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Nanocellulose hydrogels for topical wound-care applications: a study on human skin interactions

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Abstract

Nanocellulose hydrogels for topical wound-care applications: a study on human skin interactions

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The biocompatibility of nanofibrillated cellulose (NFC) hydrogels was evaluated by *in vitro* cell studies having in mind the potential use as a novel wound dressing. The NFC had undergone TEMPO-mediated oxidation and was Ca^{2+} cross-linked to create a self-standing hydrogel that was tested with human dermal fibroblasts and keratinocytes. NFC hydrogels and composites with collagen or kaolin were included in the study, as well as the commercial dressing Aquacel as a reference material. The effect of the materials on cell viability and migration was studied by measuring cell metabolic activity, investigating cell morphologies and performing a migration assay. Cells on NFC hydrogels had increased proliferation and sounder morphologies than cells grown on Aquacel, while cell migration was similar to that of cells with Aquacel. The NFC hydrogels show potential to become novel wound dressing materials and further studies will investigate their biocompatibilities further.

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Populärvetenskaplig sammanfattning

Sår drabbar de flesta flera gånger i livet och kan vara av olika allvarlighet, från ett litet skrapsår till ett stort öppet sår. För komplicerade sår krävs ett bra förband som skapar en varm och fuktig miljö samt påskyndar läkningsprocessen.

Cellulosa har i alla tider använts inom sårläkningen; från början i löv och örter, sedan i form av gasväv och slutligen i moderna förband, såsom hydrofiberförband. Skogsindustrin är särskilt intresserad av att fortsätta denna tradition av cellulosa i förband då andra applikationer av cellulosa (till exempel papper) kan börja minska med teknikens framfart. Nanocellulosa är cellulosa som separerats till mindre beståndsdelar och det har egenskaper som skulle kunna skapa ett idealt sårförband. Fibrerna i nanocellulosan binder och trasslar in sig i varandra och skapar ett nätverk som kan hålla kvar vatten (det kan bestå av runt 95 % vatten) och klassas då som en hydrogel. Nanocellulosahydrogelen har möjlighet till att uppfylla många av kriterierna för ett bra sårförband då dess höga vattenhalt kan hålla såret fuktigt, rent och hjälpa såret att dra ihop sig.

Utöver hydrogeler som endast bestod av nanocellulosa undersöktes i detta projekt även hydrogeler som blandats med kollagen eller kaolin. Kollagen är ett ämne som det finns mycket av i hud och som har viktiga roller inom sårläkning där det fyller såret och har koagulerande effekter. Kaolin är en känd blodkoagulerande mineral som inkorporerad i en hydrogel skulle kunna användas på sår med kraftig blödning. Ett kommersiellt förband (Aquacel) togs även med i experimenten för att se hur hydrogelerna förhåller sig till det.

Innan nanocellulosahydrogelerna kan användas som sårförband måste studier göras för att se hur enstaka faktorer påverkas av sårförbanden. I detta examensarbete undersöktes det hur två olika sorters mänskliga hudceller, som även har roller under sårläkning, påverkas av hydrogelerna. Utvärdering av materialet gjordes genom att celler lades på de olika hydrogelerna och under 72 h följdes cellernas aktivitet och utseende. Hydrogelernas påverkan på cellers migration har även undersökts genom att celler odlats till ett heltäckande lager. I lagret gjordes en skråma och på detta ”konstgjorda sår” lades hydrogelerna. Under 24 h utvärderades det hur snabbt cellerna migrerade för att stänga skråman.

Hydrogelerna av nanocellulosa skapade en miljö där en av cellsorterna över tid blev mer aktiva än när de var i kontakt med det kommersiella förbandet. Den andra cellsorten var lika aktiv på alla hydrogeler och Aquacel. Båda cellsorterna fick bättre och bättre utseende desto längre de var på nanocellulosahydrogelerna, vilket tyder på att de trivdes på materialet, medan celler på det kommersiella förbandet inte fick förbättrat utseende. Dessa resultat, under förhållandena i experimenten som utfördes, tyder på att nanocellulosahydrogeler är säkra att använda och minst lika bra som ett kommersiellt förband som är ute på marknaden.

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Abbreviations

a-NFC	Anionic NFC
BC	Bacterial cellulose
BPE	Bovine pituitary extract
CNC	Cellulose nanocrystals
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
hDF	Human dermal fibroblasts
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KSFM	Keratinocyte serum free medium
NFC	Nanofibrillated cellulose
PBS	Phosphate-buffered saline
TCP	Tissue culture plate
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
u-NFC	Unmodified NFC

1 Introduction

Most people have at least once in their life had a small injury that required a simple plaster. Injuries of this kind usually heal themselves without any excessive attention. In the event of a more serious injury, a great deal more attention (and not just a simple plaster) is required. The wound dressings that are applied to such wounds are specific for different phases in the wound healing and need regular changing (Sahlgrenska Universitetssjukhuset 2010). A wound dressing that needs less changing and shortens the healing time is therefore sought after.

Cellulose is a material that has long been used as a wound dressing from the form of leaves and herbs, to gauze dressings and in some of the modern dressings used today. The forest industry is keen to keep this material useful in the wound dressing market as the other applications of cellulose (for example paper) might start to decrease in demand as other technologies move forward.

Wound dressings made from nanocellulose have the potential to fulfil the characteristics of an ideal wound dressing. Nanocellulose is a natural material that can form a network in which water can be retained. This water helps to keep the wound warm and moist, which is optimal for healing (Boateng *et al.* 2008). Some studies (Lin & Dufresne 2014, Jorfi & Foster 2015) have found that some nanocellulose materials have been able to shorten healing times. This might not be true for all nanocellulose materials though, as they can have different characteristics. These differences in characteristics depend on from what, and by which processes the nanocellulose is obtained, as well as how the nanocellulose is processed or modified thereafter.

In this thesis project, the potential of wood-derived nanocellulose as an inexpensive and renewable wound healing dressing was investigated by *in vitro* cell studies where adherence, proliferation and migration experiments were performed. The purpose was to get a first indication of how the complex environment of a wound would react to the nanocellulose dressing by looking at how it affects human dermal cells.

2 Background

2.1 Wound healing

The two dermal cells that were used for the experiments were human dermal fibroblasts (hDF) and keratinocytes. To understand why these cells were suitable for this study one can look at their roles in wound healing.

Skin is mainly made up of two layers, epidermis (which is the outermost layer) and dermis (the layer under the epidermis), with a layer of collagen separating them (Figure 1). The epidermis consists of more than 90 % keratinocytes. As the keratinocytes differentiate they move towards the outside, starting from the basal layer and going through the spinous layer, granular layer and translucent layer to end up in the cornified layer (stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum). The dermis contains many different cells, of which fibroblasts are one type, that are held in place by a collagen matrix (Nguyen *et al.* 2009). Dermal fibroblasts produce collagen and thus also the dermal extracellular matrix.

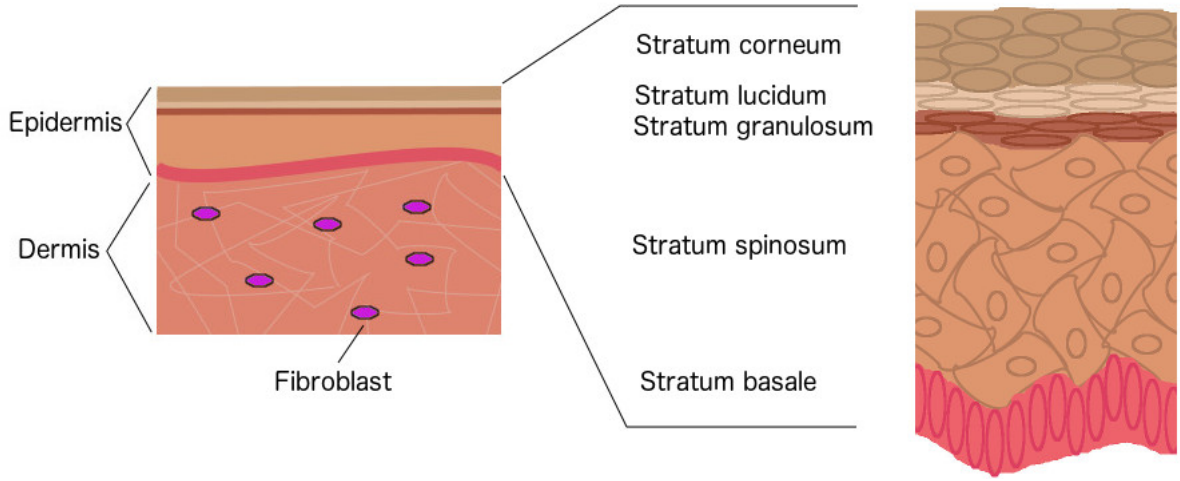

Figure 1. Cross-section of the skin. The dermis consists of the extracellular matrix with fibroblast spread through it. The epidermis consists of several layers of keratinocytes. The epidermis layers image is modified from Wikimedia Commons (Wikimedia Commons 2006).

The wound healing process itself is divided into four phases; haemostasis, inflammation, proliferation and remodelling. The division of the healing phases is fluent and sometimes they can be specified as three phases instead, where some of them have been merged. Haemostasis consists of platelets aggregating and clogging to prevent blood loss. The inflammatory phase starts by neutrophils performing phagocytosis of foreign material and dead cells. Later when macrophages arrive, they take over the phagocytosis as well as start secreting cytokines to recruit and activate other cells. This begins the proliferative phase, as fibroblasts and endothelial cells are recruited to the wound site. There, the fibroblasts proliferate and produce

components for the extracellular matrix (ECM) like collagen, while endothelial cells form microvascular structures (Nguyen *et al.* 2009). Keratinocytes also migrate to cover the wound and as they migrate the keratinocytes at the edges of the wound site will begin to proliferate. The keratinocytes also degrade dermal extracellular matrix components to facilitate the migration (Santoro & Gaudino 2005). Lastly there is the remodelling phase, where collagen fibres are cross-linked and contraction of the dermal extracellular matrix occurs to close the wound (Nguyen *et al.* 2009).

2.2 Wound dressings

Different types of wounds require different types of dressings. The two main categories of wounds are acute wounds and chronic wounds. Acute wounds can be mechanical wounds essentially caused by friction, for example scraping a knee or accidentally cutting oneself, or chemical injuries and burns. These wounds heal in the designated time, around 8-12 weeks, while chronic wounds do not. Reasons to why the chronic wounds do not heal can be that the wound suffers repeated damage, that the wound was not properly treated or because of underlying diseases the patient might have, like diabetes (Boateng *et al.* 2008).

There are several aspects that make a good wound dressing and can be applied to both types of wounds (Boateng *et al.* 2008).

