CELLULOSE HYDROGEL SHEETS FOR WOUND DRESSINGS

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Transparent sheets of regenerated cellulose hydrogels for wound dressing were prepared by hardening hydrolysed cellulose acetate in the designed forms in an acetone solution. The produced sheets were saturated with glycerol to improve their features. The moisture retention, which hinders scab formation, promotes re-epithelialization and accelerates healing process, has been studied. It was determined that the regenerated cellulose hydrogel sheets incorporated with glycerol can retain moisture for a long time. Adhesion properties for imitated wound surfaces were investigated by measuring the maximum force of adhesion, the total work of adhesion and the distance necessary to detach the hydrogel sheets from the imitated wound surface. Incorporation with glycerol lowered the adhesion parameters and showed that these hydrogel sheets would be non-adhesive. Cellulose-based hydrogel sheets were loaded with an antibiotic neomycin and showed good antibacterial properties. The sheets have potential to be used as a wound dressing material with good transparency, mechanical, antibacterial properties.

Keywords: cellulose, hydrogel, wound dressing, transparent, antibacterial

INTRODUCTION

Nowadays, there is a large supply of wound dressings for two main types of wounds distinguished: acute and chronic. Nevertheless, wounds are subdivided into smaller categories, such as pressure ulcers, venous leg ulcers, diabetic foot ulcers, traumatic wounds, surgical wounds, burn wounds, and others.[1] Wound dressings may be chosen not only according to the type of a wound, but also according to the wound healing phase, as wound healing is a dynamic and complex process. Five stages of wound healing are marked out: hemostasis, inflammation, cellular migration, protein synthesis and wound contraction, remodelling.[2] Inflammation, proliferation, and remodelling are the main stages. Using scientific knowledge, the aim is to create an ideal wound dressing, which should be non-toxic, non-adherent, transparent, allowing gaseous exchange, removing the exudate, protecting the wound from bacterial penetration, permitting thermal insulation, minimizing scar formation and maintaining the high humidity level.[3] Also, factors such as nursing costs and frequency of dressing changes are important.

Increasing attention is paid to the treatment of wounds in a moist environment. The inflammatory phase is often accompanied by the rich exudate environment, thus a dressing should generate an optimal moisture level by absorbing the exudate and protecting the wound from maceration. On the other hand, decreasing the level of the exudate requires to minimize absorption and maintain a natural moisture level for newly forming tissues.[4] Wound healing in a moist environment has several advantages, such as prevention of scab formation, faster epithelialization, reduced pH of the wound environment, which has a negative effect on bacteria.[5] Due to a big variety of wounds, diseases, patient age, complications and other features, every single case requires attention to the selection of the wound dressing. A big variety of wound dressings with different properties is distinguished: low or non-adherent contact layer dressings, hydrogel dressings, hydrofiber

dressings, semipermeable film dressings, alginate dressings, foam dressings, hydrocolloid dressings, antimicrobial dressings, deodorizing dressings.[1] Traditional dressings, which are natural or synthetic bandages and gauzes are cheap, but do not provide moisture for the wound, are adherent to the wound surface, need to be changed regularly and can dehydrate the wound. It is considered that hydrogels have the properties of an ideal wound dressing.[6] Usually, hydrogels are transparent, so wound can be observed without removing the dressing, they are soft and their rubbery nature minimizes inflammatory reactions of the surrounding cells.[7] The three-dimensional structure of hydrogels allows them to be used as a drug delivery system.[8,9] Hydrogels provide moisture and at the same time are non-adherent materials. Due to the high water content, hydrogels allow vapor and oxygen transmission and fit for pressure sores, leg ulcers, burns, necrotic, and surgical wounds.[10] Cooling and hydrating effects are very important for burn wound treatment and various additives are used to get the moisturizing effect, for example glycerol. Glycerol is non-toxic, colourless, odourless, able to attract moisture from the air and do not oxidize in the air.[11] Concentrated glycerol is antibacterial and antiviral.[12] Due to that, glycerol usually is used as a plasticizer in wound dressings.[13-15]

