Cell & Developmental Biology

Karim Z, Afrin S, Cell Dev Biol 2015, 4:2
DOI: 10.4172/2168-9296.1000154

Review Article Open Access

Nanocellulose as Novel Supportive Functional Material for Growth and Development of Cells

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Rec date: May 27, 2015; **Acc date:** June 23, 2015; **Pub date:** June 29, 2015

Abstract

The exploration of biological polymer is a new interest due to its favorable properties used in biomedical and clinical applications. Within the used biopolymer, cellulose emerged as a most exploitable functional material at nanoscale. Working with nanosize cellulose, provides some additional advantages compare to other manmade functional polymers. The fabrication of cellulosic scaffold as a platform for growth and development of cells is a thrust area of tissue engineering but this fabricated scaffold needs to have some fundamental prerequisite before implantation in donor. Thus, this short report discusses about the NC based scaffolds for the growth and development of cells and tissue. The main focus is on derived wood and microbial NCs. Thereafter; some interesting examples will be discussed to understand the necessity of cellulose-based supports in tissue engineering field.

Keywords: Cellulosic materials; Nanocellulose; Tissue regeneration

Introduction

Cellulosic materials such as wood, grass, and agricultural, forest residues and microbial cellulose (bacterial cellulose) are promising high contents cellulose resources that can be utilized to isolate nanocelluloses (NCs) using various treatment. Further isolation of NCs requires various mechanical/enzymatically as well as chemical processes [1,2]. Manipulating cellulose at the nanoscale (1-100 nm), offers the potential of novel properties that could be utilized for adhesion, growth and development of cells [3].

High specific surface area of NCs is expected to provide large number of active sites to accord cells. The specific surface area of cellulose nanofibers (CNF) (also called nanocellulose fiber, NCF) prepared using a supercritical drying process, can be as high as 480 m^2/g [4]. Thereafter, NCs has good mechanical properties; it was reported that estimated tensile modules and strength is around 145-165 GPa and 10,000 MPa, respectively [5]. Good mechanical strength and rigidity can offer the potential to use in real applications (as scaffold for bone development). Stability in water as well as hydrophilicity of nanocellulose is also of advantage while using in liquid medium (serum, synovial fluid, blood etc.). Furthermore, NCs have high crystallinity, which makes the scaffold resistant to biological corrosion in aqueous medium. Furthermore, some other properties including special morphology and geometrical dimensions, rheological properties, liquid crystalline behavior, alignment and orientation, barrier properties, surface chemical reactivity, biocompatibility, biodegradability, lack of toxicity, etc. [6-9] makes this material more advanced compare to other synthetic/manmade polymers. On the basis of these unique properties, both "nano-enhanced" and completely new "nano-enabled" products have been envisioned ranging from bulk applications like rheological modifier, composite reinforcement or paper additive, to high-end application such as tissue engineering. Tissue engineering is the study of the growth of new tissues or organs from cells on/in scaffold to produce a fully functional organ for implantation back into the donor host [3,9]. The basic requirement to make this engineering breakthrough, adhesion, growth and development of cells is compulsory (an interconnection between cells and scaffold).

Thus, before the implantation of scaffold in donor, researchers still need to understand the behavior of scaffold in vitro followed by in vivo conditions. Therefore, this report is all about the unique polymer, "cellulose" as functional material for preparation of scaffold. The use of cellulose at its nanoscale provides some sole properties those favored to apple it in medical/clinical field. The isolation of NCs using grinding and acid hydrolysis method provides two types of NCs called nanofibers (CNF) and crystal (CNC), respectively. Another cellulose, named bacterial cellulose (BC) is produced by variety of bacterial culture. The isolation of cellulose from lignolytic biomass follows top down approach except BC. The BC is pure and has greater potential to use it in medical applications compare to lignolytic NCs (impure, used harsh chemicals, loosing native structure, makes it less stable). Biocompatibility and biodegradability are two necessary properties required for implanted materials. In this short report, special emphasis has been given to discuss these properties with in vitro and in vivo examples. Finally, some more interesting and implemented research have been discussed and summarized in support of cellulose-based scaffold for cell development and growth.