- Debridement; (removal of necrotic tissue and foreign material) helps leucocytes and shortens the inflammatory phase.
- A moist and warm environment; (preferably at normal tissue temperature) is optimal for wound healing, which is why the wound dressing should provide such an environment.
- Absorb blood and have gaseous exchange; where low levels of oxygen stimulate angiogenesis (blood vessel formation) and high levels stimulate epithelisation and fibroblasts.
- Function as a barrier; bacteria should be kept away from the wound and thus prevent infection.
- Low adherence; although the dressing should stick to the wound site it should not adhere too greatly since it then becomes painful to remove and healthy cells could be removed together with the dressing.
- Dressings should also be low-cost and not require frequent change

2.3 Hydrogels

As a moist environment aids wound healing by hydrating the wound, this environment can promote wound debridement and cell migration. Hydrogels can create such an environment, which is why the nanocellulose will be made into one. Hydrogels consists of cross-linked polymers and usually more than 95 % water, which is trapped by the polymer network. They

can consist of different kinds of polymers and commercial hydrogel dressings today use synthetic polymers like poly(methacrylates) and polyvinylpyrrolidine, but dressings using alginate and cellulose also exist. Hydrogels, and other gels in general, can be loaded with compounds (for instance drugs) or be composites where other materials are mixed in to be a part of the polymer network (Boateng *et al.* 2008). These types of dressings are most suited to wounds lacking some tissue, wounds with high exudate amounts and chronic wounds.

2.4 Nanocellulose

Since cellulose has long been used as a wound dressing relatively efficiently (Broughton *et al.* 2006, Shah 2012) it is interesting to see how a different form of it, nanocellulose, would behave. Nanocellulose is derived from cellulose, which is an abundant natural resource and can be found not just in plants but also in some algae, bacteria and marine animals. Cellulose from plant-based materials, especially wood, is easiest to work with in a sustainable way (Hua 2015) since wood grows, albeit slowly, almost without any supervision, is a competent greenhouse gas consumer, and there is a lot of it. Cellulose is located in the cell wall in the form of cellulose fibres. In turn this cellulose fibre is made up by nanofibrils, which consists out of elementary fibrils that are made up by cellulose chains (Lavoine *et al.* 2012), see Figure 2. The cellulose chains consist of beta (1-4)-bound D-glucopyranose units (Figure 2 and 3) that form a polymer around 10000-15000 units long (Hua 2015).

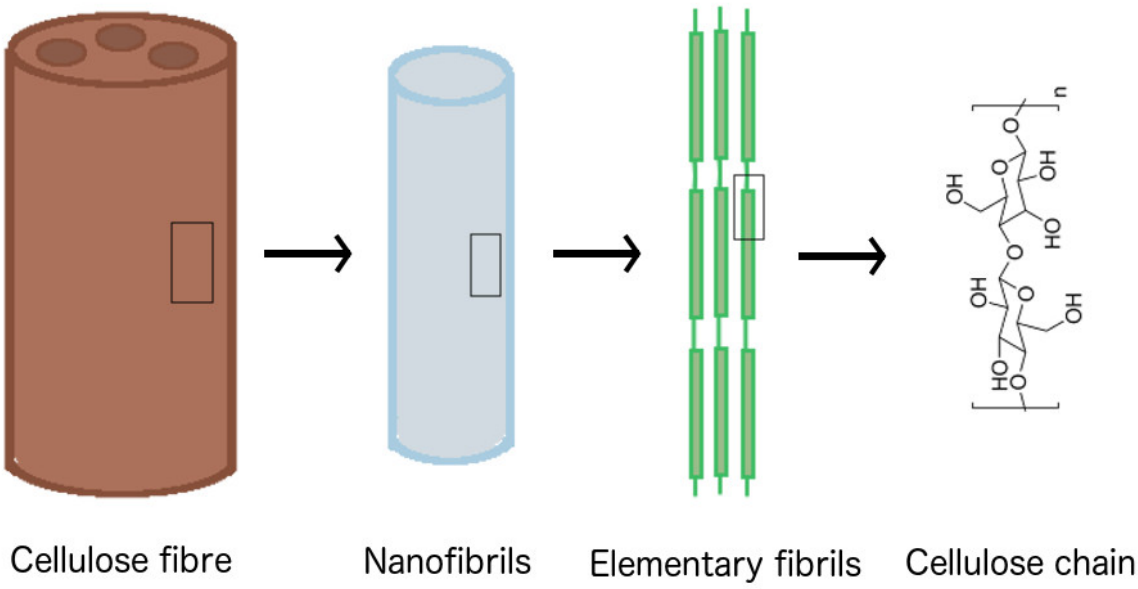

Figure 2. The structural composition of cellulose fibres. The image was adapted from (Hua 2015).

Nanocellulose are cellulose fibrils or crystallites with sizes in the nanometer spectra (Hua 2015). Nanofibrillated cellulose (NFC) is one of three types of nanocelluloses, where cellulose nanocrystals (CNC) and bacterial cellulose (BC) are the other two. NFC and CNC can be extracted from wood and other plants whereas microorganisms produce the last one

(Lin & Dufresne 2014). High-shear homogenization, chemical pre-treatments like TEMPO-mediated oxidation or mild enzymatic hydrolysis can, alone or combined with each other, mechanically treat cellulose fibres to form NFC, while CNC can be obtained by acid hydrolysis of the wood-pulp (Abitbol *et al.* 2016). NFC consists of cellulose elementary fibrils, 3-5 nm in diameter, that can form 20-50 nm thick aggregates and are around a micrometre long (0.5-1 μm). The dimensions of CNC depend on the source and preparation method; wood derived CNC are 3-5 nm in diameter and 100-300 nm in length (Hua 2015). Bacterial cellulose can be produced by bacteria like *Gluconacetobacter xylinum* and unlike cellulose from plant substrates, it does not need to be treated to remove side components like hemicellulose and lignin (Abitbol *et al.* 2016).

Since nanocellulose gets different characteristics (e.g. dimensions and surface chemistry) depending on how it is produced and from what, it can be used for many different purposes. Things like photonics, food packaging, electronics and biomedical applications are areas where nanocellulose is used or where research is being done (Lin & Dufresne 2014). In biomedical applications nanocellulose has been proposed to be used for neural interfaces, cartilage replacements, drug delivery, cardiovascular implants, tissue engineering and wound healing (Jorfi & Foster 2015). In this project the focus is on wound healing.

Each type of nanocellulose that is to be used in applications where it comes in contact with humans needs a biocompatibility analysis. There already exists some biocompatibility research on the bacterial (Sanchavanakit *et al.* 2006, Dugan *et al.* 2013, Rajwade *et al.* 2015, Li *et al.* 2015) and crystalline celluloses (Ni *et al.* 2012, Pereira *et al.* 2013, Yang *et al.* 2013, Roman 2015) but also for the fibrillated ones (Alexandrescu *et al.* 2013, Hua *et al.* 2014, Čolić *et al.* 2015). These studies generally show that the nanocelluloses are biocompatible for their intended applications. However, as mentioned above, the physicochemical properties of nanocellulose change depending of the raw material and manufacturing process, and such properties may affect the materials biocompatibility. Thus, the biocompatibility studies need to be done on a case-by-case basis and considering the foreseen biomedical application.

The natural nanocellulose network can be modified to obtain characteristics beneficial for wound healing. Functionalizing the materials through chemically charged groups or addition of bioactive compounds are some modifications that can be made.

The NFC used in this project originates from wood that has been enzymatically hydrolysed. Apart from a pure NFC hydrogel, a NFC-collagen mixture and NFC-kaolin mixture were also investigated, since collagen is an important component in wound healing and kaolin has blood-clotting properties.

2.4.1 TEMPO-mediated oxidation

The NFC was modified by TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical)-mediated oxidation leading to anionic NFC (a-NFC). This treatment oxidises the hydroxyls of C6 to carboxylates and thus makes the nanofibrils negatively charged (Hua 2015), as seen in Figure

3. The negative charge forces the fibrils apart and results in a material with individual fibrils or better dispersed fibrils, instead of aggregates (Nordli *et al.* 2016).

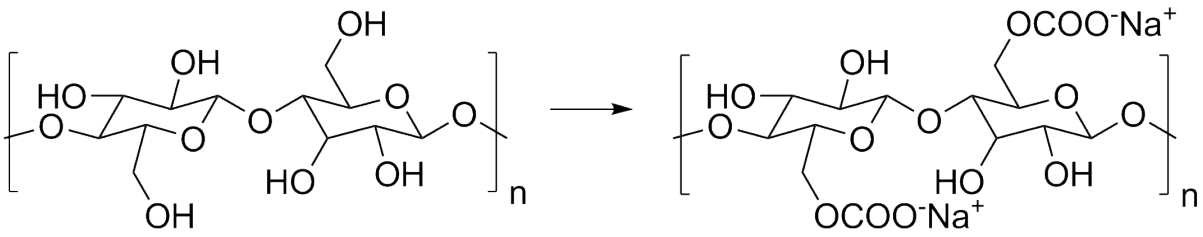

Figure 3. TEMPO-mediated oxidation of cellulose fibrils creates anionic fibrils.

2.4.2 Hydrogel cross-linking

The negative charges of the fibrils also make cross-linking with the aid of multivalent cations possible (Dong *et al.* 2013, Zander *et al.* 2014). Cross-linking leads to hydrogel contraction and a material that is easier to work with (self-standing hydrogel) for wound dressing purposes. The oxidised cellulose is at a start saturated with sodium ions. As cations are added, in this case calcium ions, the sodium ions are pushed away and replaced. The calcium ion is divalent so it can bind to the fibrils in two places. These bindings can be intra- or interfibrillar, either strengthening fibrils or aggregating fibrils together (Dong *et al.* 2013). For simplicity, the oxidised and cross-linked NFC hydrogels will be referred to as NFC.

2.5 Collagen

Collagen is the most common protein in mammals and other vertebrates. The collagen found in skin, and that aids wound healing, is a fibrillar collagen where three amino acid chains are intertwined to form a triple helix. It is the high contents of glycine, proline and alanine that make the chains flexible. In wound healing collagen captures blood platelets to form a haemostatic plug. It is also the major compound that fills the wound and forms the scar. Thus, as a wound dressing component, the collagen could aid haemostasis, attract fibroblasts together with other healing cells and make the dressing appear less foreign to the cells. It could also function as a scaffold so that cells can start producing mature collagen and aligning it faster, since the base would already be there (Purna & Babu 2000).

Type I collagen has also been proven to induce keratinocyte migration (Woodley *et al.* 1988, O'toole 2001). This quality could shorten the healing time of a wound.

Some studies with bacterial cellulose and collagen composites have not showed any definite results that the presence of collagen would aid in cell attachment or metabolic activity (Zhijiang & Guang 2011, Saska *et al.* 2012). Other studies showed indications of collagen hydrogels being able to aid cell proliferation (Dang *et al.* 2017) and increase cell adhesion (Nguyen *et al.* 2017). Thus it is necessary to investigate collagen composites on a case-by-case basis to learn if it has any effect when in that particular composite.

2.6 Kaolin

Kaolin is an aluminium silicate mineral. Although kaolin has many applications the one of interest when it comes to wound healing is its capacity to aid blood clotting. Wound dressings containing kaolin have shown to aid haemostasis in a swine model (Kheirabadi *et al.* 2009) and the US army uses a kaolin-based dressing (QuikClot) in their first aid kits (Ran *et al.* 2010). The negative charge of kaolin interacts with proteins from the coagulation cascade and promotes the blood to clot. In a study regarding blood loss during surgery it was found that a kaolin dressing decreased blood loss as well as the need for blood transfusions after the surgery (Abbott *et al.* 2014).