Cellulose, which is a natural, non-irritant, non-toxic, biocompatible and environment-friendly material, is widely used as a wound healing material. As a wound dressing, cellulose is used in various forms. In early years, cellulose was first used as gauzes. Nowadays, more improved cellulose forms for wound healing are being investigated, for example cellulose fibers,[16-18] cellulose films,[19-21] sponges.[22,23] As the hydrogel form is becoming more and more popular, cellulose hydrogels are also becoming a field of interest. Until now, some cellulose hydrogels have been investigated.[24-31] In our study, regenerated cellulose hydrogels for wound healing were created. Regenerated cellulose can be easily functionalized and various active compounds, which can accelerate and promote healing processes, can be loaded. Our regenerated cellulose sheets were saturated with glycerol to get the moisturizing effect and improve mechanical and bioadhesion properties. Additionally, to protect a wound from bacterial infection, hydrogel sheets were loaded with neomycin antibiotic.

EXPERIMENTAL

Materials

Cellulose acetate (Mn ~50.000) was obtained from Sigma Aldrich (USA). Acetone (CH_3COCH_3) was obtained from Standart (Poland), neomycin was purchased from BELA-PHARM GmbH & Co (Germany), glycerol ($CH_2(OH)CH(OH)CH_2(OH)$) was obtained from Eurochemicals (ES). All other reagents were purchased from Sigma Aldrich (USA).

Preparation of cellulose hydrogel sheets

Regenerated cellulose was obtained by the hydrolysis of cellulose acetate in acetone solution according to the patent.[32] The obtained regenerated cellulose solution was poured into plastic forms and kept at the room temperature until the solid sheets were formed. The hydrogel sheets with a thickness of 0.5 mm were rinsed with water until a neutral pH was reached. Samples marked as CHF were obtained. Another group of films was immersed into glycerol for 24 h and CHFG samples were obtained.

Mechanical properties (Tensile testing)

The tensile test was carried out using a material testing device Zwick/Roell BDO-FB0.5TH (Zwick GmbH & Co, Ulm, Germany). Dumbbell shape specimens with dimensions of 35×5 mm were punched out from the cellulose hydrogel sheets. The thickness of each sample was measured with a digital calliper. Samples were fixed with two clamps in a vertical position and preloaded at 0.02 N. After the preloading, the force was set to zero and a constant crosshead speed of 100 mm/min was determined. Data were collected till the sample's rupture. Strain-stress curves were recorded. Computer Software V11.02 TestXpert provided the tensile strength (MPa), the percentage elongation at the break moment (%) and Young's modulus (MPa). Triplicate experiments were carried out.

Mechanical properties (Rupture test)

The standard pharmaceutical drug release film test was performed with TA.TX Plus Texture Analyser (Stable Micro Systems, United Kingdom) device. Samples were cut into 30×30 mm diameter pieces. A stainless steel spherical ball probe and 5 kg load cell were used for the test. The film support rig was fitted to the heavy duty platform and positioned loosely on the machine base. The ball probe was aligned with the film support rig so that the probe could move through the aperture centre. Then the probe reached the sample, the force increased until the film broke. The test speed was 1 mm/s. The force needed to rupture the films (N) and distance to burst (mm), which is the indication of flexibility, was estimated. Triplicate experiments were carried out.

***In vitro* bioadhesion measurements**

Bioadhesion properties were evaluated with a Modular Compact Rheometer MCR 302 (Anton Paar GmbH, Austria). To imitate a wound surface, an egg shell membrane was used.[33] A cylinder probe with the diameter of 10 mm was used, the sample size was 1×1 cm. In this analysis, the force of 10 N was applied. In order to stick the egg shell membrane, a double-sided adhesive tape was used. The hydrogel sheets were fixed to the upper probe, the egg shell membrane to the bottom probe. The sample and the biological substrate were put into contact for 60 s. After this period, the upper probe was raised up at 0.05 mm/s speed till the egg shell membrane and the sample became completely unstuck. Before each measurement, the egg shell membrane was moistened with 100 µL of 0.9% sodium chloride solution. The adhesive properties were evaluated by the maximum force (F_{max}), necessary to detach the egg shell membrane from the sample, the total work of adhesion (TWA) was represented by the area under the force versus distance curve and cohesiveness (d) was expressed as the sample distance travelled till the detachment moment from the biological membrane. Triplicate experiments were carried out.

