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Highlights:

- Cellulose fiber mats were functionalized with *N*-methacrylate glycol chitosan (MGC).
- Controlled release of an antiseptic was achieved by this modification in PBS.
- Strong antibacterial efficiency of the modified mats was demonstrated.
- Preliminary in vivo tests proved their potential use as wound healing dressings.

Controlled Antiseptic/Eosin Release from Chitosan-based Hydrogel Modified Fibrous Substrates

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ABSTRACT

Fibers of cellulose networks were stably coated with *N*-methacrylate glycol chitosan (MGC) shells using subsequent steps of dip coating and photo-curing. The photo-crosslinked MGC-coated cellulose networks preserved their fibrous structure. A model hydrophilic antiseptic solution containing eosin, chloroxylonol and propylene glycol was incorporated into the shells to study the drug release dynamics. Detailed drug release mechanism into phosphate buffered saline (PBS) solutions from coated and pristine fibers loaded with the antiseptic was investigated. The results show that the MGC-coated cellulose fibers enable the controlled gradual release of the drug for four days, as opposed to fast, instantaneous release from eosin coated pristine fibers. This release behavior was found to affect the antibacterial efficiency of the fibrous cellulose sheets significantly against *Staphylococcus aureus* and *Candida albicans*. In the case of the MGC-eosin functionalized system the antibacterial efficiency was as high as 85% and 90% respectively, while for the eosin coated pristine cellulose system the efficiency was negative, indicating bacterial proliferation. Furthermore, the MGC-eosin system was shown to be efficacious in a model of wound healing in mice, reducing the levels of various pro-inflammatory cytokines that modulate early inflammatory phase responses. The results demonstrate good potential of these coated fibers for wound dressing and healing applications. Due to its easy application on common passive commercial fibrous dressings such as gauzes and cotton fibers, the method can render them active dressings in a cost effective way.

38 **KEYWORDS:** chitosan biopolymer, antiseptic/eosin release, fibrous cellulose wound dressings.

39

40 **INTRODUCTION**

41 The healing of skin wounds is a continuously investigated topic in health care science and tissue
 42 engineering. The major challenge is to achieve simple and efficient treatments able to target many
 43 tissue-related diseases (Sofokleous, Stride, Bon, & Edirisinghe, 2013). There are medical cases in
 44 which a simple cut or burn wound turns into a chronic wound if it is not treated properly(Rieger, Birch,
 45 & Schiffman, 2013). To prevent this, different wound dressings have been studied and
 46 commercialized(Broughton, Janis, & Attinger, 2006; Rieger et al., 2013; Sakai et al., 2013;
 47 Williamson & Harding, 2004). Wound dressing has a fundamental role in the healing process until
 48 complete wound closure. To design new dressing materials for effective healing of wounds, it is
 49 necessary to understand how the skin is able to repair itself after a wound is formed. The process
 50 involves four steps: (a) hemostasis (0-15 minutes), (b) inflammation (15 minutes to 6 days), (c)
 51 proliferation (6 days to 3 weeks), and (d) maturation (3 weeks to 2 years)(Broughton et al., 2006;
 52 Rieger et al., 2013; Velnar, Bailey, & Smrkolj, 2009). In some instances these stages can overlap, and
 53 in the case of chronic wounds the healing process is not completed(Gil, Panilaitis, Bellas, & Kaplan,
 54 2013; Martin, 1997; Rieger et al., 2013). Many factors such as infection, oxygenation and hormones,
 55 can interfere with the healing process resulting in improper or impaired wound healing. To promote
 56 different stages of wound healing, there are dressing products that accelerate or facilitate a specific
 57 step rather than another. In general, wound dressings can be classified into three major types: (a)
 58 passive dressings, which just cover the wound, (b) interactive dressings, which help the healing since
 59 they are not permeable to bacteria but only to oxygen and water vapor, and (c) active dressings, which
 60 deliver antibacterial agents, growth factors or drugs that contribute to the wound healing
 61 process(Hanna & Giacomelli, 1997; Paul & Sharma, 2004; Rieger et al., 2013; Stashak, Farstvedt, &
 62 Othic, 2004). The most common dressing material used is the “passive” fibrous gauze because of its
 63 readily availability and cost. However, there are other new, alternative and advanced gauzes on the
 64 market such as foams and hydrogels(Ishihara et al., 2002; Lu et al., 2010) that can adsorb minimal
 65 exudates, and films and biological dressings that incorporate epidermal and dermal components(Hanna
 66 & Giacomelli, 1997; Rieger et al., 2013; Watson & Hodgkin, 2005). Most of the recent studies for
 67 wound dressings focus on natural composite materials(Watson & Hodgkin, 2005) or electrospun