2.7 Biocompatibility

"Biocompatibility refers to the ability of a material to perform with an appropriate host response in a specific situation". This is the definition of biocompatibility given by Williams in 1987 and it can depend on many things; the characteristics of the compound, the application site or situation, reactions with tissue and possible degradation over time (Williams 2008). In the case of NFC, its production methods and source can alter the material dimension, shape, surface texture, etc. which in turn could affect its biocompatibility. The NFC material is aimed for use as a topical wound dressing, but parts of the dressing could also enter the bloodstream, which is a different environment and could cause the material to have a different effect. The material could also have different effects on different cell types and it cannot be degraded in humans as we lack the necessary enzymes. There are therefore many aspects to consider for a complete biocompatibility analysis of a material.

When cells sense a foreign material, they do not sense it directly. Instead the cells sense a layer of proteins that the material has adsorbed. Depending on the characteristics of the material different kinds of proteins will adhere, in different amounts and possibly change conformation. What proteins become adsorbed is also dependent on the environment the materials are in and what proteins are available. It tends to be hydrophilic materials that have better adsorption of adherence proteins. These proteins, like fibronectin and vibronectin, can be recognized by cells and bound to, thus binding indirectly to the material (Wilson *et al.* 2005). This property can alter how cells react to a material in different environments.

An important aspect of biocompatibility testing is to evaluate cell response to the biomaterial. The biocompatibility of a material can be tested *in vitro* in either a direct or indirect way. The direct experiment has the material in direct contact with cells, as opposed to an indirect test where only material extracts are in contact with cells. The indirect method is appropriate for toxicity testing as it can detect if any toxic compounds are released by the material (Wolf *et al.* 2013). The types of cells used in the tests are selected according to the proposed applications of the material.

2.8 Project objective

This project will contribute to obtain a biocompatibility profile of this particular nanocellulose hydrogel for its future biomedical applications. The purpose is to investigate if the produced NFC, NFC-collagen and NFC-kaolin hydrogels are biocompatible with human skin cells as well as investigating if they could aid in wound closure. The ultimate aim is to make this material an effective wound dressing. To use NFC for these purposes it must be determined that the different hydrogels are biocompatible with the human host and if they have any advantages over techniques and materials used today. This is also why Aquacel from ConvaTec, which is a carboxymethyl cellulose dressing commercially available, will be studied as a comparison to the NFC hydrogels. It is the wound dressing most similar to the NFC hydrogels as they are all made from anionic cellulose (although Aquacel is physically held together and not cross-linked as the NFC hydrogels) and have high water contents. A novel wound dressing, which the hydrogel has a possibility to become, would be a welcome addition to the repertoire of modern wound dressings.

In the present work, the biocompatibility of the NFC materials was evaluated by *in vitro* cell studies where human dermal cells (fibroblasts and keratinocytes) were cultured on the hydrogels and the cell response to the materials was evaluated in terms of cell metabolic activity (alamar blue assay) and cell morphology (fluorescence microscopy).

A cell migration assay, also known as a scratch assay, was done to see if the hydrogels could improve cell migration to close a scratch in a fibroblast monolayer. Hydrogels were put on top of the scratch, just as a dressing would be. The cell migration was measured through inverted microscopy and percentage of wound closure.

3 Materials and Methods

3.1 Production of NFC hydrogel

Unmodified NFC (u-NFC) was obtained from Innventia AB, Sweden and functionalised as previously described (Hua *et al.* 2014). Briefly, the equivalent of 2 g of never dried u-NFC was mixed with dH_2O, to a volume of 300 ml, and dispersed using a magnetic stirrer. 206 mg NaBr and 31.2 mg TEMPO were dissolved in 20 ml dH_2O. While stirring the u-NFC, the NaBr/TEMPO solution was added, as well as 20 ml of 10 wt% NaClO to start the reaction. pH was maintained at 10.3 for 2 h by dropwise addition of 0.5 M NaOH. The cellulose dispersion was centrifuged at 5500 × g for 50 min, supernatant was removed and the pellet of a-NFC was resuspended in 25 ml of dH_2O. The cellulose dispersion was dialysed using dialysis membranes with a 14 000 Da cut-off for 6 days until the conductivity of the purifying water was at most 0.001 mS/cm. The cellulose was collected and sonicated after which it was concentrated to 3 wt% by heat evaporation.

Cross-linking of the fibrils was performed as described by Dong *et al.* (2013), by dropwise addition of 1 ml of 200 mM $Ca(NO_3)_2$ for every ml of NFC dispersion, 0.1% kaolin with NFC dispersion and 0.1% collagen with NFC dispersion. The cross-linking process physically traps the kaolin and collagen into the network. From the resulting hydrogels, discs were punched, sterilized by UV light and further used in the cell studies.

3.2 Cell culture

hDF from European Collection of Authenticated Cell Cultures (ECACC) were cultured in Dulbecco's modified eagle medium (DMEM) F:12 with L-Glutamine and 15 mM HEPES supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and, 100 µg/ml streptomycin, in a humidified atmosphere, 5% CO_2, at 37°C. Cells were harvested using trypsin-EDTA treatment. Briefly, cell culture medium was removed by aspiration, cells were carefully washed with PBS. Then, 3 ml trypsin-EDTA and 3 ml PBS were added and an incubation for 5 minutes followed. Trypsinisation was stopped by addition of 6 ml DMEM and cells were transferred to falcon tubes for 5 min centrifugation at 220 × g. Supernatant was removed and cells were resuspended in 1 ml DMEM. Cell concentration was counted using Trypan blue staining and a haemocytometer. Cells were resuspended in fresh cell culture medium to continue the culture or used in the *in vitro* assays.

Keratinocyte serum free medium (KSFM) supplemented with 0.05 mg/ml BPE, 35 ng/ml EGF, 100 IU/ml penicillin and, 100 µg/ml streptomycin was used to culture the keratinocyte cell line CCD 1106 KERTr (CRL-2309) from ATCC (American Type Culture Collection). Sub-cultivation was performed similarly to the hDF, except for higher trypsin-EDTA amounts (4 ml for a T-75 flask) and centrifugation at 125 × g.

3.3 Cell response to NFC hydrogels

NFC, NFC-collagen and NFC-kaolin 8 mm hydrogel discs were placed into wells on 96-well tissue culture plates so that they covered the entire well bottom. Pieces of Aquacel (5x5 mm) were pulled apart to form thinner dressings, soaked in sterilised water and then placed in 96-well plates to cover the bottom. The hydrogels were allowed to soak in 200 µl cell culture medium while cell suspensions were prepared. Cell culture medium was removed and cells were seeded at 5000 cells/well and 6000 cells/well for hDF and keratinocytes respectively, on top of the hydrogels. Cells cultured directly on the tissue culture plate (TCP) served as a positive control. Three plates were prepared for measuring viability after 24 h, 48 h and 72 h of incubation at 37°C, 5% CO_2 in a humidified atmosphere.

The viability of the cells was assessed by the alamar blue assay. Alamar blue indicates the metabolic activity, which can be used to quantify cell growth. The active component is resazurin dye which, when taken up by cells, is reduced to its fluorescent form resofurin. The change in fluorescence correlates to cell metabolic activity in the sample (Bonnier *et al.* 2015). Cell culture supernatants were removed and 200 µl of a 1:10 alamar blue reagent

dilution in cell culture medium was added to the wells. Culture plates were then incubated at 37°C for 120 min. Blank consisted of alamar blue in cell culture medium without cells. After incubation, 100 µl aliquots from each well were transferred to a black flat-bottomed 96-well plate for fluorescence measurement in a spectrofluorometer (Tecan infinite® 2000) with 560 nm excitation wavelength and 590 nm emission wavelength.

Cell adherence and morphology were also evaluated by fluorescence microscopy, using a live/dead double staining kit (Sigma) by addition of 200 µl staining solution consisting of cell culture medium with 0.2 % calcein-AM and 0.1 % propidium iodide. Viable cells stain green because calcein-AM is modified to be fluorescent by esterases in viable cells. Dead cells stain red where propidium iodide passes through degraded membranes and interacts with the DNA helix. Following was a 30 min incubation at 37°C after which cells were imaged with a fluorescence microscope (Nikon ECLIPSE TE2000-U) at 490 nm excitation, 515 nm emission to visualise live cells and 535 nm excitation, 617 nm emission to visualise dead cells.

Interactions between the hydrogels with the alamar blue assay and the live/dead staining were investigated by performing the same procedures described above but in the absence of cells. An interaction test of the hydrogels with the reduced alamar blue reagent (alamar blue in DMEM autoclaved for 20 min at 1.5 MPa) was also performed.

3.4 Migration assay

hDF were seeded at a concentration of 50 000 cells/well in a 24-well plate and incubated at 37°C, 5% CO_2 for 24 h to form a confluent monolayer. Cut out Aquacel pieces were immersed in sterilised water and then soaked for 1 hour in cell culture medium (1% FBS). NFC hydrogel discs (10 mm) were also soaked in cell culture medium (1% FBS) for the same time. Vertical scratches were made with a 1 ml pipette tip and cell monolayers were washed twice with PBS to wash away non-adherent cells. Fresh cell culture medium (1% FBS) was added to the wells and 0 h reference images of the scratch widths were obtained by light microscopy (Nikon ECLIPSE TE2000-U) at 4x magnification. The hydrogels were then added on top to cover the scratches. The closure of scratches was imaged at 12 and 24 h using light microscopy at 4x magnification and the percentage of scratch closure was calculated. Live/dead staining of scratches at 0, 12 and 24 h was performed to obtain fluorescent images for presentation purposes.

Reference marks were made on the back of 24-well plates at the beginning of each experiment in order to facilitate the imaging of the scratches. The cell culture media had low serum amounts to inhibit proliferation, thus scratch closure would only depend on cell migration.

3.5 Statistical analysis

Data was examined using Student's t-test to find significant differences. Samples were considered statistically different at $p < 0.05$.

4 Results

4.1 Hydrogel interference

The hydrogels did not interfere with the live/dead assay, while there was interference in the alamar blue assay. The hydrogels absorbed around 20 % of both reduced and oxidised alamar blue reagent compared to the blank (Figure 4). This interference was accounted for in the cell viability assays.