Transparency/chromaticity test

Transparency/chromaticity of cellulose hydrogel sheets were analysed with a MiniScan XE Plus colorimeter (Hunter Associates Laboratory, Inc, Reston, VA, USA). Before each series of measurements, the spectrophotometer was calibrated on a standard white tile (X = 81.3, Y = 86.2, Z = 92.7). The colorimeter was set to measure total reflectance with an illuminant C and a 10° observation angle. The parameters L*, a* and b*, which mean lightness, red value and yellow value within the CIE (Commission Internationale l'Eclairage) Lab three-dimensional colour space, were measured, converted into the hue angle (h° = arctan (b*/a*)) and chroma (C = $(a^{*2}+b^{*2})^{1/2}$).[34] The hue angle is expressed on a 360º grid, where 0º = red, 90º = yellow, 180º = green and 270º = blue. Coordinates of the colour were processed with the Universal Application Software V.4-10. The white background was used for measurements. Triplicate experiments were carried out.

Dynamic-thermomechanical analysis

The dynamic-thermomechanical analysis was performed using a Modular Compact Rheometer MCR 302 (Anton Paar GmbH, Austria) device. Research was conducted using 50 mm long, 7 mm wide and 0.5 mm thick specimens. During the experiment, a temperature gradient was used. The measurements of the extensional storage modulus and extensional loss modulus were performed in a temperature range from 20 ºC to 40 ºC (heating rate of 1 ºC/min). The specimens were tested in air atmosphere and treated with a constant mechanical vibration under the tensile mode at a frequency of 0.1 Hz (oscillation amplitude of 2 mm).

Water evaporation rate from hydrogel or moisture retention ratio

In order to prevent variations of humidity, a desiccator filled with a saturated magnesium nitrate solution was used. The desiccator was kept in a drying oven at 35 ºC. The relative humidity in the desiccator after equilibration was of approximately 50%. The hydrogels prepared as described earlier were slightly dried up with a filter paper and weighed (W_0), then placed into the desiccator and after certain intervals of time, the samples were weighed again (W_t). The evaporated water content was calculated by the equation:

$$W_E = \frac{W_0 - W_t}{W_0} \times 100, \% \quad (1)$$

Triplicate experiments were carried out.

Fluid uptake ability of hydrogels

Cellulose sheets (sample size of 2×2 cm) were dried to a constant weight (M_0) in a drying oven at 105 ºC. Samples were incubated in 10 mL of pseudo-extracellular fluid (PECF) buffer at 37 ºC for 3 h. PECF buffer simulates the wound fluids and was prepared by dissolving 0.68 g of sodium chloride (NaCl), 0.22 g of potassium chloride (KCl), 2.5 g of sodium hydrogen carbonate ($NaHCO_3$), and 0.35 g of sodium dihydrogen phosphate (NaH_2PO_4) in 100 mL of distilled water. The pH of PECF was adjusted to 8 ± 0.2. The swollen weights of the samples were determined by draining the sample surface with a filter paper and weighing the sample (M_t). The weight of swollen samples was recorded every 30 min. The PECF absorption was calculated from the equation:

$$S_A = \frac{M_t - M_0}{M_0} \times 100, \% \quad (2)$$

Triplicate experiments were carried out.

Cytotoxicity test

Cytotoxicity test was carried out for rat hepatocytes. The hepatocyte stock from a healthy 3 month old rat was isolated. The primary cell counting was performed: the cell number in stock was about 10^8 cells/mL, viability ~95%. Further, hepatocyte cells were resuspended in a culture medium. 2×10^7 cells/mL hepatocytes were resuspended in 199 mL of a buffered medium with HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) at pH 7.4, where cells can maintain high viability and normal metabolic rates for a long time, up to 24 hours. After resuspension, the cells were dispensed on Petri dishes. Milled samples at a concentration of 50 mg/mL were added to Petri dishes with hepatocytes and incubated for 90 minutes at 37 °C. After incubation, the cells were transferred to centrifuge tubes. Aliquots for cell count and viability testing were dispensed. Cell viability was valued by

staining with trypan blue, triplicate experiments were carried, ≥600 cells for every test were used. The cells were counted in two chambers of a standard hemocytometer, 50 squares each time for every sample. Moreover, to determine if the hepatocyte membrane can be damaged by the CHF and CHFG samples, lactate dehydrogenase (LDH) activity of the samples was measured in the incubation medium by means of a bioassay kit for LDH determination.