nanofibers that can offer structural and functional advantages, although their clinical application and commercialization are not yet fully feasible (Gil et al., 2013; Liakos et al., 2014; Liu, Thomopoulos, & Xia, 2012; Rieger et al., 2013). However, there have been recent advances on electrohydrodynamic fiber fabrication techniques with portable devices that allow the fibrous wound dressing mats to be applied in a much more flexible and versatile manner (Lau, Sofokleous, Day, Stride, & Edirisinghe, 2014). It has been shown that cellulose fibers when used as a scaffold support injured limb or wound, absorbs blood and secretions exudates and provide pain relief (Miao et al., 2011). It serves as the protective dressing for wound, thereby preventing wound contamination from outside environment (Maneerung, Tokura, & Rujiravanit, 2008).

Photo-cross-linkable hydrogels have been commonly used for three-dimensional (3D) encapsulation of cells (Poon, Cao, Liu, Chan, & Chan-Park, 2010). Poon et al. proposed an alternative method of making hydrogel-encapsulated cell constructs through layer by layer (LBL) buildup of alternating layers of cells and hydrogel. They used dual-curable (thermal and UV) chitosan-graft-polyethylene glycol-graft-methacrylate copolymer for encapsulation of smooth muscle cells with very good degree of viability (Poon et al., 2010). Song et al. used a novel method to encapsulate nanofibers deposited on micro-patterned metal surfaces as templates to embed the nanofibers into hydrogels when the hydrogels were pressed against the patterned metal surface covered with cell laden nylon nanofibers (Song et al., 2014). They reported that nanofibers not only enhanced the integrity of the hydrogel as a whole for durability and easy handling but also improved the mechanical robustness of individual micro-topographical hydrogel structures. The cells also transferred over into the hydrogels maintaining a good degree of viability (Song et al., 2014). Very recently, it was demonstrated that aqueous based nano-sized curcumin impregnated gelatin cellulose fibers showed effective antimicrobial activity. The process adopted was a green process and the materials employed were of natural origin. The developed curcumin loaded gelatin impregnated cellulose fibers showed more pronounced antibacterial activity than curcumin alone (Raghavendra & Jayaramudu, 2014).

In the present study, we report a novel wound dressing material, which is based on an easy to implement method able to transform common passive fibrous mats (including passive dressing materials) into active. This was achieved by dip coating the mats in *N*-methacrylate glycol chitosan solutions and subsequent UV polymerization of it over the fibers forming shells. The polymer was water soluble before light-induced crosslinking hence facilitated coating of the fibers easily. After the photocuring the material turns into hydrogel resisting dissolution in water. The process is

schematically shown in Figure 1. Fibrous cellulose sheets were used as model passive dressing substrates. The *N*-methacrylate glycol chitosan solutions were fortified by dispersing a commercial antibacterial topical solution eosin (Johnson, Muthukrishnan, & Pellois, 2013; Massimine et al., 2006). They were rendered active after the coating and photocuring process (Amsden, Sukarto, Knight, & Shapka, 2007) releasing the active agent slowly in moist media. To evaluate the potential applicability as a biomedical device, the new material has been also studied in an *in vivo* model of wound healing on mice after a single application during the early inflammatory phase.

EXPERIMENTAL

Cellulosic substrates

As fibrous substrates, pure cellulosic (Whatman, Grade 105) thin sheets 100 mm×150 mm in size were used. They were cut into smaller square pieces during the experiments. According to the manufacturer, the sheets are made up of pure cellulose fibers free from additives such as silicones and sizing agents. Typical thickness was within 35 to 40 μ m range. The sheets leave no fiber residues when rubbed against surfaces.