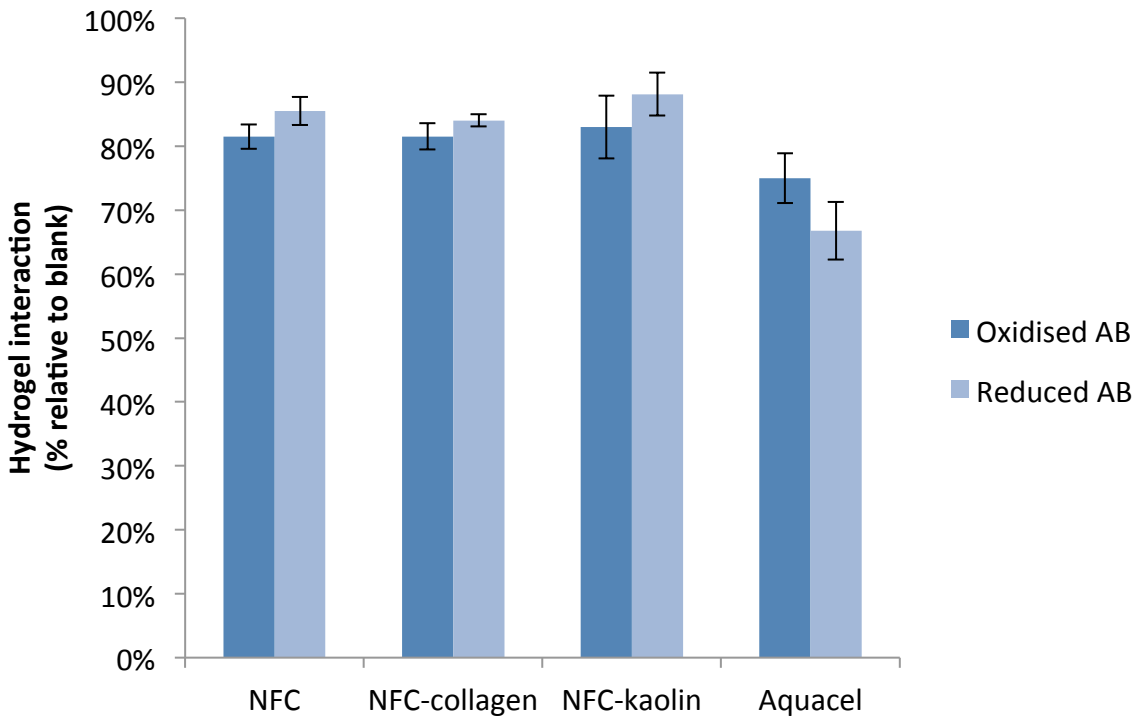

Figure 4. Percentage of alamar blue fluorescence that hydrogels absorb compared to the blank (alamar blue reagent in cell culture medium).

It should be mentioned that alternative methods of cell quantification were investigated as well. Hydrogel incubation in PBS with cellulase was used to enzymatically degrade the cellulose in the hydrogels and liberate the hDF. The interference of the hydrogels should become minimal and the amount of DNA and protein of the cells would become quantifiable. These alternative quantifications did not conform well with the mode of hydrogel

degradation. The amount of cellulase was too great to correctly quantify the protein amounts from the cells and there was DNA contamination in the cellulase, which interfered with the DNA assay. Hydrogel degradation with EDTA was also investigated, since it had been stated that EDTA could break down cation cross-linked gels (Dong *et al.* 2013). After a 40 min incubation period in 37°C with a trypsin-EDTA mixture (380 mg/L EDTA) the hydrogel conformations were not altered significantly. This indicates very sturdy hydrogels, which of course is a good quality for a wound dressing.

Thus, being aware of the hydrogel interference with the alamar blue reagent, the results of the alamar blue assay were verified by fluorescence microscopy images (see section 3.3).

4.2 Cell adhesion and proliferation

The metabolic activity of hDF cultured on the hydrogels can be seen in Figure 5. All the hydrogels showed similar cell adhesion (24 h time points) and proliferation (increase in metabolic activity over time), however much lower activities than that of cells cultured on the positive control. At the 72 h time points for hydrogels the activities were significantly higher compared to the previous time point, i.e. cells slowly adapt and proliferate when cultured on the hydrogels.

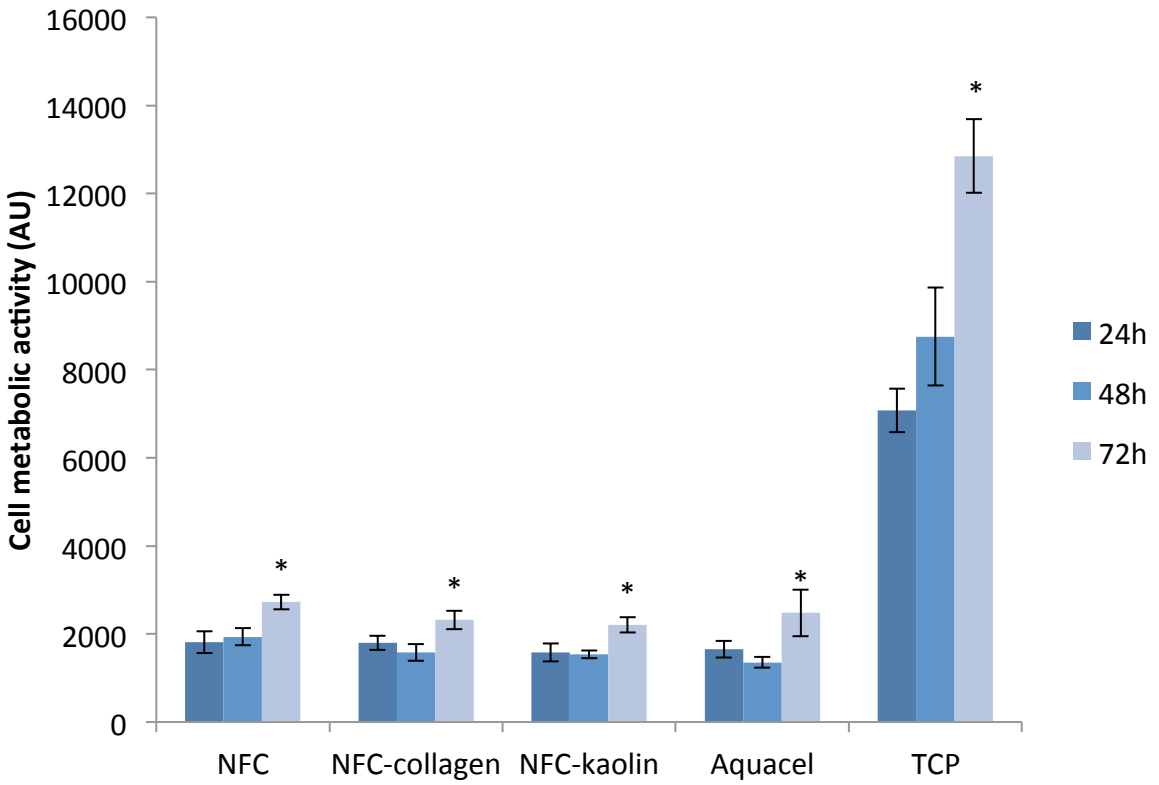

*Figure 5. Metabolic activity of hDF cultured on the different hydrogels and control. Columns marked with * were significantly different ($p < 0.05$) from the previous time point, indicating cell proliferation. The data represents the mean ± SEM for $n = 6$.*

Keratinocytes cultured on NFC hydrogels had similar metabolic activities, however more rapidly increasing than when cultured on Aquacel (Figure 6). The cell adhesion (at 24 h) is the same for NFC-collagen and TCP, which indicates cells being as prone to bind to the material as they are to a specified cell-binding surface. Cell activities on NFC hydrogels were significantly different between each time point, a strong indication of proliferation, while cell activities on Aquacel at 48 and 72 h were only significantly different from the 24 h time point. Cells on NFC-collagen also had significantly higher metabolic activities at 24 and 48 h than cells on the other NFC hydrogels, but this difference was lost at 72 h, which implies that the collagen aids cell adherence and proliferation for the first two days.

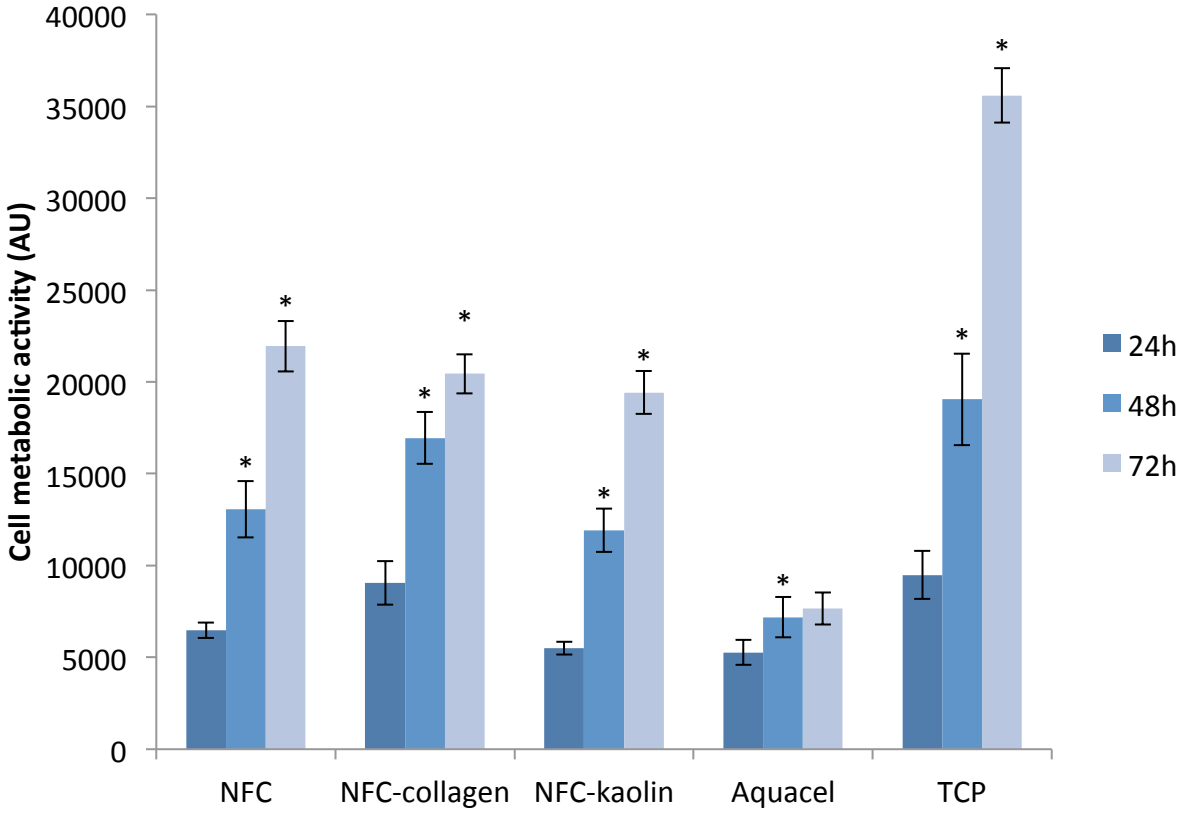

*Figure 6. Keratinocyte metabolic activities on the different hydrogels and control. At 48 h and 72 h cells on NFC hydrogels had significantly higher activities than on Aquacel ($p < 0.05$). Columns marked with * were significantly different ($p < 0.05$) from their previous time point. The data represents the mean ± SEM for $n = 6$.*

4.3 Cell visualisation

To assess cell adhesion patterns and cell morphology a live/dead staining was performed and cells were observed under fluorescence microscopy. Representative images of live cells are presented in Figures 7 - 10. There were minimal amounts of dead cells, thus such images are not shown.

When looking at the numbers of adherent hDF (Figure 7) they seem to have increased over time on NFC, NFC-collagen and the TCP control, confirming the alamar blue results. It was difficult to visualise cells on Aquacel since there were not many cells and the structure of Aquacel allowed cells to be in several layers, which made it difficult to focus the microscope lens on the cells. Thus the Aquacel images might not correspond with the alamar blue assay.

NFC-kaolin images are not presented together with the other hydrogel images, as cells on NFC-kaolin could usually not be discerned, this because the hydrogel did not let enough light through to make the cells visible.