Neomycin loading

A bath process for loading the cationic antibiotic neomycin was used. Water-based and glycerol-based solutions of concentrations of 40 mg/mL were used for antibacterial activity evaluation. Samples of hydrogel sheets were cut into 15×15 mm pieces and immersed for 24 h into a water-based or glycerol-based neomycin solutions with the concentration mentioned above. The samples saturated with the aqueous neomycin solution were named as CHF-N, the samples saturated with glycerol based neomycin solution as CHFG-N. After 24 h, the samples were removed from the solutions of the active substances and further were used for the antibacterial activity test.

Antibacterial activity

Gram-negative *Escherichia coli* (DH5 α) and Gram-positive *Bacillus subtilus* (ATCC 6051) bacteria strains were used for the test. 250 μL (0.5-2.5 × 10^{-8} cells/mL) of each bacterial suspension was flooded on Petri dishes with LB agar (Lennox, USA). The CHF and CHFG samples were cut to 15 mm side squares, placed on Petri dishes with agar and incubated for 18 h. Antibacterial activity was assessed by measuring the inhibition zone, measuring from the edge of the disk tothe end of the clear zone.

RESULTS AND DISCUSSION

Hydrogel films (CHF), as inert matrices with a three-dimensional structure, were formed from regenerated cellulose. Another group of films (samples CHFG) were saturated with glycerol to get the moisturizing effect and improve mechanical and bioadhesion properties. Due to their high water content and flexibility, the hydrogels are very similar to a natural tissue. The sheets could be loaded with different active compounds. Due to the high porosity, the transport of various active substances can be easily achieved. These and many other features make hydrogel sheets promising as a wound dressing. In this work, mechanical properties, bioadhesion, transparency, moisture retention, cytotoxicity, as well as antibacterial properties of cellulose-based hydrogel sheets were studied.

Mechanical properties

In order to achieve a successful wound treatment, elasticity and resiliency are necessary properties for dressings. The elasticity helps to retain the shape of a dressing and its use is extended as it does not need to be changed often. Young's modulus (YM) specifies the stiffness or rigidity of the film, strain at break (TS) indicates the tensile strength of the film to resist breaking and elongation at break (E) describes the flexibility or extensibility of the films.[35]

Tensile strength, elongation at break and Young's modulus of the cellulose sheets are listed in Table 1. The samples saturated with glycerol showed better mechanical properties than the cellulose sheets with water. They demonstrated more than twice higher tensile strength. Also, elongation at break and Young's modulus increased significantly. Sheets designed to be used as wound dressings should be sufficiently robust and fictile, although a too high value of Young's modulus could bring to rigidity and stiffness, thus giving an unpleasant sensation to the patient. According to the rupture test, loading with glycerol strengthens the cellulose sheets by almost 4 times. Considering the distance to the burst, we can see that the elasticity decreases. CHFG sheets as wound dressings could be robust enough for a long time and also could have good enough stretching properties, compared with CHF. The higher values of the mechanical parameters in the case of the cellulose sheets loaded with glycerol suggest that this kind of sheets would be strong enough to withstand the pressure from a compressive or tensile force and would be easy to handle as a wound dressing.

Table 1
Mechanical properties of CHF and CHFG samples obtained from the tensile test and rupture test

Sample	Tensile strength, MPa ± SD	Elongation at break, % ± SD	Young's modulus, MPa ± SD	Rupture force, N ± SD	Distance to burst, mm ± SD
CHF	2.73 ± 0.66	13.81 ± 0.58	5.76 ± 0.61	10.09 ± 1.58	7.65 ± 1.23
CHFG	6.33 ± 0.98	16.00 ± 0.57	7.50 ± 0.55	39.77 ± 1.58	5.50 ± 1.11

Table 2
Bioadhesion measurements (maximum force (F_{max}) necessary to detach the egg shell membrane from the sample, the total work of adhesion (TWA) and cohesiveness (d))

Sample	F_{max}, N ± SN	TWA, mJ ± SN	d, mm ± SN
CHF	1.6 ± 0.39	0.35 ± 0.10	2.8 ± 0.27
CHFG	0.7 ± 0.10	0.2 ± 0.01	1.5 ± 0.02