Hydrogel preparation

The polymer is a modification of water soluble glycol chitosan, (polymerization degree > 400, 90% of deacetylation) which is methacrylated at 7% by glycidyl methacrylate (Amsden et al., 2007; Hatefi & Amsden, 2002; Hayami, Waldman, & Amsden, 2011; Sukarto, Yu, Flynn, & Amsden, 2012), in order to obtain a photo-crosslinkable polymer, namely *N*-methacrylate glycol chitosan (MGC). The polymer was synthesized according to a method presented in a previous work (Amsden et al., 2007). Briefly, purified glycol chitosan was dissolved in distilled water with a concentration of 1.9% and it was reacted with glycidyl methacrylate for 24h at pH 9. After that, the solution was neutralized with 0.1M hydrochloric acid, dialyzed against distilled water in 15 kDa membrane for 24 hours and finally lyophilized into *N*-methacrylate glycol chitosan (MGC) white powder. The methacrylation degree was controlled by ^1H NMR technique a priori (Romano et al., 2015). The photocurable resin was prepared as a solution of 3% w/w MGC in water with 1% w/w of photoinitiator (Irgacure 2959). All the commercial materials were purchased from Sigma Aldrich and used as is.

129 *Cytocompatibility*

130 Cell cytocompatibility of the hydrogel after photocuring the resin with UV light (without fibers) was
 131 verified by Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Life technologies L-3224) to
 132 test biocompatibility and cell growth by confocal microscope. The Live/Dead® Viability/Cytotoxicity
 133 Kit for mammalian cells treatment uses the green-fluorescent calcein-AM to indicate intracellular
 134 esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane
 135 integrity. The Assay was tested after culturing human keratinocytes NCTC 2544 cell line, predominant
 136 (95%) cell type in the epidermis (see Figure and Supporting Information, Figures S1) on the cross
 137 linked polymer.

138 *Functionalization of cellulose sheets with MGC*

139 The wound dressing samples were produced by impregnation of the cellulose fibers in the MGC
 140 solution described above for 1 min and subsequently leaving them to dry overnight. When the coated
 141 cellulose samples were completely dry, they were irradiated with UV light (Lightningcure™ L9588
 142 lamp, coupled with a dichroic mirror that selects the light below 405 nm) for 10 minutes on both sides
 143 for crosslinking with an irradiance of 20 mW/cm². Figure 1 shows photographs of the process in
 144 rendering the inactive fibrous sheet active with the application of the drug loaded hydrogel, in the
 145 figure the hydrogel was loaded with the antiseptic instead in order to demonstrate their excellent
 146 miscibility and compatibility.

147 *Characterization of the MGC-cellulose system*

148 FTIR studies were performed by FTIR Vertex, from 4000 to 400 cm⁻¹ spectral range in absorbance
 149 mode. Mechanical tensile tests were performed with Instron, model 3365 with a load head from 500N
 150 in tensile mode. Thermogravimetric analysis (TGA) was carried out by a Q500 TA apparatus (TA
 151 Instruments, USA). A nitrogen flow, with a rate of 50 mL/min, a heating rate of 10 °C/min and
 152 platinum pans were used. The samples were analyzed from 30°C to 600°C.

153 *Drug Release Experiments*

154 To prepare the samples for drug release and antibacterial tests both bare cellulose fibers and cellulose
 155 fibers coated by MGC were soaked for 1 min in a commercial antiseptic solution, Neomercurocromo®
 156 (Laboratorio Farmaceutiche Specialità Igienico Terapeutiche S.I.T. s.r.l.), which is an aqueous solution

of 2.0% eosin. More specifically, 100 mL of the aqueous topical solution contains 2 g eosin, 0.3 g chloroxylenol, and 30 g of polypropylene glycol. SEM-EDX analysis was conducted on the samples impregnated by the antibacterial solution in order to analyze their surface chemical composition. Dried samples for drug release tests were made by cutting 2x1cm² pieces (weighing approximately 2mg) from larger drug and drug/polymer impregnated sheets. The eosin drug release from these samples was monitored by performing UV-visible absorption measurements on the fibrous samples after different soaking times in 10 ml of phosphate buffer saline (PBS) solution with a Varian Cary 6000i spectrophotometer in absorbance mode from 200 to 800 nm spectral range. The measurements were recorded as a function of time. The characteristic absorption peak of the eosin around was determined to be at 540 nm.