The morphology of hDF cells on NFC hydrogels improved over time (Figure 8). The longer hDF were on NFC hydrogels, the more morphologically sound they became. The cells started off with spheroid forms at 24 h on the NFC hydrogels, at 48 h they had started to elongate slightly and had maybe one extension per cell or the start of extensions. At 72 h most cells were elongated and had more than one extension. hDF in Aquacel were of a spheroid shape and at 72 h the healthiest cell might have evidence of beginning of extensions.

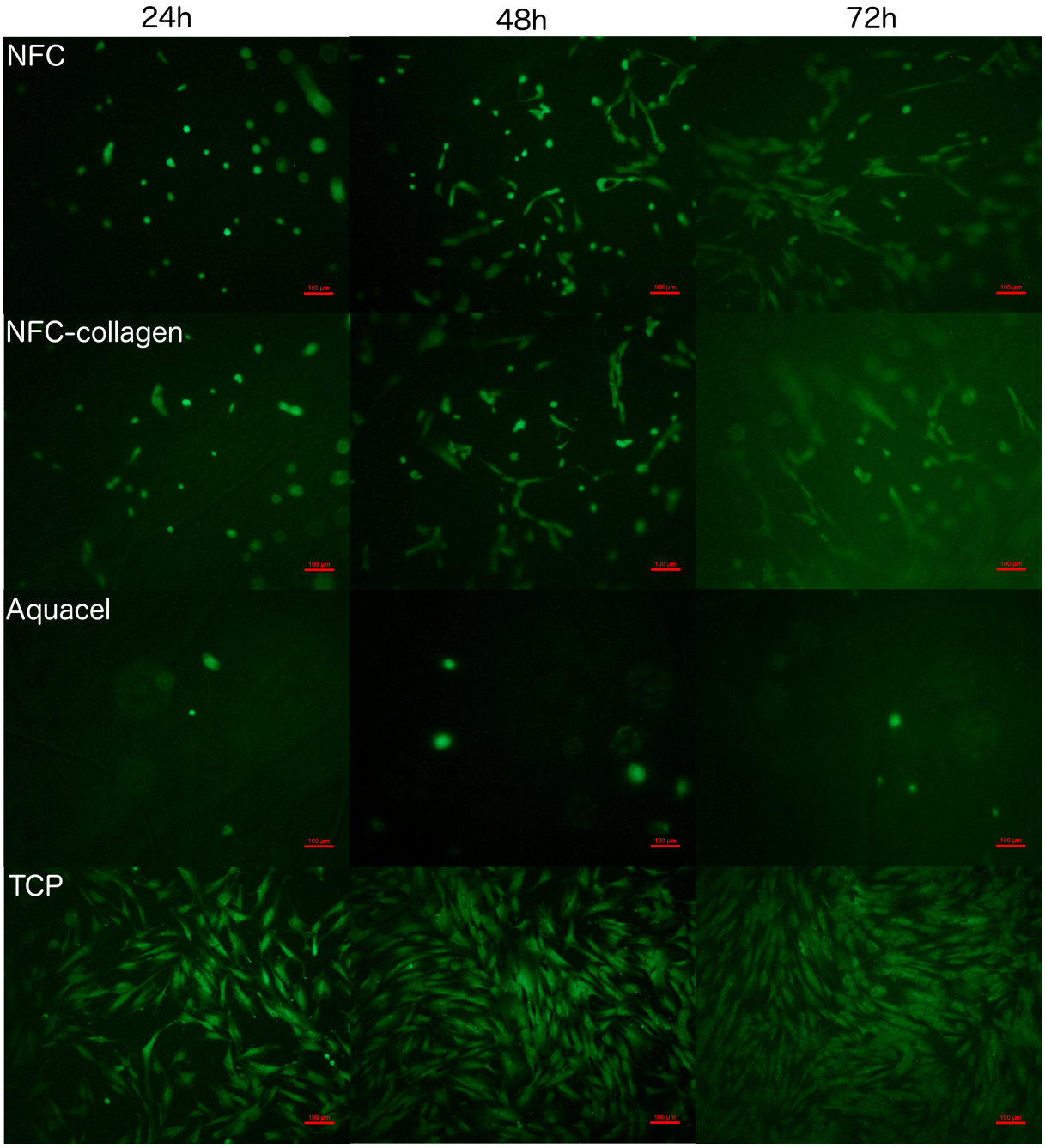

Figure 7. Low magnification images of hDF on hydrogels and in control at different time points, where cell adhesion patterns and proliferation can be observed. TCP is a positive control where cells were grown without hydrogel. Scale bars represent 100 µm.

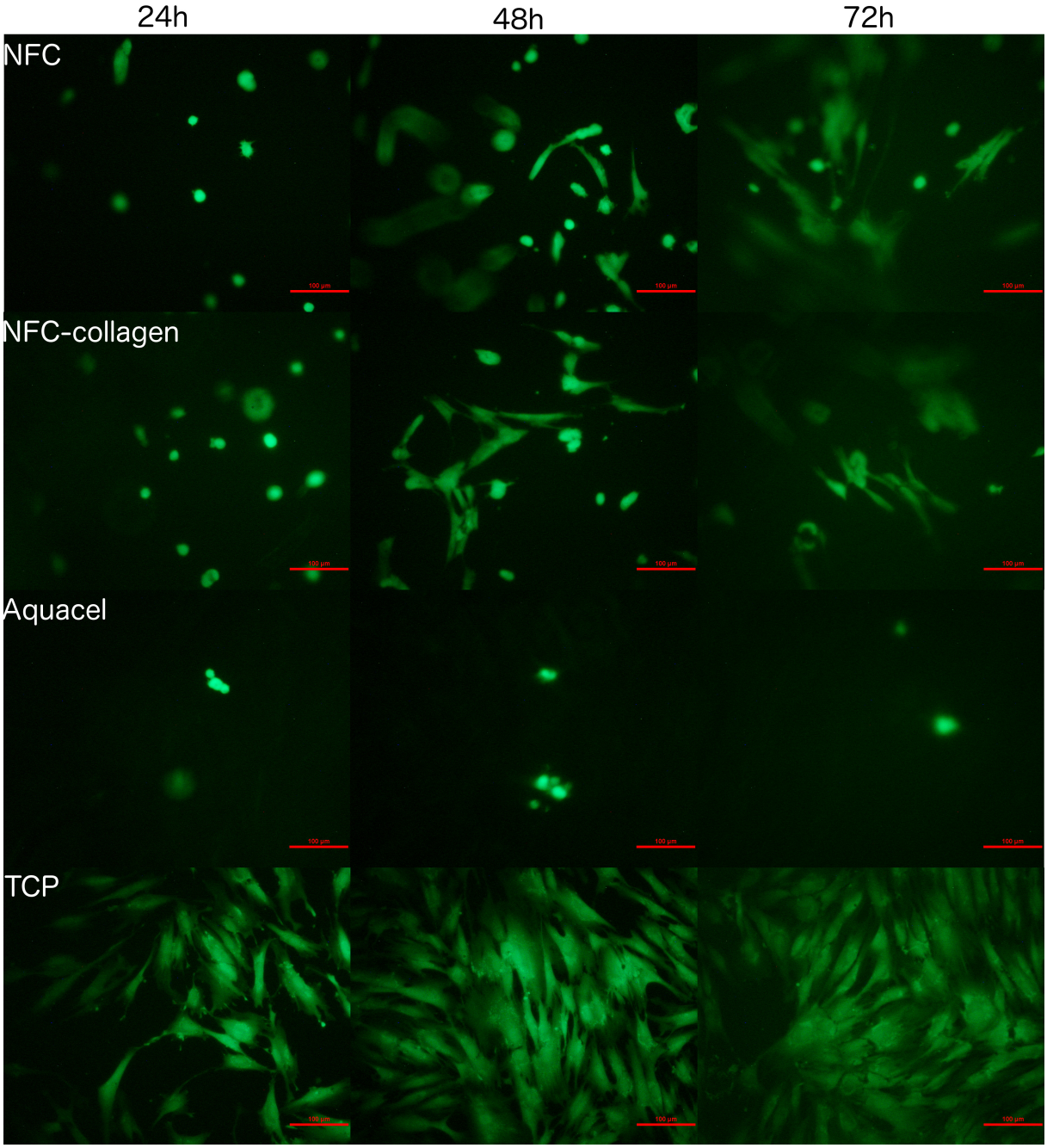

Figure 8. High magnification images of hDF cultured on hydrogels and control, depicting cell morphology. TCP is a positive control where cells were grown without hydrogel. Scale bars represent 100 μm.

From the fluorescence microscopy images of keratinocytes (Figure 9) it is evident that the cells fused together. These fused cell areas got larger as time went on so cell numbers should therefore increase, thus reflecting the cell number increase in the alamar blue assay. There was minimal keratinocyte fusion in Aquacel and none on the TCP.

Figure 10 shows a closer look at the fused cells on NFC hydrogels and how they increased over time. In Aquacel some cells formed small cluster of at most four cells, but the majority of them were not adhered to each other. Typically keratinocytes have a polygonal shape, as shown in the TCP control. It would seem that the cells at 24 h, that were not fused, either had spherical or polygonal shapes, and that as time went on most of the single cells had fused.

