induced by Na⁺, Ca²⁺ and Mg²⁺ were evaluated after each processing method. The fast washings did not alter the compactness of the CNC-HMs. The prolonged washing caused instead a physical weakening of the membranes, which eventually become shapeless in the case of Na⁺-CNC-HMs. Conversion to alcoholgel determined a slight decrease in volume (about 10% in diameter and 20% in thickness) which persisted after long time soaking in aqueous solution. The conversion hydrogel-alcoholgel-hydrogel increased the Ca²⁺-CNC-HMs and Mg²⁺-CNC-HMs compactness and stability for long term storage in aqueous solution. In particular, CNC-HMs converted into alcoholgels and then soaked again in water, maintained the original shape and compactness for more than three months. In the case of

Na⁺, instead, the membranes were almost completely dissolved by long permanence in water both, in the case of Na⁺-hydrogels and Na⁺-alcoholgels. The mild drying (procedure d) allowed water evaporation and membrane compaction, independently of the cation used for gelification and increased the long-term stability in aqueous environment.

Cytotoxicity tests. Taking into account the ISO 10993-5:2009 suggestions for the biological evaluation of medical devices,¹⁸ we performed three types of tests: a) tests of the molecular components of the device, b) the direct contact assay and c) the indirect contact assay. In view of possible applications of CNC-HMs as medical device for melanoma phototherapy, we performed extensive cytotoxicity tests of CNC-HMs and their components *in vitro*, with melanoma cell cultures (A375). These tests were performed by monitoring the morphological changes by phase contrast microscopy and the alteration of the cell growth and metabolic properties by MTT assay. The most significant tests were repeated with a cell line from a different human melanoma cells (M14) and on normal human fibroblasts.

Toxicity of hydrogel molecular components. It has been suggested that the toxicity of hydrogels could arise from their soluble precursors which are loosely bound to the hydrogel network or progressively released by degradation. In particular, toxicity could increase if chemical modification of the precursors and incorporation of reactive side groups onto these molecules have been performed.^{28,29} In our case, the precursors are the CNCs bearing carboxylic groups in C6' position of the cellobiose units and the gelling cations. We studied the effect of the CNC solution and of a CNC film which is made of pure CNCs self-assembled in a compact layer and not crosslinked by cations.²⁷ In the first experiment, 5 mL of suspension of CNC (8mg/mL) were added to the cell culture containing 7mL of cell medium. The CNC film was directly laid over a culture of A375 cells and 7mL of cell medium were added to cover the film surface. In both cases, we did not observe a substantial toxicity, as it can be seen in Figure 3 (panel A) which is the histogram of the cell survival

after 24 hours of incubation in the presence of 3.3 mg/mL of CNC and of a CNC film, with respect to the control. To monitor the alteration of cells morphology induced by CNC and/or the up-take of CNCs by melanoma cells, trafficking and the intracellular localization of the FITC -labelled CNCs have been investigated by confocal microscopy. Panel A and A1 of Figure 4 show the A375 cells treated with 0.13 ± 0.03 mg/mL of FITC-labelled CNCs in the cell culture medium. CNCs location is visible only marginally in some cell membranes; nuclei stained with PI are negative and no CNCs are visible inside (A1). Figure 4, panel B and B1 (magnification of B), corresponding to the cells treated with 0.4 ± 0.1 mg/mL FITC-labelled CNCs, show an increase in the positivity in the sub-membrane part as well as in the cytoplasm (indicated by the arrows). At 0.53 ± 0.13 mg/mL FITC-labelled CNCs (Figure 4, panel C and C1) we observed a positivity in the cytoplasmic area showing numerous positive vesicles for CNCs, clearly visible in the zoom-in image (panel C1). At difference from previous published data,³⁰ the negatively charged CNCs used in this study undergo substantial cell internalization and negligible adverse effects were observed in cells incubated with CNC as well as with FITC labelled CNC in agreement with the 94% cell viability of A375 cells treated with CNC, shown in Figure 3 (panel A).