Antibacterial tests

For antibacterial efficiency tests a common testing code *Assessment of Antibacterial Finishes on Textile Materials* by American Association of Textile Chemists and Colorists (AATCC)(Chemists, 2013) was performed in duplicate. *Escherichia coli* (gram-negative), *Staphylococcus aureus* (gram-positive), *Candida albicans* (fungi) were used as bacteria and fungus media. The bacteria count was made at time zero (t₀) and after 20 hours (t₂₀). To evaluate antibacterial efficiency the following equation was used(Chemists, 2013).

$$AE = (bnc_{20h} - bns_{20h} / bnc_{20h}) * 100 \quad (1)$$

where 'AE' is the antibacterial effect, "bnc20h" is the bacterial number on the control sample at 20 hours and "bns20h" is the bacterial number on the test sample at 20 hours. The control samples were the cellulose fibers after immersion in Neomercurocromo and the test samples were the cellulose-MGC fibers also impregnated in the antibacterial agent.

Wound healing model

Male C57BL/6J mice, weighing 22–24 g (Charles River, Calco, Italy) were used. Procedures were performed in accordance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.L.G.S. 116/92 and 26/2014). Animals were maintained in a room under a 12 h light/dark cycle (lights on at 08:00 a.m.), at controlled temperature (21±1 °C), and relative humidity (55±10%). They were anesthetized with isoflurane 1.5-2% from a nose cone. Mice were individually anesthetized using an intraperitoneal injection of ketamine (10%) and xilazine (5%). The

dorsal surface were shaved and rinsed with an alcohol swab. A sterile 4mm punch biopsy tool was used to make a full-thickness excisional wound (Gerharz, Baranowsky, Siebolts, & Eming, 2006). Mice (n=6-8 per group) were dressed with MGC or MGC/eosin coated cellulose fibers, while the control group was left undressed, and then housed individually. Mice were euthanized after 6 and 20 hours and their wounds were excised and stored, until processing, in a deep freezer (-80°C).

Measurement of cytokine levels

Each sample was homogenized in phosphate-buffered saline (PBS) (pH 7.2–7.5) containing 0.4 M NaCl, 0.5% of bovine serum albumin (BSA), 0.05 % Tween 20 and protease inhibitor. Then the homogenate was centrifuged at 4 °C for 1 h at 15,000g. The concentration of cytokines interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) were measured by using a commercially available enzyme-linked immunosorbent assay kit (R&D, Systems, Minneapolis, MN) according to the manufacturer's instructions.

Data analysis

For the observations based on ELISA determination, the value obtained from each wound sample was calculated as pg of cytokine/mg of protein. Values obtained from each group were averaged and were presented as means $\pm$ SEM for each examined cytokine. The significance of variations was determined using One-way ANOVA followed by the Bonferroni's Multiple Comparison Test. Graphpad Prism 5 were used for all statistical analysis (GraphPad Software Inc. San Diego, CA, USA).

Figure 1. Photographs of (a) untreated fibrous cellulose sheet used as the base substrate, (b) MGC polymer before photo-crosslinking, (c) MGC polymer containing mixed with the antiseptic containing eosin (d) MGC polymer treated cellulose sheet (after dip coating and photo-crosslinking) and (e) MGC polymer/drug treated cellulose sheet (after dip coating, photo-crosslinking the polymer loading it with Neomercurocromo by soaking in the antiseptic solution).

RESULTS AND DISCUSSIONS

The chitosan polymer, by virtue of its inherent biodegradability, cytocompatibility, homeostatic activity, has been extensively studied for various biomedical applications, including tissue adhesives, hydrogel dressings, tissue engineering scaffolds (Croisier & Jérôme, 2013; Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Giri, Thakur, Alexander, Badwaik, & Tripathi, 2012; Kim et al., 2008; Scanga, Goraltchouk, Nussaiba, Shoichet, & Morshead, 2010; Takei, Nakahara, Ijima, & Kawakami, 2012), and carrier for rapid drug release (Ishihara et al., 2002). In the present case, the photocurable chitosan derived hydrogel forms a shell around each individual cellulose fiber of the passive substrate, preserving its fibrous structure. Subsequently eosin, a prototype hydrophilic antibacterial drug, is incorporated into the newly formed shell of the fibers. Herein, the development of the wound dressing material is presented and consecutively the release mechanism of the antibacterial agent as well as its antibacterial and wound healing (based on animal data) effects are studied and discussed in detail. As control materials, cellulose sheets wet by the antibacterial agent, eosin, without the polymer coating, are used. Besides the cellulose fibers proposed in the present investigation, other fibrous commercially available passive dressing materials like gauzes and tulle can also be used. Remarkably, the presence of the modified chitosan hydrogel over the fiber surfaces allows a slower and gradual release of the drug, resulting in much higher antibacterial effect (Guarino, Alvarez-Perez, Borriello, Napolitano, & Ambrosio, 2013; Uchegbu et al., 1998; Vogt et al., 2006) compared to the uncontrolled immediate drug release from untreated, bare cellulose fibers. Therefore, the proposed method can lead to novel and effective wound healing products with rapid and inexpensive commercialization potential.