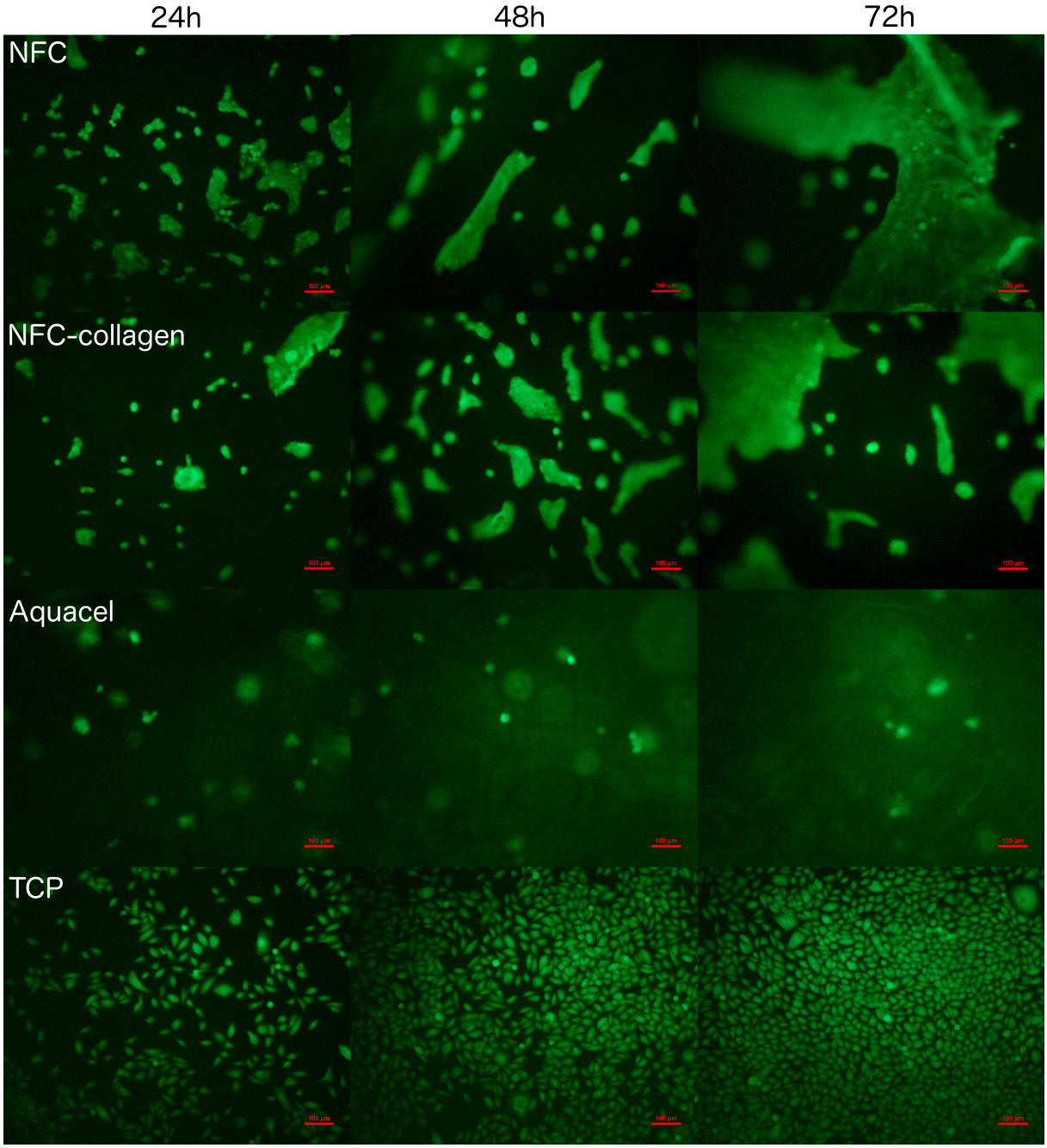

Figure 9. Low magnification images of keratinocytes cultured on hydrogels and control at different time points, showing cell adhesion patterns and proliferation. TCP is a positive control where cells were grown without hydrogel. Scale bars represent 100 µm.

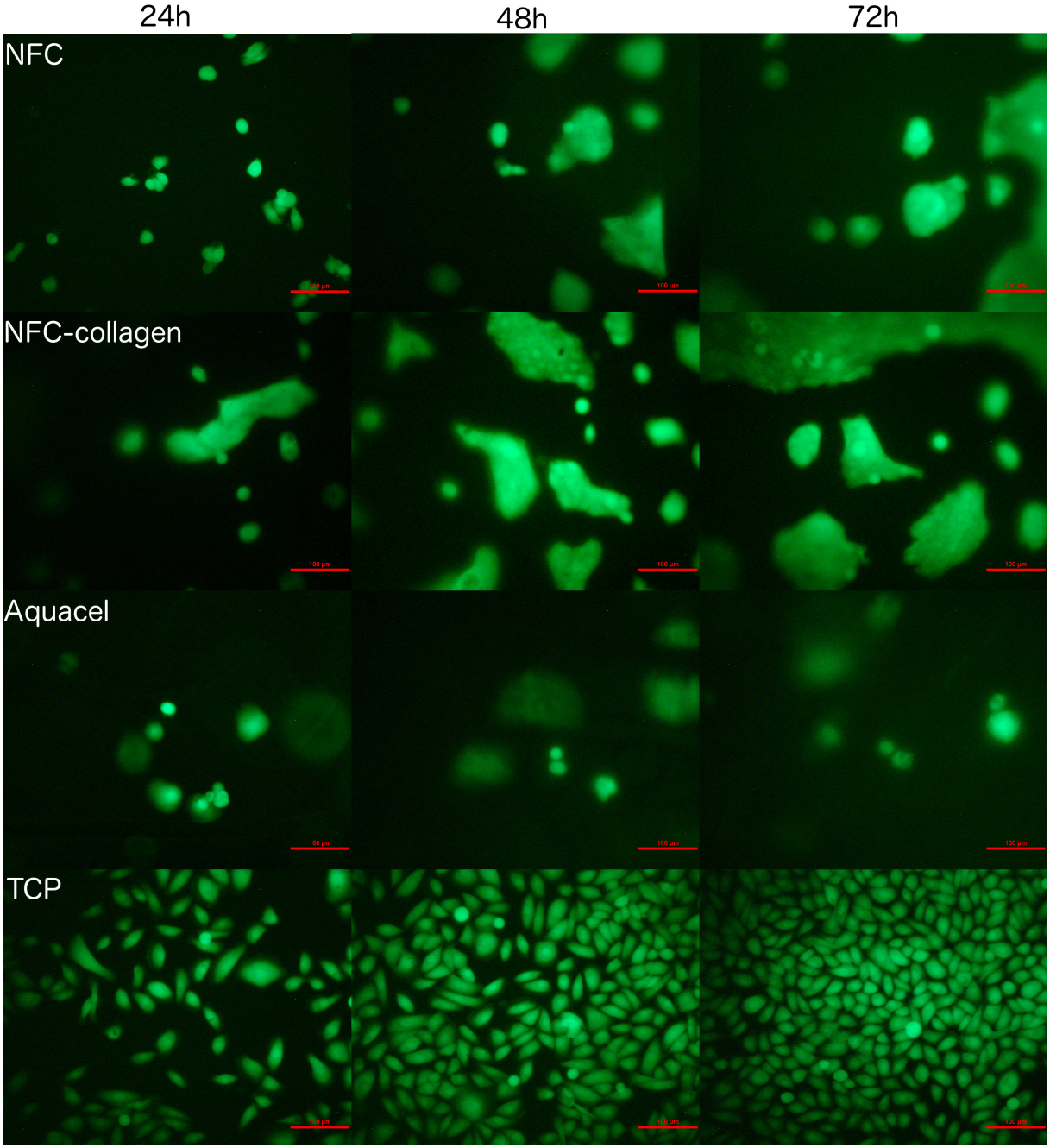

Figure 10. High magnification images of keratinocytes cultured on hydrogels and control at different time points, showing the morphology of cells. TCP is a positive control where cells were grown without hydrogel. Scale bars represent 100 µm.

Occasionally cells were visible on NFC-kaolin hydrogels (Figure 11). The cells were similar in amount and morphology to their counterparts on NFC and NFC-collagen. At 72 h on a NFC-kaolin gel hDF were elongated and had more than one extension for most of them (Figure 11A). At 48 h keratinocytes exhibited cell fusions and similar amounts to the other NFC hydrogels (Figure 9 and 11B).

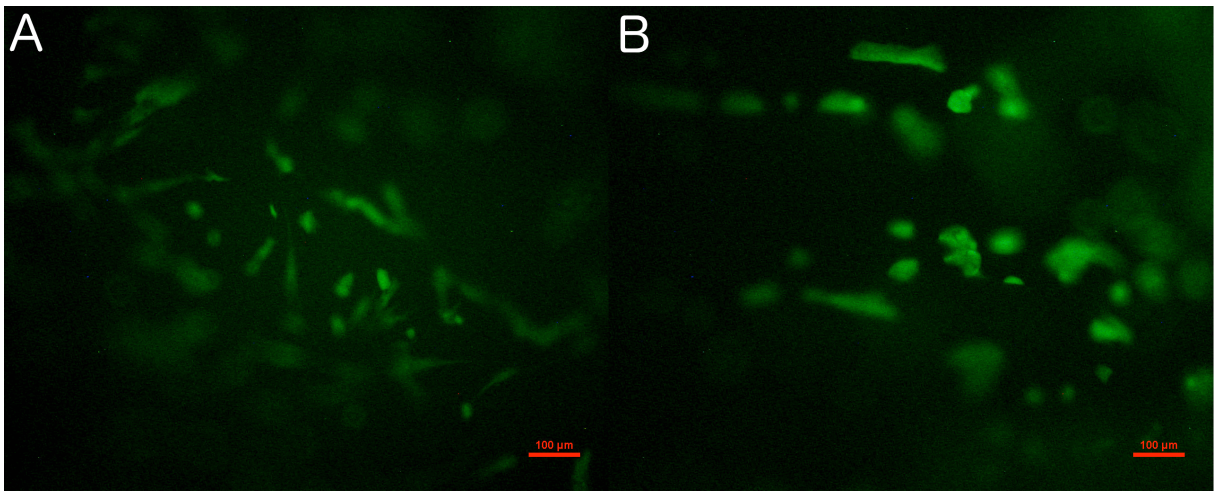

Figure 11. Cell adhesion and morphology on NFC-kaolin hydrogel. ***A.*** *hDF at 72 h and* ***B.*** *keratinocytes at 48h. Scale bars represent 100 µm.*

4.4 Cell migration

The migration assay retails how cell migration rates are affected by the different hydrogels. Compared to the scratch wound at zero hours (Figure 12) it seems that NFC-collagen and cells without hydrogel (TCP) have the most rapid wound closure. The hydrogels presence on top of the cell monolayer also did not have any negative effect on the cell morphologies or the integrities of the cell layers.

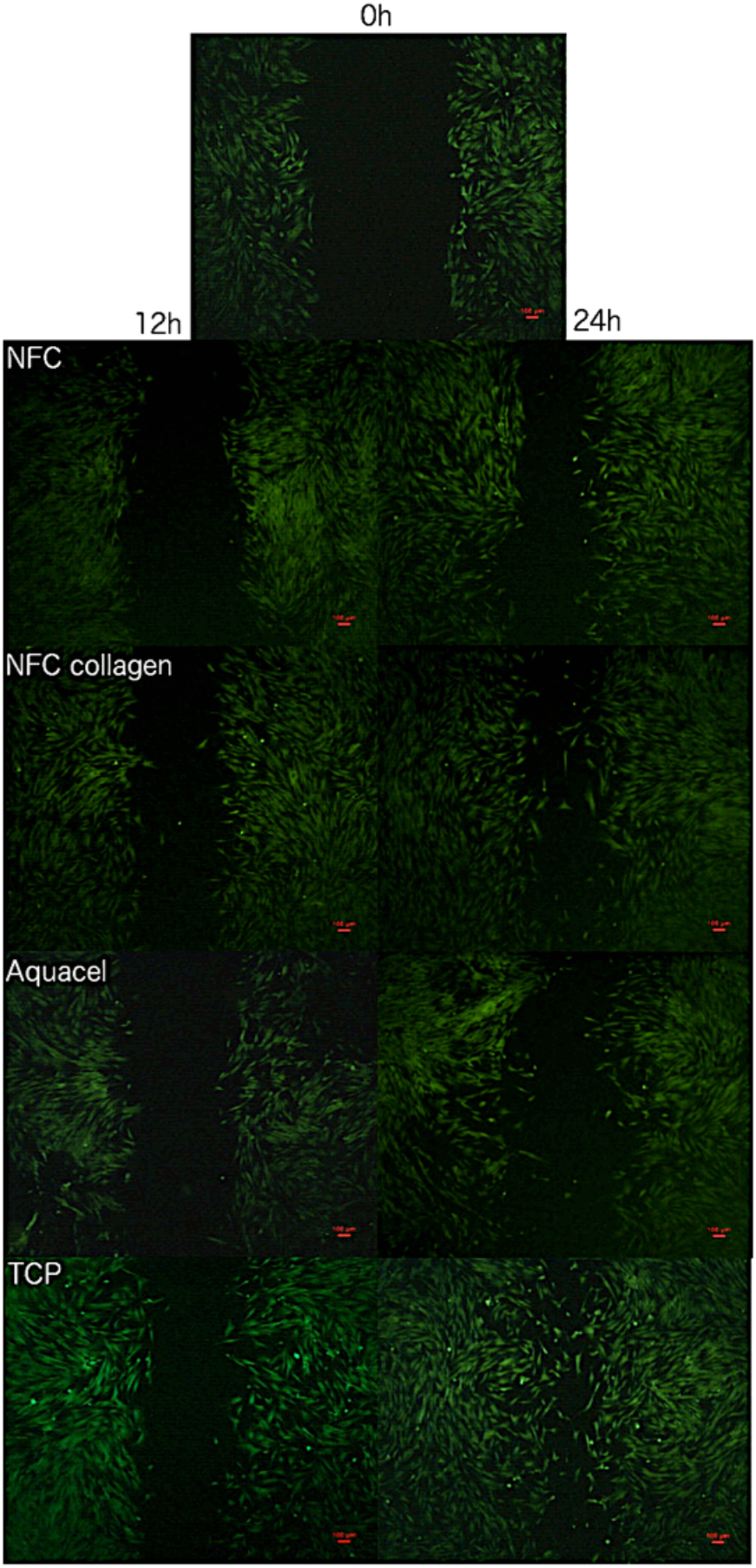

Figure 12. hDF migration under hydrogels and in control to close a scratch made in cell monolayers. Cells were imaged at 0, 12 and 24 h after the scratch was made. Cells in the absence of hydrogels serve as control (TCP). Scale bars represent 100 µm.