The gelling agents used in the present study are the chloride salts of Na^+ , Ca^{2+} and Mg^{2+} , which are ubiquitous components of biological fluids and of the cell culture media. In particular, Na^+ , the concentration of which in RPMI 1640 is 90.75 mM, plays a fundamental role to retain the osmotic pressure of the cells, while Ca^{2+} and Mg^{2+} concentration in RPMI 1640 is 0.42 mM and 0.41 mM, respectively. Since only a significant leakage of these ions from the hydrogels is expected to produce toxicity, we firstly considered the worst case of release of the whole cation content from the CNC-HMs. As the estimated cation content in the membranes after 5 minutes rinsing in water is around 125 mM (see Table 1, rinsed-CNC-HMs), the upper limit of cation release in cell culture media is estimated to be around 160

μmoles ,¹³ which corresponds to an increase of the final cation concentration in the cell culture medium of 19.6 mM. The melanoma cell (A375 and M14) survival when 19.6 mM Na^+ , Ca^{2+} and Mg^{2+} are added to the culture medium is shown in Figure 3 (panels B and C, respectively). The survival data confirm a modest toxicity of Na^+ and Mg^{2+} and a higher toxicity of Ca^{2+} . However, this toxicity should be observed if the whole cation content of a just formed hydrogel, without any rinsing or soaking treatment, is released. More realistic data are obtained after evaluating the actual amount of the cations present and possibly released in/by the membranes after the different processing strategies reported in Table 1. (see later on).

b) Direct contact assay CNC-HMs in direct contact with the confluent A375 cell monolayer induced morphological perturbations which were more evident for the cells lying under the CNC-HM, while the morphology of the cells in proximity with the membranes were less affected. Figure 5, panel B is the representative image of A375 cells incubated in contact with a Ca^{2+} rinsed-CNC-HM. This image shows a remarkable inhibition of the growth and cell body retraction compared to the control cells (Figure 5, panel A). Moreover, cell suffering overt signs are detectable by the presence of surface blebs (Figure 5, panel B), not visible in the CNC-HM non-contact zone (Figure 5, panel C). Taking into account that CNCs do not decrease the cell viability (see Figure 3), and that this macroscopic effect is cation-independent, we argued that a cell response to a mechanical shear contributes to the change in morphology. MTT assay confirms a decrease of cell survival after 24 h of contact with Ca^{2+} -CNC-HMs, as shown in Figure 6 (white columns). From this Figure it appears that the survival of cells incubated in contact with a Ca^{2+} rinsed-CNC-HM (crosslinked by around 125 mM Ca^{2+}) decreased up to 22% of the control cells and was only slightly lower than the survival of cells in contact with a Ca^{2+} water-CNC-HM (crosslinked by around 38 mM Ca^{2+}). The cell death was independent of careful rinsing and soaking the membranes in

pure water, suggesting a predominant effect of a physical trauma to the cells due to hydrogel shearing which could eventually mask the cytotoxicity of the gelling cations.

c) Indirect contact assay A simple set-up for the quantitative evaluation of functionality of cells in indirect contact with CNC-HMs, was obtained by using a microporous cell culture insert (PET track-etched membrane 8.0 μm pore size) suspended over a confluent cell monolayer by small plastic supports, as shown in Figure 1. This set-up allows the free exchange of the molecules between the cell monolayer and suspended CNC-HM but avoids any shear effect. In this way, the only toxic effect is due to the leakage from the CNC-HMs. Ca^{2+} concentration-dependent toxicity comes into play when the coarse mechanical effect is removed, as shown in Figure 6 (grey columns). In fact, we observed a comparable survival between the cells in direct and indirect contact with CNC-HMs containing around 125 mM Ca^{2+} (around 22% cell survival of samples Ca^{2+} rinsed-CNC-HM with respect to their control). Conversely, a cell survival of 53% with respect to the control of the indirect contact test was higher than that of the direct contact one (28%) for membranes containing around 38 mM Ca^{2+} (Ca^{2+} water-CNC-HMs). This suggests that the mechanical shearing levels the cell survival to a minimum value, independently of Ca^{2+} toxicity, that indeed emerges when the first cause of death is removed. The surviving population (22%) probably corresponds to cells, which can respond to stress by activation of efficient survival pathways. Since these data demonstrate that the release of un-bound cations could actually make a substantial contribution to the toxicity of CNC-HMs, different cations and different CNC-HMs washing procedures were tested. In particular, the toxicity of CNC-HMs listed in Table 1 was evaluated with A375 cells and the observed behaviour was confirmed with M14 melanoma and normal fibroblast cell lines. The cell viability of melanoma A375 cells, shown in Figure 7, confirms that Na^+ is a safe gelling agent (survival > 80% even after a short rinsing procedure: Na^+ rinsed-CNC-HMs). However the Na^+ -CNC-HMs are fragile and even