Isolation Procedures of NCs

Lots of cellulosic materials are available as discussed previously; one of most studied is wood due to high contents of cellulose (47%) compare to other lignocellulosic biomass [5]. A hierarchical structure of wood from macro to nano is mentioned in Figure 1a. This image confirms the top down approach for the isolation on NCs from cellulosic biomass. Isolation of NCs from cellulose is a complex process due to breaking of linking bonds, which makes cellulose a stable biopolymer. Microfibers are joined laterally by means of

hydrogen bonding, as the microfibrils were generated, they were found to coalesce laterally through interfibrillar hydrogen bonding to form bundles. The bundles associate with neighboring bundles to produce a composite ribbon of cellulose microfibrils. The glucose and cellulose chain structures show the presence of several hydroxyl radicals in the cellulose chain and all these hydroxyl groups participate in hydrogen bonding. The interfibrillar hydrogen bonding energy has to overcome in order to separate the microfibrils into individual entities. Any attempt to loosen up the microfibrils by either complete or partial destruction of hydrogen bonds before the mechanical process would be a step forward in the quest for energy efficient generation of NCs (Figure 1b). The use of acids and bleaching agents convert the cellulosic biomass into the washed cellulose (by dissolving hemicellulose and lignin). The fibrillation of washed cellulose using mechanical procedure gives CNF and the hydrolysis of cellulose microfibers in the presence of acids dissolve the amorphous part and separate the crystalline part, thus the crystalline part of cellulose in nanoscale is called CNC as mentioned in Figure 2a.

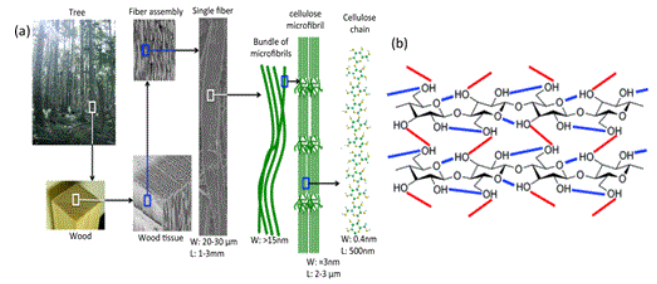

Figure 1: A hierarchical structure of wood from macro to nano (a), reprinted from Isogai et al. [10]. The molecular structure of cellulose showing the β-D-glucopyranose molecule connected through β-1,4-glycosidic bonds. The inter- and intra-chain hydrogen bonds are shown by red and blue solid lines, respectively (b) Adopted from Thiruvengadam and Vitta [11].

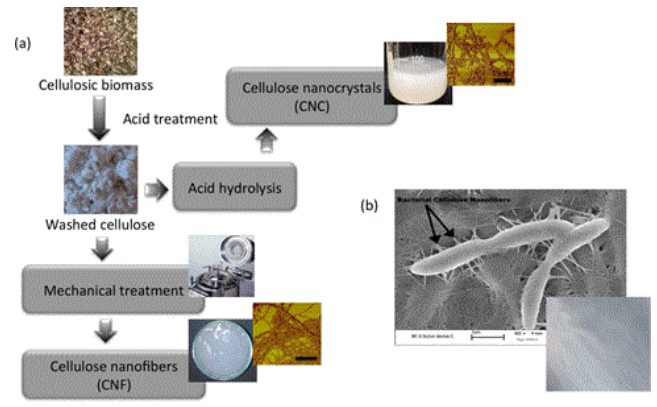

Figure 2: A top down approach for the isolation of NCs from wood (a) and *a cellulose producing bacteria, Gluconacetobacter Xylius (b), from the cover of Bacterial Nanocellulose, Edited by Miguel Gama, Paul Gatenholm and Dieter Klemm, CRC Press 2013.*

Cellulose is also synthesized extracellularly by several bacterial species such as Gluconacetobacter, Agrobacterium, Pseudomonas, Rhizobium, and Sarcin. In general, nanocellulose of bacteria (BNC) has the same chemical composition as that from plants but it is produced in the absence of other polymers (such as hemicelluloses or lignin), which makes it chemically pure (Figure 2b).