The drug-carrying polymer is a modification of water soluble glycol chitosan by glycidyl methacrylate (Amsden et al., 2007; Hatefi & Amsden, 2002; Hayami et al., 2011; Sukarto et al., 2012) in order to obtain a photo-crosslinking polymer, namely *N*-methacrylate glycol chitosan (MGC). The polymer is synthesized according to a method presented in a previous work (Amsden et al., 2007), and its cytocompatibility is verified through interaction with human keratinocytes cell line (see Figure 2 and Supporting Information, Figures S1). Cell adhesion on the substrate was monitored by wide field and confocal microscope. Cell culture on the hydrogel was stained with HOECHTS 33342, FITC, Alexa and Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Life technologies L-3224) to test biocompatibility and cell growth. Confocal microscope images shown in Figures 2a and 2b, show cells on the cross linked polymer after Live/Dead® Viability/Cytotoxicity Kit for mammalian cells treatment was performed. Live cells were stained by calcein-AM in green and dead cells were stained

246 by ethidium homodimer-1 to in red. The image shown in Figure 2c were taken using markers like
 247 HOECHTS 33342 and FITC. In this case, as seen in Figure 2c, cells were able to survive and
 248 proliferate on the sample surfaces.

249
 250 **Figure 2.** (a) Cells on the hydrogel sample observed by the Live/Dead Assay Kit, (b) Magnification
 251 showing a group cells in healthy condition, (c) cells grown on the surface of the hydrogel (left bumpy
 252 region) and on microscope glass slide surface from which the growth started.

253
 254 The wound dressing samples were produced by impregnation of the cellulose fibers in the water
 255 solution of MGC (3% w/w of MGC and 1% w/w of photoinitiator in water), subsequently allowing
 256 them to dry overnight. When the coated cellulose samples were completely dry, they were irradiated
 257 with a UV light source. As a result of photocuring the hydrogel forms a stable coating around each
 258 fiber. MGC is thus anchored to the cellulose surface. The morphology of the untreated and MGC
 259 coated cellulose fibers were investigated by scanning electron microscopy (SEM) (Figure 3). The
 260 fibrous network of the substrate is still clearly visible after the application of the MGC polymer. This
 261 indicates that after application of the polymer, the initial and apparent fibrous nature of the sheets is
 262 preserved further confirming that the polymer forms a thin shell around the fibers instead of forming a
 263 compact coating over the sheets (coating thickness was around 20 μm).

Figure 3. Representative scanning electron microscopy (SEM) images. (a) Commercial cellulose fibers as received. (b) Commercial cellulose fibers coated by the modified chitosan, MGC hydrogel. (c) Untreated commercial cellulose fibers as in (a) at higher magnification. (d) Hydrogel treated commercial cellulose fibers as in (b) at higher magnification.

FTIR measurements (see Supporting Information, Figure S2) verified the existence of the photocured polymer on cellulose fibers at the end of the photocuring process. Peaks due to the methacrylation are identified by O-H and N-H stretching vibrations at 3300 cm^{-1} ; C-H stretching of CH_2 and CH_3 groups and C=C stretching at 2900 cm^{-1} ; O-C=O and C=O stretching and also N-H bending vibration (shifting due to methacrylation) around $1700\text{-}1600\text{ cm}^{-1}$. Typical peaks of vibration of polysaccharide structure are seen at $1500\text{-}1600\text{ cm}^{-1}$. Mechanical tests were performed with Instron 3365 with a load head starting from 500N, in tensile mode. Mechanical tensile tests showed that the strain at break of the treated samples increased by three times with respect to bare cellulose sheets, and the stress at break of the cellulose-MGC samples was twice compared to pure cellulose ones (see Supporting Information, Figure S3). Although not the main focus of the present study, the MGC coating was found to enhance the mechanical properties of the fibrous support, making the handling of the wound dressing substrates a less delicate procedure. Thermo-gravimetric analysis (TGA) was also performed on cellulose, MGC and cellulose-MGC systems to investigate the presence of interaction between both components. However, the degradation peak of the MGC ($\sim 285^\circ\text{C}$) was found to shift towards higher temperatures (close to 305°C) in the presence of the cellulose fibers, hinting interaction between the MGC shell and cellulose (see Supporting Information, Figure S4).