Table 3
Transparency/chromaticity of CHF and CHFG samples

Sample	L*±SN	a*±SN	b*±SN	C*±SN	h°±SN
CHF	88.61±0.77	-1.19±0.01	2.57±0.20	2.84±0.18	114.99±1.63
CHFG	79.54±0.10	-0.43±0.02	12.76±0.07	12.76±0.0	91.95±0.09

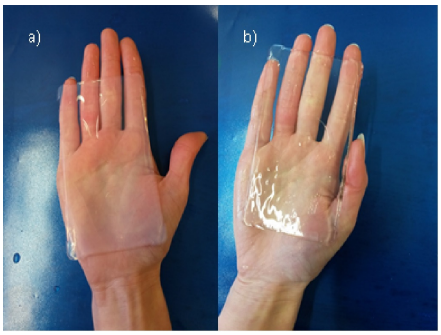

Figure 1: Demonstrative transparency of CHF (a) and CHFG (b) samples

***In vitro* bioadhesion measurements**

Table 2 shows the results of bioadhesion measurements. Impregnation with glycerol had a positive effect on bioadhesion properties. The maximum force necessary to detach cellulose sheets from a biological membrane, the total work of adhesion and cohesiveness decreased significantly. The values of this test are close to or even better than those mentioned in the literature.[36,37]

The typical wound dressings after the contact with the wound become soft and could stick to the wound. Removing such a wound dressing causes discomfort to the patient and becomes painful. To keep the wound clean, traditional wound dressings as gauzes should be changed once a day, or twice a day, while hydrocolloids need to be less frequently changed, thus cause less pain, also they are less painful during the removal process.[38] The lower the adhesiveness of a wound dressing, the less damage is done to a newly formed wound bed.

Transparency/chromaticity test

Transparency of wound dressings has gained more and more attention in recent years. Transparency in wound healing has many advantages, as the wound can be observed externally during the healing period and unnecessary ligation of wound dressings is avoided, so the patient feels less pain and more comfort.

Index L* refers to the white and black colour ratio (brightness) and ranges from 0 (pure black) to 100 (pure white). Here, the CHF sample has a closer value to pure white (88.61 ± 0.77) than the CHFG samples (79.54 ± 0.10). The negative values of index a* refer to the intensity of green colour opposite to red, as well as the positive b* index to the intensity of yellow colour opposite to blue. The CHFG samples have a more intensive yellow colour, compared with CHF, also the chromaticity index C* increases in the CHFG samples (see Table 3). As the C* value increases, the colour becomes more intense. The hue angle in the CHFG samples confirms that the samples saturated with glycerol appear more yellow and the colour gets more pure. As may be seen from Figure 1, both films have very good transparency.

Moisture retention ratio

Wound dressings that can retain moisture generate a protective barrier, help to reduce scar formation, accelerate wound re-epithelialization, which results in a faster healing process compared to a treatment in a dry environment.[39] The results of the study show that the regenerated cellulose sheets loaded with glycerol would be appropriate to use not only due to their improved mechanical properties, but also because of the moisture remaining in the wound dressing for a long time. After 4 h, the CHF samples retain only 18% of the initial sample weight, 82% of the moisture is lost, while the samples loaded with glycerol even after 3 days lose only 17% of their moisture. As CHFG samples are transparent and can retain moisture for a long time, a frequent dressing replacement could be avoided.

Figure 2 shows the amount of water evaporated from the CHF and CHFG samples over time.

Fluid uptake ability of hydrogels

The fluid absorption capacity of a wound dressing is a very important parameter for maintaining a moist environment over the wound. The swelling of hydrogel sheet samples in PECF buffer is shown in Figure 3. Dried cellulose sheets reached the swelling equilibrium after approximately one and a half hour. These samples were able to absorb up to 167% (±4%) of the PECF buffer, but already after 30 min the hydrogel sheets were able to absorb up to 133% (±3%) of the buffer.