dissolved after long contact with water. Traces of Ca^{2+} were clearly toxic, and a survival comparable to the control has been obtained only after a long lasting incubation in water of the Ca^{2+} -CNC-HMs (Ca^{2+} water-CNC-HMs). However, hydrogels crosslinked by Ca^{2+} showed a good stability in water and in the culture medium. The Mg^{2+} -CNC-HMs gave the best performances, since these membranes combine quite low toxicity with good stability. In fact, if Mg^{2+} rinsed-CNC-HMs produced a low cell survival (37.5%), it increased to about 90% after washing and thermal treatment (Mg^{2+} 60°C-CNC-HM) and 130% after long-lasting soaking in water (Mg^{2+} water-CNC-HMs). Finally, when 1 μM STS was used as positive control, we observed a vitality reduction comparable to that induced by the hydrogel containing 125 mM Ca^{2+} (30% of cell survival with respect to the control cells). The survival trend of M14 (Figure 8) was in line with the behaviour of A375, however the absolute values of viability (%) of M14 were lower than that of A375, for all the tested CNC-HMs. The lack of normal tissue toxicity of the most effective CNC-HMs was proved with a normal fibroblast line. The survival of normal fibroblasts incubated with the HMs was comparable to that of the control (Figure 9). Finally, in Figure 10 we report the viability of the A375 cells exposed to the CNC-HMs gellified by Na^+ , Ca^{2+} and Mg^{2+} , and soaked 5 days in ethanol (final cation concentration in the Ethanol-CNC-HMs estimated to be 38 ± 4 mM) and then again in water. The slightly lower viability with respect to CNC-HMs soaked for long times in water suggests that some ethanol traces are still present. However, the intermediate washing step in ethanol makes the CNC-HM more stable and compact independently of the cation used for gellification.

Discussion

This research addresses the problem of the cytotoxicity of skin hydrogel patches derived from cation-induced assembly of CNC. Notwithstanding cellulose is routinely used in drugs/products approved by Government health agencies,³¹ and, neither toxic solvents nor

aggressive reagents are required for the preparation of these devices, a reliable hazard assessment *in vitro* of CNC hydrogel patches is still lacking. Conversely, this information is mandatory to enable regulatory approvals for nanomaterial applications.³² Cell lines in monoculture systems were preferred for *in vitro* assessment as they are suitable for a wide-ranging toxicological analysis and allow a good experimental reproducibility. It is known that cell lines are genomically and biologically heterogeneous and only partially mirror the aberrations that are present in primary tumors.³³ For this reason, we performed a detailed toxicological investigation with A375 melanoma cells, but we confirmed the melanoma cell response to the nanomaterial with M14 cell line. Moreover, the possible toxicity for the normal tissue surrounding melanoma was investigated with normal human fibroblasts. MTT assay and cell morphology observation demonstrate that small CNCs, with average cross-sectional dimension of 3-5 nm and length of 200-500 nm, bearing a high density of negative charges, are not toxic to melanoma cells in culture. Furthermore, observations using confocal microscopy (Figure 4) showed the up-take of CNCs labelled inside the vesicles, both in the area of the cell membrane and scattered in the cytoplasm. The presence of internalized CNCs further supports the quantitative data of Figure 3, which demonstrate the non-toxicity of CNCs. Sonication and cross-linking by cations entangle CNCs in soft hydrogels, the elastic modulus of which depends on the ratio charge/radius of the positively charged crosslinker.¹² Ca^{2+} has been extensively used as crosslinker of polysaccharides and, recently, to produce CNC hydrogels.³⁴ For the present study we decided to use Ca^{2+} together with Mg^{2+} and Na^+ , which are present in biological fluids and are common components of the cell culture media. The first issue was the set-up testing procedures which allow the evaluation of the toxicity of the hydrogel itself, without any interference by side-effects. We enlightened that the mechanical shearing of CNC-HMs on confluent cell monolayers induced a severe reduction of cell viability, independently of the cation used as hydrogel crosslinker. This effect is