BNC is produced by bacterial cultivation in aqueous culture media containing glucose, phosphate, and oxygen. It has a ribbon like shape (less than 100 nm wide) and high crystallinity index. Due to the replication of bacteria, nanocellulose fibrils in the BNC pellicle form a randomly assembled tangled structure, which has good mechanical properties even in the wet state. Thus, such properties make it a novel biological material that is biocompatible and functionally competent for various biomedical applications. It has also been shown to promote chondrocyte adhesion to determine its potential for ear cartilage [3].

Biological Properties of NCs

The development of bioabsorbable and biocompatible tissue scaffold materials is a topic of intense research. Such a material is sought for many medical applications, ranging from wound healing to skin, bone, cartilage, nerve and other tissue regeneration [12,13]. Synthetic polymers have been extensively examined and implemented as tissue scaffold materials [14], but many exhibit undesirable characteristics including non-bioabsorbability and low biocompatibility. Although naturally derived polymers are being implemented as alternative materials due to their high biocompatibility, some are not bioabsorbable or elicit undesirable side effects including infection and immune response [15]. Some improved materials examined, such as siliceous fiber [16,17] and naturally derived materials such as small intestine sub mucosa membranes [18,19], offer improved performance, but still exhibit several issues including possible side effects of impurities or vestigial degradation products in the body, non-controlled degradation periods, and unwanted heterogeneous compounds that may result in a rejection response.

Cellulose is a biopolymer having unique properties at nanoscale making it a promising candidate for cell growth and development. Chemical (functional), physical (surface properties) have been discussed in a very elegant literature [20]. Biological properties are very interesting and unique for natural polymers. We have discussed some of these properties in this report to understand the compatibility of natural polymers with living bodies.

NCs have been investigated as a promising substrate for regenerative medicine and wound healing such as scaffolds for tissue-engineered meniscus, blood vessels, and ligament or tendon substitutes [21,22]. The applicability of CNFs as hemodialysis membranes [23] and in the films for long-lasting sustained drug delivery [24] has also been documented. Furthermore, the hydrogels of CNFs can be used as potential cell culture scaffolds because it provides the desired 3D environments for the growth and differentiation of human hepatic cell lines [25], as well as human pluripotent stem cells [26]. Thus, the investigations of biological properties of NCs or their scaffolds are compulsory before the implantation. It is obvious that biocompatibility is not only one important parameter but biodegradability is also other key parameter. These properties completely depend on nanostructural properties of NCs, applied concentrations, study models, cell types and exposure times, but many other factors may influence this process such as shape, surface area, charge, source of nanocellulose and the mode of its preparation,

degree of agglomeration in culture media, examined biological parameters and assays [27,28].

Biocompatibility

Biocompatibility is referred to as the ability of a foreign material implanted in the body to exist in harmony with tissue without causing deleterious changes, which is an essential requirement for biomedical materials. Biocompatibility does not only refer to the quality of not having toxic effects on biological systems, but also to the need of having an appropriate host response to ensure satisfactory performance on a specific application. The initial biocompatibility evaluation is based on in vitro testing of different responses to the material in model systems under controlled conditions, which, however, may not necessarily reflect the true response in the body, therefore in vivo testing is also required at later stages. Usually the toxicity profile of a material is established before more application specific responses are investigated. The cytocompatibility (a type of biocompatibility), of a material can be assessed directly or indirectly with cultures of model cell lines, of e.g. fibroblasts, macrophages or stem cells, and the observed effects can vary depending on cell type. The two tests differ in the manner in which the test material is exposed to the cells. In direct tests, cells are in direct contact with the studied material, for example the material is used as cell culture substrate. In indirect tests, also called elution tests, cells are exposed to an extract solution of the material. The tests are evaluated through changes in cell proliferation, viability and morphology.