Eosin, a molecule used in dermatological therapy with bacteriostatic action on gram-positive bacteria and fungi, was used as a model hydrophilic antibacterial drug for release kinetics experiments (Giray, Bal, Kartal, Kızılel, & Erkey, 2012). Eosin also accelerates re-epithelialization and healing of injured skin and has been approved by FDA (Federal Register, 1982). In fact, a recent study on giant omphaloceles patients concluded that eosin significantly promotes epithelialization of un-ruptured omphaloceles reducing hospitalization times (Bertin et al., 2013). To prepare the samples for drug release and antibacterial tests, both bare cellulose fibers and cellulose fibers coated with MGC were soaked for different times in order to ensure the maximum drug uptake by the fibers. The maximum drug uptake was obtained after one minute of soaking in commercial Neomercurocromo® (Laboratorio Farmaceutiche Specialità Igienico Terapeutiche S.I.T. s.r.l., Italy), a water solution of 2.0% (2g/ml) eosin. At the end of the 1 minute dip coating process, a five percent increase in weight of the samples was recorded. This was very repeatable and was controlled for each sample before release experiments were done. A similar uptake was measured for control samples (untreated cellulose) as well. This is attributed to the similar chemical structure between cellulose and

chitosan, both having a polysaccharide backbone (see Supporting Information, figure S5). SEM-EDX analysis was conducted on the samples impregnated by the antibacterial solution in order to analyze their composition (Figure 4 and Supporting Information, Figure S6).

Figure 4. SEM imaging and EDX analyses; (a) cellulose fibers coated with MGC (b) cellulose fibers coated with MGC-eosin (Neomercurocromo).

Although the morphology of the cellulose-MGC fibers seems to remain unaltered before and after their impregnation with the antibacterial solution, the new peaks appearing in the EDX spectra after the treatment indicate the presence of the antibacterial agent. The bromine signal appearing in the EDX spectrum in Fig. 4b, the form of eosin in the commercial medicine is Eosin Y. Comparison of EDX signals of eosin functionalized cellulose fibers and cellulose-MGC fibers indicate presence of eosin in both cases (Supporting

315 Information, Figure S6). Presence of the hydrogel shell around the fibers does not alter or
 316 change the eosin related signals such as bromine which affirms the fact that MGC is a highly
 317 suitable matrix for eosin.

318

319 **Figure 5.** UV-visible absorption spectra of the eosin, untreated, eosin coated, MGC coated
 320 and MGC-eosin coated cellulose fiber networks obtained by measuring dry samples. The
 321 broad peak with a maximum around 540 nm is mainly attributed to the absorption of eosin in
 322 the drug Neomercurocromo. Pure eosin in solution generally displays the absorption
 323 maximum within the range 525-535 nm depending on its molecular structure and functional
 324 groups such as Br and NO₂.

As seen in Figure 5, the samples impregnated with the antibacterial agent show a new broad band in their UV-visible absorption spectra (black and dashed lines), with a maximum around 540 nm that is attributed to eosin (short dashed line). This band is responsible for the characteristic intense red color of the Neomercurocromo solution. The eosin release from these samples was investigated by performing UV-visible absorption measurements on the fibrous samples after different soaking times in 10 ml of phosphate buffer saline (PBS) solution. The experiments were conducted during a period of four days and the results are shown in Figure 6a. For the pristine cellulose-eosin system, a fast (uncontrolled) release of the antibacterial agent took place and it was already completed after 1-2 minutes. For the cellulose-MGC-eosin system, the release took place gradually during the four day period at the end of which only 4% of the initially loaded eosin remained on the samples. This behavior was also clearly noticed by naked-eye inspection of the experiments over time. Photographs of the samples taken at different time intervals are presented in Figure 6b. The change of color from intense red to light pink and eventually to white indicates the decrease of eosin content. After a few seconds in PBS solution, the cellulose-eosin sheets start losing their color, eventually turning into white in just one minute, indicating a radical fast release of eosin. On the other hand, the cellulose-MGC-eosin samples retain their color, changing tone gradually during the course of the experiments. The results were further supported by Fourier Transform Infrared Spectroscopy (FTIR) analysis, in which it was found that after one day in 10 ml of PBS solution the signal strength attributed to eosin was still significant and measurable on fibers coated by MGC (see Supporting Information, Figure S7) whereas it was absent in the case of eosin loaded-pristine cellulose fibers.