probably due to activation of programmed cell death as revealed by the positive control performed with STS which is an inducer of cell apoptosis. Micro-environmental mechanical stress has been demonstrated to induce apoptosis in cancer cells³⁵ and warnings about physical trauma have been reported on the European Standards EN ISO 10993-5 for the evaluation of Biomedical devices by direct contact tests in vitro¹⁸. Enhanced physical stress can be expected for CNC-HMs used for the present study since they are made of a homogeneous and soft material, as indicated by its rheological parameters (storage and elastic moduli) and the shear-thinning behaviour.¹² These characteristics make the CNC hydrogels easily injectable and suitable for in vivo applications such as drug and cell delivery, absorption of exudates and exchange of fluids. However, when utilized in cell cultures, soft hydrogels stay in close contact with cells causing mechanical stress.³⁶ Conversely, direct toxicity test performed with hydrogels obtained by crosslinking coarse NCF with Ca^{2+} have been reported to induce only small, but not quantified, disruptions of a fibroblast cell layer.¹⁶ These hydrogels, at difference from CNC-HMs, are solid-like materials as evidenced by the values of storage and elastic moduli, which are one order of magnitude higher than those of Ca^{2+} -CNC-HM of comparable cellulose concentration.¹² The reduced adhesion of solid-like devices and the decreased susceptibility of normal cell lines to physical stress could give rise to a reduced apoptosis.^{36,37} To evaluate the toxicity of CNC-HMs in indirect contact with cells, an original method based on the use of a commercial insert with a microporous (8 μm), permeable polyethylene terephthalate (PET) membrane was used. The most popular approach to perform indirect toxicity tests is the agar overlay assay, where a sub-confluent cell culture is overlaid by a thin layer of agar on top of which is placed the test material. This method suffers from several limitations, among which is the slow diffusion of molecules through the agar membrane, so that only acute cytotoxic effects are detected.³⁸ The innovative protocol here proposed offers two main advantages: a) the

high porosity and the large pore size of the PET insert allow the free permeation of solutes, included micro-sized CNC eventually released by the hydrogel; b) the support is hanging over the cell layer, so that any physical stress is prevented.

By using appropriate testing conditions, we investigated the specific toxicity of CNC-HMs and the processing which reduces the release of individual hydrogel components, namely the cations, since CNCs have been demonstrated to be safe. High cation concentration decreases cell survival, being divalent cations more toxic than Na^+ as shown by the data of Figure 3 (panel B and C), where Ca^{2+} , Mg^{2+} and Na^+ were directly added in the culture medium and from Figure 6 where the concentration-dependent toxicity of Ca^{2+} was investigated. This reduced survival can be ascribed in part to the unbalance of osmotic pressure which is more evident in the presence of divalent cations. However, the divalent cations coordination by carboxyl groups is strong and, if most of the unbound ions were removed during the wash steps (as for water-CNC-HM), a very small leakage occurs during the incubation in culture medium as shown in Figure 7. The stronger coordination of divalent cations preserves also the hydrogel structure after long-lasting incubation in aqueous solution. Conversely, the poor stability of the HMs gellified by Na^+ is in accord with the weak coordination of this cation by TEMPO oxidized CNC, which results in a larger mesh size and lower stiffness with respect to the hydrogels obtained by divalent cations¹². An intermediate washing step in ethanol, which strengthens the hydrogel network by favoring hydrogen bonds between adjacent CNCs, makes all the CNC-HMs more stable and compact, irrespectively of the charge of the cation. The higher toxicity of Ca^{2+} with respect to Mg^{2+} in spite of their chemical similarity could be due to the role of Ca^{2+} as signalling molecule involved in all the mechanisms of cell death. Ca^{2+} concentration is maintained within specific limits to ensure adequate homeostasis.³⁹ Conversely, cytosolic Mg^{2+} concentration can be as high as in the mM range and an up-take of this ion does not perturb substantially the intracellular processes.⁴⁰

Conclusions

Nanocellulose possesses a large structural versatility, which allows tunable material development and gives superior properties to nanocellulose-based biomedical devices for tissue engineering and cell or drug delivery. In this paper we utilized rod-like CNC rather than NCF to obtain hydrogel membranes and Na^+ , Ca^{2+} , Mg^{2+} as gelling cations. Accurate washing procedures are necessary to remove the excess of salt and increase the stability of CNC-HM in water solutions. Careful evaluation of testing protocols must be done to assess CNC-HM properties *in vitro* and the results of test performed on precursors, in direct and indirect contact with cell cultures, must be considered all together to assess the safety of the nanomaterial and of the device. Apparent cytotoxicity due to physical phenomena noticed by the testing under *in vitro* conditions must be identified and correctly evaluated. To this purpose we set-up an innovative protocol which is of widespread interest for *in vitro* tests of biomedical devices. We report evidence that CNC-HM fabricated with cation –mediated gelation, lack of cytotoxicity, in particular when the gelling cation is Na^+ or Mg^{2+} being Mg^{2+} -CNC-HM also stable after long-term storage in aqueous solution. Due to the processing capabilities, biocompatibility, and efficacy of CNC materials it is envisaged their large application in biomedicine for new biomaterial development with unique and interesting properties.

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Captions for Figures

Figure 1. Sketch of the experimental set-up of indirect contact toxicity tests.