Petersen and Gatenholm, [29] have pointed out that biocompatibility of BC for tissue engineering applications can be related to the fact that its structure shows similarities with extracellular matrix components, such as collagen. In fact, collagen and BC nanofibers have similar diameters (around 100 nm) and are extracellularly assembled from precursor molecules into polymer chains. However, it is well known that the human body does not readily degrade cellulose because it lacks cellulolytic enzymes, which will inevitably cause some incompatibility. It is a disappointment that direct investigations on the biocompatibility of CNC and CNF are rare. Some studies on CNC-based materials (such as hydrogels) only report experiments of cell cultivation, through the growth, propagation and activity of cells to evaluate the conditions of material biocompatibility. Recently, wood CNC have been isolated from an integrated part of bioethanol production process, called bioethanol CNCs. Cytocompatibility, test have been performed using ASCs (primary human cells) and cell line L929. The result confirms the cell adhesion and growth as shown is Figure 3 [2].

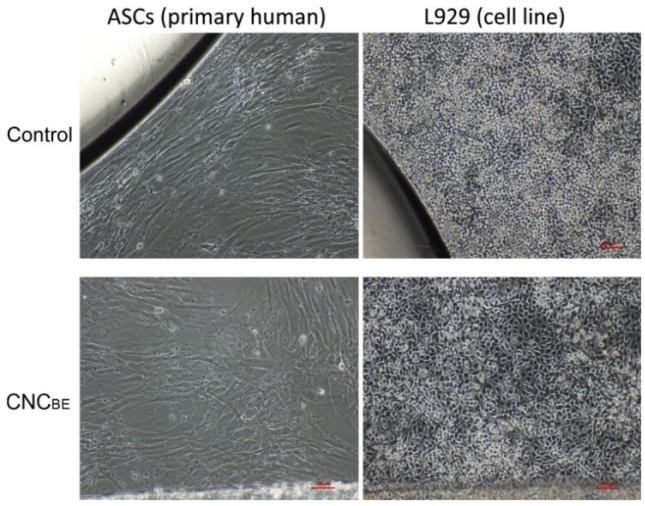

Figure 3: Images showing adhesion and growth of primary human cells and L929 cell lines on the negative control and films of CNC from bioethanol after 15 days of incubation.

In a recent study, six different types of modified NCs have been fabricated for the measurement of cytocompatibility. The differences between samples were surface charges and porosity. CNC and Enzyme-NCF do not carry significant number of surface charges, TEMPO-CNC and Carboxy-NCF have anionic surface charges and (EPTMAC-CNC and EPTMAC-NCF have cationic behavior as confirmed by zeta-potential values. The porosity of CNC samples was less compared to CNF. The cell viability and adherent percentage of human dermal fibroblast (HDF) have been determined. For the CNC-based films (Figure 4a), only TEMPO-CNC, i.e. sample featuring anionic and co-axially aligned fibril aggregates, possessed good cytocompatibility and is comparable to the negative control (Thermanox disc, TMX). For the samples composed of NCF (Figure 4b), only EPTMAC-NCF, i.e. samples comprising cationic fibrils, showed significantly higher number of adhered cells than the positive control (cells cultured on TMX in the presence of 5% DMSO), indicating that the EPTMAC-NFC was more cytocompatible than the other NFC samples. From these results it have been confirmed that the charges not only have a high impact on the compatibility but on the other hand the porosity have drastic effect on the growth and development of HDF.