When looking at the percentage of wound closure at 12 h compared to 0 h (Figure 13), all the hydrogels are in the same range. At 24 h, the general similarity in range is still there except for a significant difference between NFC and NFC-collagen. NFC-collagen did seem to have slightly better wound closure than the other hydrogels because of a significant similarity to TCP at 12 h and the difference to NFC at 24 h. The significant similarity between NFC-collagen and TCP did not continue because after 24 h, cells without hydrogels (TCP) had significantly higher wound closure.

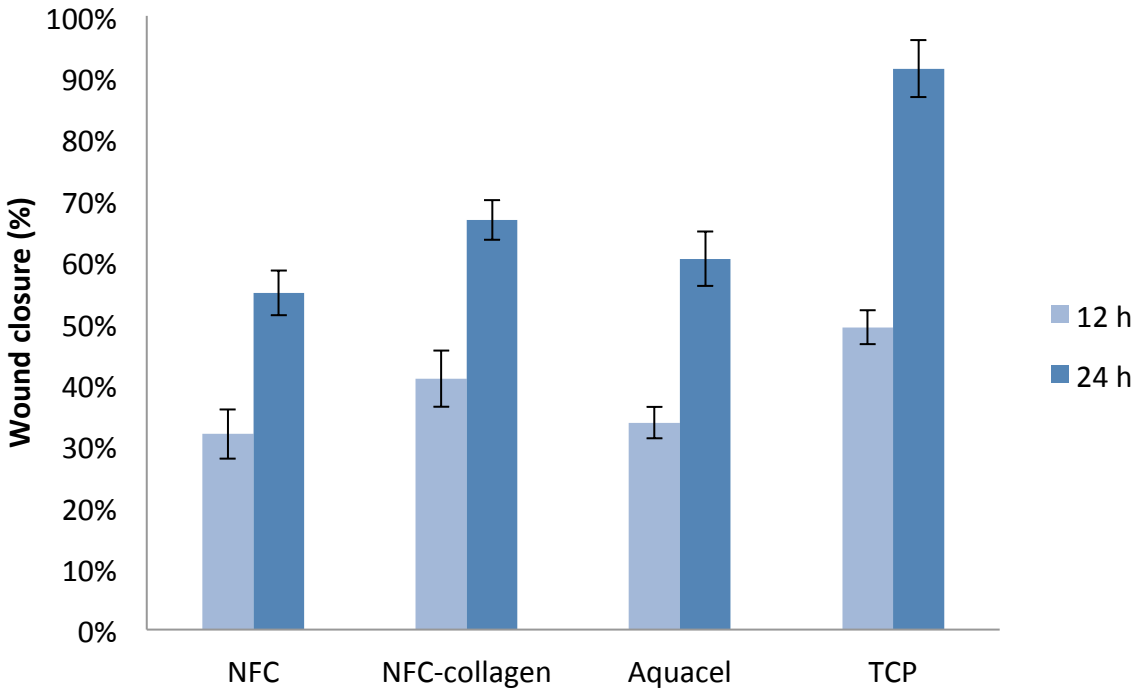

Figure 13. Percentage of wound closure in hDF cell layers after a 12 and 24 h incubation with hydrogels compared to scratches at 0 h. The control was hDF without hydrogel (TCP). All hydrogels promoted wound closure ($p < 0.05$) between the two time points, but at a lower rate than the control. At 12 h NFC-collagen is not significantly different from TCP ($p > 0.05$) while it becomes different ($p < 0.05$) at 24 h. The data represents the mean ± SEM for $n = 3$.

5 Discussion

The *in vitro* studies performed here show that the average metabolic activity of hDF cultured on the NFC hydrogels was much lower than that of the positive control, but comparable to the commercial dressing (Figure 5). This low metabolic activity, especially at 24 h, signifies low cell adhesion to the materials. When looking at the cell proliferation i.e. the increase in cell viability/number over time, significant increases in metabolic activity only occurred at 72h for the NFC hydrogels. This means that even if hDF have poor adhesion to the material they do seem to adapt and proliferate on it. Besides, the fluorescence microscopy images confirmed these results and showed that the hDF get more and more elongated morphologies the longer

they were cultured on NFC and NFC-collagen (Figure 8). It would seem that cells are reluctant at first to bind fully to the material and spread out, but as time goes on the amount of cells increases as well as they acquire typical fibroblast morphology. This is a good indication that the materials are not toxic to cells and that they might not hinder the proliferative phase in wound healing.

Authors have investigated the biocompatibility of various forms (hydrogel, air dried films and freeze dried aerogels) of TEMPO-mediated oxidised NFC towards mouse and human fibroblasts showing that cell number/viability/metabolic activities of cells with such samples were closer to that of the positive controls (Alexandrescu *et al.* 2013, Nordli *et al.* 2016, Liu *et al.* 2016, Rashad *et al.* 2017). The reason for the difference between the results presented here and those found by others could be that different quantitative assays, different types of cells or NFC materials (source and type; aerogel, film, hydrogel) were applied in the studies. The experiment protocols also matter, for example in how the cells are allowed to adhere to the material either by allowing cells to adhere in the absence or presence of cell culture medium covering the samples. As the results in this thesis seem to suggest that when cells reach a certain number they adapt to environment, the difference in cell adhesion method could cause the differences seen. An intrinsic low hDF adherence to these specific NFC materials can also be an explanation for the low activities. One study has shown low adhesion of hDF on air-dried carboxylated NFC films (Hua *et al.* 2014).

For NFC-kaolin and Aquacel the alamar blue assay results could not be verified by the fluorescence microscopy images since cells could not be visualised so well (Figures 7 and 8). The few images obtained with NFC-kaolin seem to support that the hydrogel behaved similarly to the other NFC materials in terms of cell amount and morphology (Figure 11A). The few hDF that have been imaged on Aquacel did not have elongated morphologies, which could mean that the cells did not like the material. Since cells have been seen to maintain their viability well under all materials (Figure 12), the materials themselves were not toxic, and the reason for hDF to avoid Aquacel are possibly more structural.

The adherence of keratinocytes onto the hydrogels was greater than for the fibroblasts since the activities at the 24 h time points for keratinocytes were closer to TCP (Figure 6). Especially NFC-collagen had high adherence, as at early time points there was no significant difference in cell activities between it and TCP, indicating that collagen improves keratinocyte adherence. Overall, keratinocytes cultured on NFC hydrogels have higher proliferation rates than on the commercial Aquacel dressing. This apparent difference in proliferation is also enforced by the fluorescence microscopy images (Figure 9) where there is no clear indication of cell number increase over time for Aquacel.

In the fluorescence microscopy images of keratinocytes on NFC hydrogels (Figure 9), it looks like cell amount increased over time, but the most striking thing is that cells fused together. Cell fusions increased over time i.e. bigger fusions were observed at longer incubation times. A reason for this fusion of keratinocytes may lie in the calcium present in the cross-linked

hydrogels. Addition of extracellular calcium has shown to produce cell fusions *in vitro* (Rekus 2000, Deyrieux & Wilson 2007). As cell fusions have been shown to be a tendency of differentiated cells (Hennings & Holbrook 1983), it could be speculated that the cells in the experiment performed here have started the terminal differentiation process. However, the differentiation process starts with growth inhibition (Rekus 2000) which is something not seen here, as cells showed proliferation patterns in the alamar blue assay. Thus it might be more likely that cells were in the beginning stages of cell differentiation as a cell can only be in one state at the time; proliferating, migrating or differentiating. From the results discussed here it can be said that there was cell proliferation as well as morphological changes. The two factors related to fusing could be beneficial in wound healing as small clusters of keratinocytes in the wound can aid in wound closure by being linked together by proliferating cells, and thus diminishing the area needed to be covered by the proliferating cells (O'toole 2001). Further experiments and research is needed to fully investigate the state of the keratinocytes and the hydrogels effect on them, for instance by looking at proliferation and differentiation factors.

Aquacel did not have major cell fusions, which supports the notion of calcium ions from the hydrogels inducing the morphological change. Fused keratinocytes could be discerned on NFC-kaolin (Figure 11B), with amount and morphology reminiscent of that of the other NFC hydrogels (Figures 9 and 10). Since metabolic activity was in the same range as well (Figure 6), the NFC-kaolin hydrogel would appear to have the same qualities as the other NFC hydrogels.

These experiments were performed *in vitro,* which is a simpler environment than *in vivo*, but at the same time more controllable. One example of this is that the calcium ions *in vitro* are more accessible than *in vivo* because of ion exchange with the cell culture medium. The hydrogels also physically adsorb proteins, depending on what proteins are adsorbed the cells will sense the hydrogels differently and either prefer or reject adhering to them. *In vivo*, the protein amounts and types available can differ from the conditions during the *in vitro* experiment. These experiments are only guidelines to get a first estimation of how cells behave in the presence of the hydrogels, but are still essential to get an understanding of different processes. The main task of the *in vitro* experiments is to screen new materials and get an understanding of the underlying processes of the *in vivo* environment. Since there are many simultaneous events occurring during *in vivo* conditions, the *in vitro* experiments can be designed to provide an opportunity to investigate isolated events, and thus gain understanding of them.

Regarding the migration assay, the materials did not negatively affect the cell layers (Figure 12). This is important, as too strong cell adhesion is undesirable in a wound dressing. When removing the hydrogel dressing it could result in pain for the patient, as newly proliferated healthy cells would be ripped off together with the dressing (Boateng *et al.* 2008). Cells exposed to the hydrogels generally migrated slower than cells without the materials (Figure 13). At the 12 h measurement point, the migration of cells exposed to NFC-collagen was not

significantly different from cells without dressing. This could signify that this hydrogel has no negative effect on migration for 12 h. The cell migration on NFC-collagen also improves over time compared to NFC, so the collagen in it seems to make a difference in wound closure. Type I collagen has been proven previously to induce keratinocyte migration (Woodley *et al.* 1988), but it did not have the same effect on fibroblasts (O'toole 2001). However, since collagen is known to interact with fibroblasts it is not surprising that some effects of it could be seen. Throughout the experiments, the migration of cells on NFC hydrogels was similar to cell migration on the commercial Aquacel dressing. The slower migration for cells under hydrogels could be because of the steric hindrance the hydrogel poses, since cells would have to push it away during migration. Future experiments should include a migration assay with keratinocytes.