(b)

name	0sec	20sec	1min	5min	15min	1h	20h	96h
Cellulose/ eosin								
MGC/ cellulose/ eosin								

Figure 6. (a) Absorption intensity of the samples (untreated and MGC-coated cellulose) impregnated with Neomercurocromo at 540 nm at different time intervals of immersion in PBS medium. The decrease in the intensity indicates the release of the active principal, eosin, from 0 to 4 days. The uncertainty in all of the measurements was within 0.5%. (b) Photographs of the same samples showing loss of reddish color as a function of time indicating release of eosin from treated fibers.

To study the mechanism of drug release in PBS solution from the cellulose-MGC-eosin system, qualitative confocal microscopy analysis was also performed on samples immediately after their preparation and also after four days in PBS. The results are presented in Figures S8, S9 (Supporting Information). Confocal images show the polymer coating around all the cellulose fibers at the beginning of the tests and its partial dissolution after 4 days in PBS. By comparing confocal images with spectrophotometric data, it can be argued that both the swelling and the

gradual dissolution of the polymer are responsible for the controlled release of the antibacterial agent, while for the cellulose-eosin system the release mechanism is almost immediate. The slow dissolution of MGC shell in water was monitored by FTIR and TGA measurements spanning a 3 day period (see Figures S10, S11, Supporting Information). In particular, decrease in the intensity of N-H stretching band in Figure S10 towards day 3 indicates dissolution of MGC shell. It is highly likely that the uptake process is merely absorption of the antiseptic during dip coating. The release, on the other hand (as seen in the Supporting Information Figure S10) is controlled by the partial dissolution of the polymer in the PBS solution. It is also likely that eosin can establish chemical interactions such as hydrogen bonding with the polymer and because of the COO^- moieties in its structure. This behavior will be studied further as future work. Furthermore, the disappearance of the thermal degradation shoulder at 305°C near day 3 can indicate partial dissolution of the MGC shell encapsulating the cellulose fibers. On the other hand, after 3 days of release in PBS medium, the maximum degradation temperature of cellulose fibers decreases from 356°C (degradation of raw cellulose fibers) to 327°C (Fig. S11). This decrease in T_{max} can be explained by the decrease of intermolecular interactions between cellulose fiber chains due to swelling when MGC is partially dissolved during drug release. The gradual release allows the antibacterial agent to be effective for a long period of time compared to the instantaneous effect of the cellulose-eosin system. Referring to the main steps of wound healing, after the first hemostasis step, the phase of inflammation can last from 15 minutes to six days. Therefore, the cellulose-MGC-eosin system can act very efficiently at this stage (the critical inflammation phase), in which the wound has to be continually protected against bacterial infections. It should be noted that the rate of swelling and dissolution of MGC can be tuned by altering the methacrylation degree of the hydrogel. Therefore, it can be adapted to different wound healing phases and different active agents.

To investigate the antibacterial effect of the developed wound dressings, a common testing code, *Assessment of Antibacterial Finishes on Textile Materials*, by American Association of Textile Chemists and Colorists (AATCC)(Chemists, 2013) was performed in triplicate. In particular, two different bacteria and one fungus were used, namely *Escherichia coli* (E-coli; gram-negative), *Staphylococcus aureus* (gram-positive), *Candida albicans* (fungi), respectively. As control samples cellulose fiber sheets immersed in Neomercurocromo solutions were used. Before performing the antibacterial tests, these samples (eosin coated cellulose) were

sterilized with ultraviolet germicidal light exposure and all samples were immersed in sterile water for a few seconds. Since a huge amount of eosin is released uncontrollably as soon as these samples are immersed in liquid or placed in bacteria populated Petri dishes, such few second immersion was necessary to reduce this effect. As seen in Figure 7, cellulose fibers treated with MGC-eosin display a significantly higher antibacterial efficiency compared to cellulose fibers treated only by eosin for *Staphylococcus aureus* and *Candida albicans*. In the former case, an antibacterial efficiency of 85% and 90% was achieved respectively, while for the cellulose-eosin system the efficiency was negative, indicating bacterial proliferation. For *E. coli*, the results show that there is not a significant difference between cellulose-eosin and cellulose-MGC-eosin systems. This is consistent with the fact that eosin is known to be not effective against gram-negative bacteria regardless of its release dynamics.