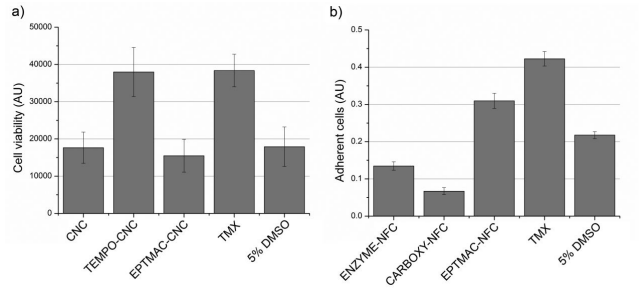

Figure 4: Cell viability of HDF cells cultured on CNC, TEMPO-CNC and EPTMAC-CNC films (a), and number of adherent cells on ENZYME-NFC, CARBOXY-NFC and EPTMAC-NFC films (b). Corresponding values for cells cultured on TMX (negative control) and cells cultured on TMX in the presence of 5% DMSO (positive control) are shown in each panel. Data represent the mean ± standard error (n=5). Adapted from Carlsson, [30].

In order to access blood compatibility, NCs samples were used where the material was brought in contact with blood or plasma under controlled conditions. Thereafter, the events associated with the activation of the cascade systems in the blood were studied (such as protein adsorption, fibrin and thrombin formation, and platelet and leukocyte adhesion/activation, as well as complement system activation) by observing the material surface and by determining the levels of associated components in the blood. Thus, hemocompatibility (or blood compatibility) is another significant property of biocompatibility, especially for blood contacting biomaterials and artificial organs, such as artificial blood vessels, pumps, and artificial hearts. Interestingly, recent study reported the regulation of blood metabolic variables by the presence of TEMPO-oxidized CNF. The oral administration of TEMPO-oxidized CNF to mice was proved to be effective for reducing the postprandial blood glucose, plasma insulin, glucose-dependent insulinotropic polypeptide, and triglyceride concentrations. It seems that TEMPO-oxidized CNF have both promising hemocompatibility and unique biological activities [31]. In another report thrombogenic and complement activation properties of the nanocellulose composite were determine. To increase the thrombogenic property an automatically charged heparin conjugated layer was applied in the composite. These results were confirmed by a significantly larger reduction in platelet number in the blood following incubation of non-heparinized composites compared to heparinized ones. For the heparinized composites, no significant differences could be observed in relation to the reference materials. This was also true in terms of thrombin formation, as determined by measuring the levels of thrombin-antithrombin complex (TAT) in the blood plasma after whole blood contact with the materials.

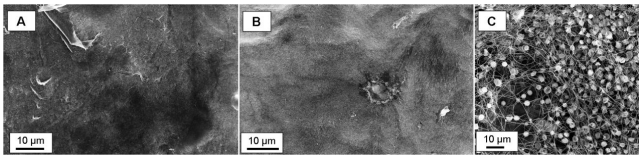

Figure 5: SEM micrographs showing extent of platelet adhesion after whole blood incubation with heparinized composites before (a) and after cyclic voltammeter cycling (b) and with non-heparinized composite (c). Reprinted with permission from Carlsson, [30].