6 Conclusion

The experiments performed in this master thesis suggest that cells in contact with NFC hydrogels proliferate, although at a slower rate than cells on the positive control, with morphologies resembling those of typical fibroblasts and forming cell clusters in the case of keratinocytes. Cells on NFC hydrogels are metabolically in the same range as the commercial dressing Aquacel, while their morphologies appear to be sounder than cells on Aquacel. NFC hydrogels do not accelerate the wound closure compared with the experiments in absence of the materials, neither negatively affect the cell layer. A similar effect was observed with the reference material Aquacel. There might also be more positive effects of NFC-collagen than the other NFC hydrogels on keratinocyte adhesion and fibroblast migration.

The *in vitro* experiments are only indications of what could happen *in vivo*, since only single factors of the skin and wound healing were investigated. The conditions of these experiments also differ from those of a practical application of a wound dressing; where the materials will be exposed to a protein and cell milieu much more complex than the one given in the *in vitro* conditions. It would seem, under the conditions of these experiments, that NFC hydrogels are just as biocompatible as Aquacel if not better in certain aspects. Further research is needed to investigate the many effects hydrogels could have on the complex wound healing process.

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References

Abbott EM, Nandyala SV, Schwend RM. 2014. Does a Kaolin-Impregnated Hemostatic Dressing Reduce Intraoperative Blood Loss and Blood Transfusions in Pediatric Spinal Deformity Surgery? Spine 39: E1174–E1180.

Abitbol T, Rivkin A, Cao Y, Nevo Y, Abraham E, Ben-Shalom T, Lapidot S, Shoseyov O. 2016. Nanocellulose, a tiny fiber with huge applications. Current Opinion in Biotechnology 39: 76–88.

Alexandrescu L, Syverud K, Gatti A, Chinga-Carrasco G. 2013. Cytotoxicity tests of cellulose nanofibril-based structures. Cellulose 20: 1765–1775.

Boateng JS, Matthews KH, Stevens HNE, Eccleston GM. 2008. Wound healing dressings and drug delivery systems: a review. Journal of pharmaceutical sciences 97: 2892–923.

Bonnier F, Keating ME, Wróbel TP, Majzner K, Baranska M, Garcia-Munoz A, Blanco A, Byrne HJ. 2015. Cell viability assessment using the Alamar blue assay: A comparison of 2D and 3D cell culture models. Toxicology in Vitro 29: 124–131.

Broughton G, Janis JE, Attinger CE. 2006. A Brief History of Wound Care: Plastic and Reconstructive Surgery 117: 6S–11S.

Čolić M, Mihajlović D, Mathew A, Naseri N, Kokol V. 2015. Cytocompatibility and immunomodulatory properties of wood based nanofibrillated cellulose. Cellulose 22: 763–778.

Dang Q, Liu K, Zhang Z, Liu C, Liu X, Xin Y, Cheng X, Xu T, Cha D, Fan B. 2017. Fabrication and evaluation of thermosensitive chitosan/collagen/α, β-glycerophosphate hydrogels for tissue regeneration. Carbohydrate Polymers 167: 145–157.

Deyrieux AF, Wilson VG. 2007. In vitro culture conditions to study keratinocyte differentiation using the HaCaT cell line. Cytotechnology 54: 77–83.

Dong H, Snyder JF, Williams KS, Andzelm JW. 2013. Cation-Induced Hydrogels of Cellulose Nanofibrils with Tunable Moduli. Biomacromolecules 14: 3338–3345.

Dugan JM, Gough JE, Eichhorn SJ. 2013. Bacterial cellulose scaffolds and cellulose nanowhiskers for tissue engineering. Nanomedicine 8: 287–298.

Hennings H, Holbrook KA. 1983. Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture: an ultrastructural study. Experimental cell research 143: 127–142.

Hua K. 2015. Nanocellulose for Biomedical Applications: Modification, Characterisation and

Biocompatibility Studies. Doctoral thesis, Uppsala University.

Hua K, Carlsson DO, Ålander E, Lindström T, Strømme M, Mihranyan A, Ferraz N. 2014. Translational study between structure and biological response of nanocellulose from wood and green algae. RSC Adv 4: 2892–2903.

Jorfi M, Foster EJ. 2015. Recent advances in nanocellulose for biomedical applications. Journal of Applied Polymer Science 132: 1–19.

Kheirabadi BS, Scherer MR, Estep JS, Dubick MA, Holcomb JB. 2009. Determination of Efficacy of New Hemostatic Dressings in a Model of Extremity Arterial Hemorrhage in Swine. The Journal of Trauma: Injury, Infection, and Critical Care 67: 450–460.

Lavoine N, Desloges I, Dufresne A, Bras J. 2012. Microfibrillated cellulose - Its barrier properties and applications in cellulosic materials: A review. Carbohydrate Polymers 90: 735–764.

Lin N, Dufresne A. 2014. Nanocellulose in biomedicine: Current status and future prospect. European Polymer Journal 59: 302–325.

Liu J, Chinga-Carrasco G, Cheng F, Xu W, Willför S, Syverud K, Xu C. 2016. Hemicellulose-reinforced nanocellulose hydrogels for wound healing application. Cellulose 23: 3129–3143.

Li Y, Wang S, Huang R, Huang Z, Hu B, Zheng W, Yang G, Jiang X. 2015. Evaluation of the Effect of the Structure of Bacterial Cellulose on Full Thickness Skin Wound Repair on a Microfluidic Chip. Biomacromolecules 16: 780–789.

Nguyen B-NB, Moriarty RA, Kamalitdinov T, Etheridge JM, Fisher JP. 2017. Collagen hydrogel scaffold promotes mesenchymal stem cell and endothelial cell coculture for bone tissue engineering. Journal of Biomedical Materials Research Part A 105: 1123–1131.

Nguyen DT, Orgill DP, Murphy GF. 2009. The pathophysiologic basis for wound healing and cutaneous regeneration. In: Orgill D, Blanco C (ed.). Biomaterials for treating skin loss, pp. 25–57. Woodhead Publishing Limited, Cambridge.

Ni H, Zeng S, Wu J, Cheng X, Luo T, Wang W, Zeng W, Chen Y. 2012. Cellulose nanowhiskers: preparation, characterization and cytotoxicity evaluation. Bio-medical materials and engineering 22: 121–127.

Nordli HR, Chinga-Carrasco G, Rokstad AM, Pukstad B. 2016. Producing ultrapure wood cellulose nanofibrils and evaluating the cytotoxicity using human skin cells. Carbohydrate Polymers 150: 65–73.

O'toole EA. 2001. Extracellular matrix and keratinocyte migration. Clinical and experimental

dermatology 26: 525–530.

Pereira MM, Raposo NRB, Brayner R, Teixeira EM, Oliveira V, Quintão CCR, Camargo LSA, Mattoso LHC, Brandão HM. 2013. Cytotoxicity and expression of genes involved in the cellular stress response and apoptosis in mammalian fibroblast exposed to cotton cellulose nanofibers. Nanotechnology 24: 075103.

Purna SK, Babu M. 2000. Collagen based dressings--a review. Burns : journal of the International Society for Burn Injuries 26: 54–62.

Rajwade JM, Paknikar KM, Kumbhar JV. 2015. Applications of bacterial cellulose and its composites in biomedicine. Applied Microbiology and Biotechnology 99: 2491–2511.

Ran Y, Hadad E, Daher S, Ganor O, Kohn J, Yegorov Y, Bartal C, Ash N, Hirschhorn G. 2010. QuikClot Combat Gauze Use for Hemorrhage Control in Military Trauma: January 2009 Israel Defense Force Experience in the Gaza Strip—A Preliminary Report of 14 Cases. Prehospital and Disaster Medicine 25: 584–588.

Rashad A, Mustafa K, Heggset EB, Syverud K. 2017. Cytocompatibility of Wood-Derived Cellulose Nanofibril Hydrogels with Different Surface Chemistry. Biomacromolecules 18: 1238–1248.

Rekus MT. 2000. Characterization of growth and differentiation of a spontaneously immortalized keratinocyte cell line (HaCaT) in a defined, serum-free culture system. Doctoral thesis, Heinrich-Heine-Universität Düsseldorf.

Roman M. 2015. Toxicity of Cellulose Nanocrystals: A Review. Industrial Biotechnology 11: 25–33.

Sahlgrenska Universitetssjukhuset. 2010. Sår och Sårbehandling. Sahlgrenska Universitetssjukhuset

Sanchavanakit N, Sangrungraungroj W, Kaomongkolgit R, Banaprasert T, Pavasant P, Phisalaphong M. 2006. Growth of Human Keratinocytes and Fibroblasts on Bacterial Cellulose Film. Biotechnology Progress 22: 1194–1199.

Santoro MM, Gaudino G. 2005. Cellular and molecular facets of keratinocyte reepithelization during wound healing. Experimental Cell Research 304: 274–286.

Saska S, Teixeira LN, Tambasco de Oliveira P, Minarelli Gaspar AM, Lima Ribeiro SJ, Messaddeq Y, Marchetto R. 2012. Bacterial cellulose-collagen nanocomposite for bone tissue engineering. Journal of Materials Chemistry 22: 22102.

Shah JB. 2012. The History of Wound Care. The Journal of the American College of Certified Wound Specialists 3: 65–66.

Wikimedia Commons. 2006. Skinlayers. https://commons.wikimedia.org/wiki/File:Skinlayers.png# Retrieved 2017–05–18.

Williams DF. 2008. On the mechanisms of biocompatibility. Biomaterials 29: 2941–2953.

Wilson CJ, Clegg RE, Leavesley DI, Pearcy MJ. 2005. Mediation of Biomaterial–Cell Interactions by Adsorbed Proteins: A Review. Tissue Engineering 11: 1–18.

Wolf MF, Coleman KP, Lewerenz GM. 2013. In Vitro Assessment of Cell and Tissue Compatibility. Biomaterials Science, Ratner BD, Hoffman AS, Schoen FJ, Lemons JE (ed.), pp. 593–608. Academic Press, Oxford and Waltham.

Woodley DT, Bachmann PM, O'Keefe EJ. 1988. Laminin inhibits human keratinocyte migration. Journal of Cellular Physiology 136: 140–146.

Yang X, Bakaic E, Hoare T, Cranston ED. 2013. Injectable Polysaccharide Hydrogels Reinforced with Cellulose Nanocrystals: Morphology, Rheology, Degradation, and Cytotoxicity. Biomacromolecules 14: 4447–4455.

Zander NE, Dong H, Steele J, Grant JT. 2014. Metal Cation Cross-Linked Nanocellulose Hydrogels as Tissue Engineering Substrates. ACS Applied Materials & Interfaces 6: 18502–18510.

Zhijiang C, Guang Y. 2011. Bacterial cellulose/collagen composite: Characterization and first evaluation of cytocompatibility. Journal of Applied Polymer Science 120: 2938–2944.