Figure 2. Pictures of CNC-HMs and CNC-FL. Panel a: CNC-HMs obtained with 1M NaCl (left disk), 1M CaCl₂ (central disk) and 1M MgCl₂ (right disk). Top panel and down panel show respectively the top view and frontal view of the membranes. The salt concentration in the CNC-HMs after 5 minutes of rinsing in water is estimated to be around 125 mM. Panel b: CNC-FL obtained by casting a CNC solution on a petri dish and drying it at 60°C. Panel c: CNC-FL shown in panel a) after soaking it in water for 24 hours.

Figure 3: Toxicity of the CNC hydrogel precursors. Melanoma A375 (panels A and B) and M14 (panel C) cell viability (%) after 24 hours of incubation, in the presence of 3.3 mg/mL of CNC suspension and a CNC-FL (panel A), 19.6 mM NaCl, CaCl₂, MgCl₂ (panels B and C). Untreated cells, that is cells incubated with medium only, were assayed as negative control (columns C). The experiments were performed in triplicate and the SD of the mean was < 0.1% (not noticeable as error bar in the histogram).

Figure 4: Representative confocal fluorescent images of melanoma cells incubated with A) 0.13±0.03 mg/mL B) 0.4±0.1 mg/mL C) 0.53±0.13 mg/mL FITC-labelled CNCs. A1, B1, C1(scale bar: 10 µm) zoomed-in images of A, B, C (scale bar: 10 µm), respectively. All cells were treated for nuclear staining with PI, 1µg/mL for 30 minutes and then incubated with FITC-labelled CNCs for 24 h, at 37 °C in a 5% CO₂ atmosphere.

Figure 5: Phase Contrast Microscopy. Representative images of the morphological changes induced by CNC-HMs on melanoma cells. (A) Control of A375 cells. (B) Image of A375 cells lying under the CNC-HM. (C) A375 cells outside of CNC-HM area. The CNC-HM utilized was obtained by using Ca^{2+} as gelling agent and was rinsed by short washings in water (Ca^{2+} rinsed-CNC-HM).

Figure 6. Melanoma A375 cell survival at 24 h in response to direct (white column) and indirect (grey column) contact with Ca^{2+} -CNC-HMs subjected to two different washing processes. Rinsed-CNC-HM refers to CNCHM rinsed by 5 min washings in water and with a Ca^{2+} content estimated to be 125 ± 17 mM. Water-CNC-HM refer to CNC-HM left in water for 5 days after gelation, so that the estimated Ca^{2+} content in the hydrogel volume was lowered to 38 ± 4 mM. Untreated cells were used as negative control. The experiments were performed in triplicate and the SD of the mean was $< 0.1\%$ (error bar not visible in figure).

Figure 7. Cell viability of A375 cells exposed (24 h) to CNC-HMs gellified by Na^+ , Ca^{2+} and Mg^{2+} and undergoing different washing protocols. Untreated cells were used as negative control (Histogram columns C). STS: staurosporine. The CNC-HMs were suspended according to the indirect contact assay protocol and the viability was evaluated by MTT test. A main effect emerged for treatment ($p < 0.001$). Post hoc analysis maintained significant differences for the control cells compared to Rinsed-CNC-HMs (salt concentration in the hydrogel 125 ± 17 mM) Ca^{2+} ($p < 0.001$) and Mg^{2+} ($p < 0.001$), Water-CNC-HMs (salt concentration in the hydrogel 38 ± 4 mM) Mg^{2+} ($p = 0.009$), and 60°C -CNC-HMs (salt concentration in the hydrogel 54 ± 16 mM) Ca^{2+} ($p < 0.001$). The asterix indicates set of

more than 6 data, for which ANOVA test was performed. The other columns correspond to set of 4 measurements. The error bar is the SD.

Figure 8. Cell viability of M14 cells exposed (24 h) to CNC-HMs gellified by Na^+ , Ca^{2+} and Mg^{2+} and undergoing different washing protocols. C: untreated cells. STS: staurosporine.

The assays were performed in triplicate. The reported values are the mean and the error bar is the SD. All the other experimental details as in Figure 7.

Figure 9 Cell viability of human fibroblasts exposed (24 h) to CNC-HMs gellified by Na^+ , Ca^{2+} and Mg^{2+} and undergoing different washing protocols. C: untreated cells. STS: staurosporine. The assays were performed in triplicate. The reported values are the mean and the error bar is the SD. All the other experimental details as in Figure 7.

Figure 10. Cell viability of A375 cells exposed (24 h) to CNC-HMs gellified by Na^+ , Ca^{2+} and Mg^{2+} and soaked 5 days in ethanol (final cation concentration in the CNC-HMs estimated to be 38 ± 4 mM). C: untreated cells. The experiments were performed in triplicate. The reported values are the mean and the error bar is the SD.