Biodegradability

Various recent research articles have discussed about the in vitro biodegradability of NCs [32,33] but very few reports have been published regarding the in vitro degradation of NCs. In an early, Miyamoto et al. [34] found that the degradation of cellulose and cellulose derivatives in canine specimens depended significantly on the cellulose crystalline form and chemical derivatization. Regenerated cellulose prepared by deacetylation of cellulose acetate (presumably the highly crystalline cellulose II polymorph) did not measurably degrade over the course of the 6-week experiment. Contrarily, however, up to 75% (w/w) of equivalent samples of amorphous regenerated cellulose were degraded and absorbed over the same experimental period. Another study reported that CNC was actually more biodegradable than fullerenes and carbon nanotubes in aqueous environments, but without the *in vivo* investigation of biodegradability [35]. Recently, oxidized cellulose was rendered more vulnerable to hydrolysis and therefore potentially degradable by the human body. Based on this strategy, researchers attempted to enhance the biodegradability of nanocellulose through oxidation, such as the report of improving BC degradability *in vitro* (in water, phosphate buffered saline, and simulated body fluid) through periodate oxidation [36,37]. With the pre-γ-irradiation and sodium periodate oxidation treatments on BC membranes, it was reported that *in vitro* degradation of oxidized BC involved two major phases, (1) initial rapid degradation of about 70–80% of the entire sample; (2) slower degradation of an additional 5–10% which eventually levels off leaving a small amount of nonresorbable material. Further experiments on *in vivo* degradation (male New Zealand White rabbits) showed the marked degradation of oxidized BC membranes at all-time points, with the most rapid degradation occurring in the first 2–4 weeks [38]. As discussed previously, the absence of enzymes in vivo system makes the cellulose degradation approximately negligible. A very interesting research has been performed to increase the degradation rate of cellulose for in vitro condition. In this work, buffer ingredients were incorporated into the bacterial cellulose suspension in order to create a more optimal pH microenvironment for the preferred acid cellulases, which are significantly less active at the biological pH 7.4. The results demonstrated show that incorporation of buffer ingredients helped to retain the activity of the cellulases. The glucose released from degraded materials was also increased from 30% without incorporation of buffer ingredients to 97% in the presence of incorporated buffer ingredients at the suboptimal pH environment of 7.4 [32]. This strategy was further supported in a study, which reports cellulase dependent biodegradability of cellulose acetate with cardiac fibroblast (CF) and cardiac myocytes (CM) as shown in image given below (Figure 6). The

effect of cellulase was cell type specific, with the FB being more sensitive to the action of the enzyme than CM. Figure 6 (left) shows representative fluorescence structural images of monolayers of cardiac fibroblasts after 24 h of cellulase treatment. FB adhesion to the surface was affected in a dose dependent manner enzyme units were used in Figures. 6A-6C, respectively [23,39]. This resulted in regional cell sheet detachment from the scaffold. CM, treated with the same cellulase concentrations were much more resistant to detachment and showed no apparent differences from control samples in cytoskeletal structure and macroscopic cell organization (Figure 6D). Cell viability, probed by Trypan Blue, was unaffected by the cellulase treatment. Nuclear images of control (Figure 6F) and treated (Figure 6E) cells showed similar cell densities [39].

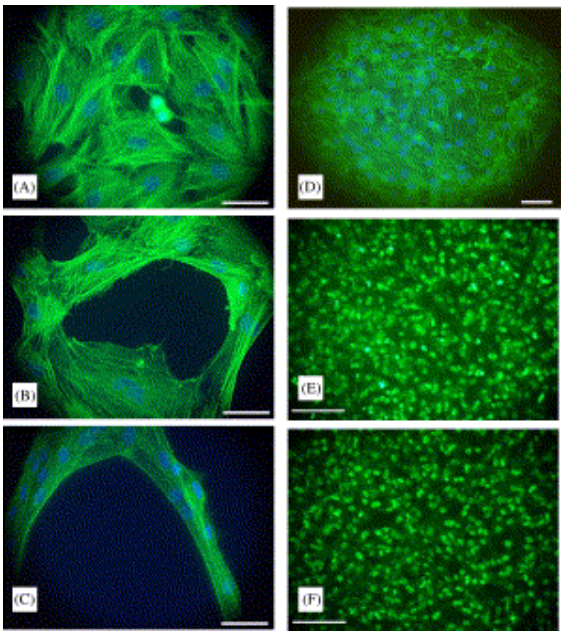

Figure 6: Dose-dependent cellulase effects on cardiac cells: Cardiac fibroblasts were treated for 24 h with 23 units (A), 46 units (B) and 92 units (C) of cellulase, respectively. While at the lowest dose, a cell cytoskeleton was normal and undistinguishable from a control, at higher concentrations fibroblasts' adhesion to the matrix was affected, and cells were peeling off, forming empty holes. At the same time cardiac myocytes, treated with the same cellulase concentrations, preserved their macroscopic integrity at all doses. D shows a CM sample treated with 46 units for 24 h. Images (E) and (F) show live myocytes (nuclei labeled with SYTO-16) on cellulase-treated (92 units) and control sample, respectively. Scale bar is 50 μm (A-D) and 100 μm (E, F). Reprinted from ref. [39].