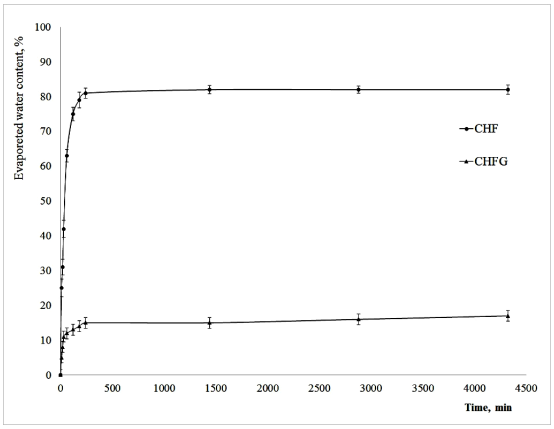

Figure 2: Kinetic curves of water evaporated from CHF and CHFG samples at room temperature

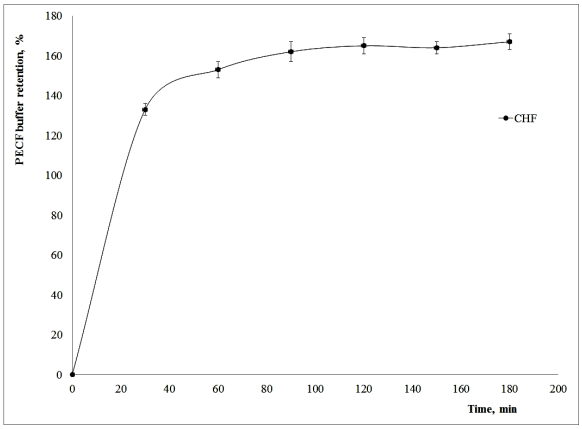

Figure 3: Kinetic curve of PECF buffer retention by cellulose hydrogel sheets

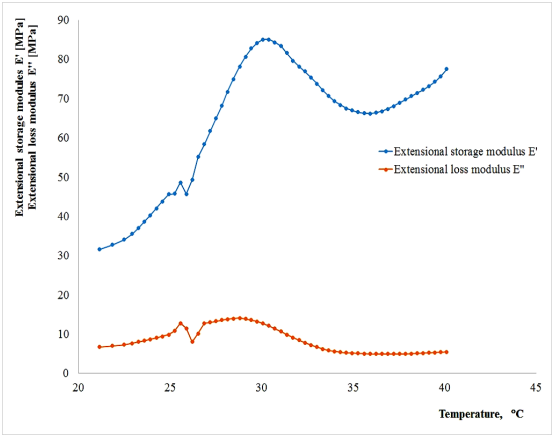

Figure 4: Dynamic thermomechanical analysis of storage and loss modulus *vs*. temperature of CHF samples

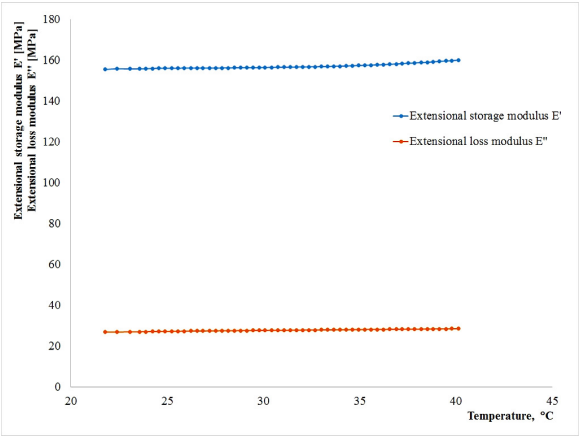

Figure 5: Dynamic thermomechanical analysis of storage and loss modulus *vs.* temperature of CHFG samples

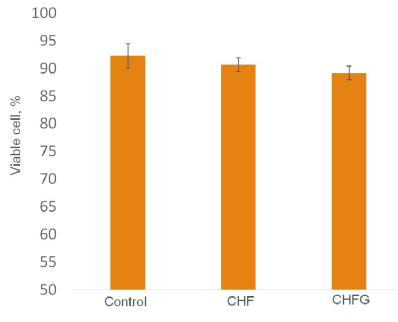

Figure 6: Viability of isolated hepatocytes after incubation with samples CHF and CHFG

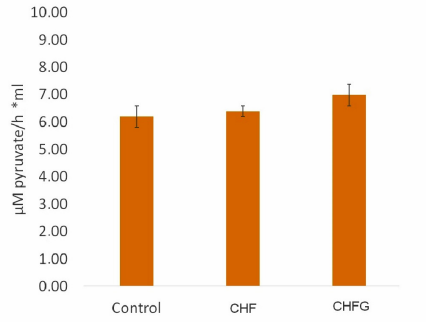

Figure 7: LDH activity in a culture medium of hepatocytes after incubation with CHF and CHFG samples

Such liquid absorption of a wound dressing is efficient, effective and would encourage the wound to heal more successfully by moving quicker to the scar maturation stage.