Figure 7. Antibacterial/antifungal effect of the eosin and MGC/eosin coated cellulose fibers tested against *E. coli* and *S. aureus* bacteria and *C. albicans* fungus. Negative values indicate bacterial proliferation.

Finally, induction of cutaneous wound resulted in a significant increase of the pro-inflammatory cytokine IL-6, in contrast to its very low profile in normal condition, i.e., naïve animals (see Figure 8). When the wound area was covered with the cellulose-MGC fibers loaded with eosin, levels of this cytokine were significantly reduced by at least 50%, indicating a potent anti-inflammatory action (Werner & Grose, 2003) of this wound dressing after a single application (Figure 8). This showed that the MGC-cellulose-eosin system was able to reduce the cytokine

successfully when applied on the wound of mice during the first stages of inflammation. On the contrary, when the wound area was dressed by MGC-cellulose fibers, IL-6 levels were similar to the wounded group (Figure 8), indicating that the differences in the cytokine profile could be attributed mainly to the eosin action.

Figure 8. Effects of a single application of MGC/cellulose fibers or MGC/eosin coated cellulose fibers on the expression of IL-6 after 6h and 20h post wound induction. Data are expressed as mean $\pm$ SEM (n=6 per group). *** p <0.001 vs wound group; # p <0.001 vs MGC/cellulose 20h.

Table 1. Effects of a single application of MGC/eosin coated cellulose fibers on wound inflammation in mouse skin 20 hours after injury. Data are expressed as pg/mg protein (mean $\pm$ SEM; n=8 per group). *** p <0.001 vs wound group.

<i>Cytokine concentration</i>	<i>Naïve</i>	<i>Wound</i>	<i>Wound + MGC/cellulose/Eosin</i>
<i>IL-1β concentration (pg/mg protein)</i>	27.03 $\pm$ 5.71	423.80 $\pm$ 15.32	142.50 $\pm$ 15.60***
<i>TNF-α concentration (pg/mg protein)</i>	3.98 $\pm$ 1.22	12.44 $\pm$ 0.87	4.77 $\pm$ 0.82***

Similarly, levels of other two pro-inflammatory cytokines, TNF- α and IL-1 β were monitored. Significant decreases of these factors were also observed as a result of wound area coverage by the proposed active gauzes (Table 1). This preliminary set of data demonstrates that the cellulose-MGC fibers application is safe and biocompatible for the animal skin and it is able to release eosin within the first 20 hours after a single application. Further studies are needed to better clarify the combined action of polymer plus eosin in the different phases of the wound healing including long-term evaluation and multiple applications. However, the measured decline in the various cytokine levels within the first 20 hours is highly encouraging and appears to be mainly correlated with the release of eosin from the hydrogel shells.

CONCLUSIONS

In conclusion, the present results demonstrate that photo-crosslinkable *N*-methacrylate glycol chitosan coatings applied on micron sized cellulose fibers as drug containing hydrogel shells permit gradual drug release during several days of application compared to the drug coated bare cellulose fibers for which the release is almost instantaneous (few minutes). Preliminary tests on their application as wound dressing on mice indicated substantial decrease in IL-6 and TNF α as well as IL-1 β cytokine levels within the first 20 hours of application, suggesting their suitability as wound healing gauzes with drug release capability within several hours of single application. Moreover, noteworthy is the fact that the drug loaded *N*-methacrylate glycol chitosan solutions can also be applied to other commonly used commercial fibrous materials, such as synthetic gauzes and cotton fibers in a similar way described in the present study. The process could also be implemented to various biopolymer nanofibers matrices as matrix chemistry, surface functionality, and mat degradation rate of nanofibers can be better controlled for tuning materials–biology interface interactions. The ease of application, rapid polymerization and gradual drug release renders the present method as a highly potential new generation wound dressing material which can be easily commercialized.

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