NCs Based Scaffolds and Cells Survival

Scaffolds represent important components for tissue engineering. However, researchers often encounter an enormous variety of choices when selecting scaffolds for tissue engineering. A noble scaffold should have some unique properties that make them eyes catcher; for example, (a) Scaffolds should provide void volume for vascularization, new tissue formation and remodeling so as to facilitate host tissue integration upon implantation. (b) Scaffolds should provide support for either extraneously applied or endogenous cells to attach, grow and differentiate during both in vitro culture and in vivo implantation. (c) Scaffolds may interact with the cellular components of the engineered tissues actively to facilitate and regulate their activities. (d) Scaffolds provide mechanical and shape stability to the tissue defect [40].

Cellulose based scaffold for the growth and development have all these properties as discussed previously, which make them suitable candidate in tissue engineering. In recent publication non-woven cellulose II fabrics were used as scaffolds for in vitro cartilage tissue engineering [41]. The scaffolds were activated in a saturated $Ca(OH)_2$ solution and subsequently coated with a calcium phosphate layer to increase the adhesion properties of scaffold. Chondrocyte cell response and cartilage development were investigated. The cell adherence was significantly improved compared to untreated cellulose fabrics, and the proliferation and vitality of the adhered chondrocytes were excellent, indicating the biocompatibility of these materials.

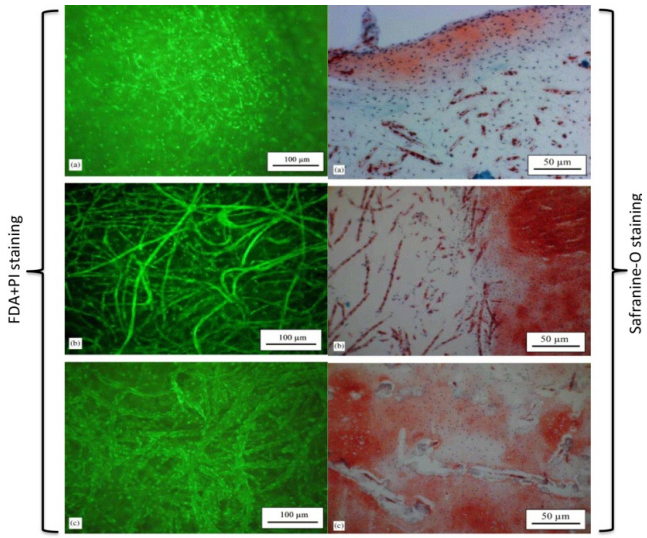

Figure 7: Scaffolds seeded with primary chondrocytes, 1 week in culture, FDA+PI staining: (a) untreated, (b) Ca(OH)2-treated and (c) CaP-coated cellulose. Safranine-O staining of cellulose scaffolds seeded with primary bovine chondrocytes, in vitro cultured for 6 weeks: (a) untreated, (b) Ca(OH)2-treated and (c) CaP-coated samples. Reprinted from ref. [41].

NEWS of Chalmers University indicates a very interesting research in collaboration with Gothenburg University, that NC stimulates the formation of neural networks (Figure 8). Such a model could elevate brain research to totally new levels, with regard to Alzheimer's disease and Parkinson's disease, for example (as shown below in image). Over a period of two years the research group has been trying to get human nerve cells to grow on nanocellulose and finally they get succeed. In the future this method may be useful for testing various pharmaceutical candidates that could slow down the destruction of synapses. In addition, it could provide a better alternative to experiments on animals within the field of brain research in general.