Dynamic-thermomechanical analysis

Article I. The dynamic properties of CHF and CHFG samples are expressed in terms of the storage modulus and the loss modulus.

Figure 4 and Figure 5 show the storage and loss modulus *vs.* the temperature. Increasing the temperature, the storage modulus and the loss modulus of CHF samples also increase, showing a loss of elastic properties, thus a sample is becoming more rigid. Meanwhile, during the whole experiment, CHFG samples maintain a constant storage and loss modulus. CHFG samples have higher values of the loss and storage modulus, compared with CHF, which shows higher stiffness of CHFG samples. Used as a wound dressing, CHFG samples could retain their shape and mechanical properties for a long time despite an increased temperature and motion, and a frequent change of a dressing would be unnecessary.

Cytotoxicity test

The viability of hepatocytes incubated with milled CHF and CHFG samples was compared with the control, *i.e.* cell viability without the samples. Control cells viability reached over 92%. Viability of the control hepatocytes did not reduce significantly through the incubation time. High viability in the control group indicated that all necessary conditions for the hepatocyte incubation, such as pH, temperature, culture medium type, were satisfied in the best way. The samples CHF and CHFG also showed excellent viability of hepatocytes with values close to those of the control group (Figure 6). Moreover, the lactate dehydrogenase (LDH) activity in the culture medium of the control and CHF and CHFG treated hepatocytes was studied. LDH is found in many body tissues, including the liver. Elevated levels of LDH in the blood serum may indicate liver damage. LDH test is a common clinical procedure to estimate liver damage. If hepatocyte membrane is damaged, LDH can come out through the membrane to the incubation medium and stay in the supernatant, where it can be detected by the biochemical assay. The results obtained demonstrated that pre-treatment of cells by the CHF or CHFG did not increase the LDH release from the hepatocytes, compared to the control cells. The results revealed that none of the hydrogels was cytotoxic.

Antibacterial activity

Healing of open wounds is a complex process, which is often impeded by infections with various microorganisms. Such complications may prolong the healing process, that is why treating with antibiotics may be required. Local delivery of a drug accelerates wound healing and usually has no side effects.[40] An aminoglycoside antibiotic neomycin has a strong activity against Gram-negative bacteria and a partial activity against Gram-positive bacteria.[41] It is known that glycerol also possesses bacteriostatic properties.[12] The antibacterial activity of cellulose sheets saturated with water or glycerol based solutions of the antibiotic neomycin was tested. The bacterial growth inhibition zone was measured (in mm) for

evaluating the antibacterial activity. Results are shown in Table 4.

Table 4
Results of bacterial growth inhibitory zone measurements

Sample	Gram-negative bacteria *Escherichia coli* (DH5α)	Gram-positive bacteria *Bacillus subtilus* (ATCC6051)
	Bacterial growth inhibitory zone diameter, mm	
CHF	-	-
CHFG	7	4
CHF-N	20	18
CHFG-N	25	21

Antimicrobial activity results indicate that regenerated cellulose sheets loaded with neomycin showed good antibacterial properties. CHF samples without active ingredients demonstrated no antibacterial activity, while cellulose sheets with glycerol showed antibacterial activity against both gram-negative and gram-positive bacteria. The best results were obtained for CHFG-N samples. The inhibitory zone of the bacterial growth for gram-positive bacteria was 21 mm, while against gram-negative it was larger and reached 25 mm. CHF-N samples demonstrated a slightly lower antibacterial activity, compared with CHFG-N.

CONCLUSION

Regenerated cellulose hydrogel sheets were created for a wound dressing application. Loading with glycerol significantly improved mechanical, thermomechanical, bioadhesion parameters of the sheets. Such dressings would adapt well to the wound surface, would be easily removed and the routine procedure of changing dressings would be facilitated. The samples saturated with an antibiotic neomycin solution showed good antibacterial activity against both gram-negative and gram-positive bacteria. The prepared hydrogels are not cytotoxic. An additional advantage of such wound dressings is their transparency, which allows monitoring the wound healing process without unnecessary wound ligation.

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