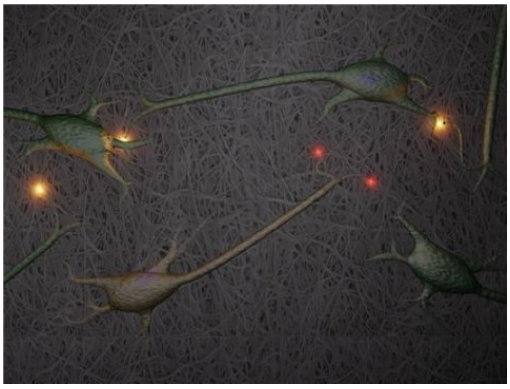

Figure 8: A model shows the growth of nerves cell on the cellulose-based scaffold to understand the function of brain in disease.

Providing a conclusive microenvironment for cell growth, proliferation and differentiation is a major developmental strategy in the tissue engineering and regenerative medicine. Here, authors describe the fabrication of a cellulose scaffold for tissue engineering purposes from cellulose fiber using a salt leaching method. Cellulose scaffolds were implanted subcutaneously in mice for 5 days to check in vivo tissue-matrix interactions. The results confirm the proliferation and differentiation after implantation of scaffold in vivo (Figure 9).

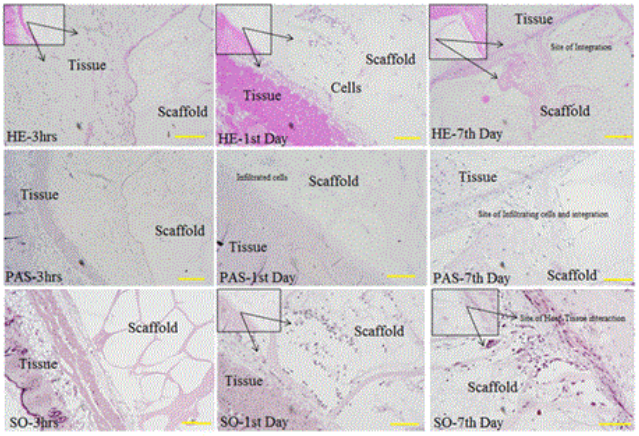

Figure 9: In vivo testing was performed to investigate real time interactions between host and scaffold. Hematoxylin and Eosin (HE) staining was performed to determine the distribution of check cell nuclei. Cellulose was stained with Periodic acid–Schiff (PAS) and mast cell granules with Safranin O. Scale bar100lm. Adopted from ref.[42].

Conclusion

The aim of this article is to demonstrate the current state of research and future development of NC in the application of cell growth and development through the discussion of selected examples. Undoubtedly, NC has great potential for the breakthrough of a novel generation of biomedical materials. Reported studies on NC have led to significant advancement with the promise of even greater advances likely to come in the future. Overall, creating controlled properties, reliable and reproducible production techniques for biocompatible NC (not only for BNC) will be essential and beneficial to pave the way for greater acceptance of NC as a commercially available material in biomedical applications. Lots of green processes for isolation of NCs have been discussed and reported by Lee et al. (43) but still researcher needs some more advanced routs for isolation of chemical and defect free NC.

Specifically, regarding cellular scaffold, the mechanisms for cells and NC interaction remain unclear and require intensive in vivo study. Furthermore, it is possible for future study to regulate the interactions between cells and NC through controlling the macro- and microstructure of NC. The studies on the development of tissue substitutes and repair biomaterials have made positive progress (especially with BNC), which promotes the launch of several commercial products and practical usage in clinic. On the other hand, covalent attachment of biologically active ligand molecules to the NC framework can enhance and alter its characteristics for specific applications, which may improve interactions between materials and human tissues.

From both scientific and economic viewpoints, NC, the resource and gift provided by Nature, is on the threshold of a breakthrough driven by recent extraordinary activities in the field of biomedical applications.

Acknowledgment

Authors thank colleagues for reading this report and point out mistakes for further improvement.

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