Nanocellulosic Materials in Tissue Engineering Applications

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Abstract

This chapter deals with an overview of design and fabrication of three-dimensional (3D) scaffolds for tissue engineering (TE) applications using the electrospinning technique. A general introduction to cellulose, a short overview of sources and methodology for the production of cellulose nanocrystals (CNCs), and principles of tissue engineering and the electrospinning technique will be given. Applications for CNCs are manifold and range from super water absorbent, drug delivery, packaging, personal care to pharmaceuticals. However, in this chapter the application in tissue engineering will be discussed in detail.

Keywords: Cellulose nanocrystals, tissue engineering, electrospinning, three-dimensional scaffold

1. Introduction

Cellulose, the most ubiquitous natural biopolymer, is considered as a virtually inexhaustible source of raw material for the increasing demand of environmentally friendly, sustainable and biocompatible products [1, 2]. Despite its ubiquity in essentially every traditional and long-established scientific and industrial field, cellulose permanently occupies prominent positions in the most advanced and emerging technologies, as well [3]. It has been used either in native or regenerated form in several applications including paper, board, fibers, films, textiles,

packaging materials, hygienic products, and serves as a support material in chromatography and many other life science applications[1]. The most common attributes when describing the importance of cellulose are hydrophilicity, high reactivity, low fouling properties, renewability, nontoxicity, and the compatibility with many other organic and inorganic materials. This facilitates the creation of hybrid materials and nanocomposites with unique properties such as conductive fibers, papers with printed circuits, flame retardancy, and wound dressing materials [1].

Cellulose is the major component in the rigid cell walls of wood, other bio-plants and certain bacteria, algae, fungi, and some marine animals (e.g., tunicates). Cellulose is a linear syndiotactic homopolymer consisting of D-anhydroglucopyranose units (AGU), which are covalently connected through β-(1 → 4)-glycosidic bonds between the carbon atoms C(1) and C'(4) of adjacent glucose units, resulting in a cellobiose unit (see Figure 1), which is the main building block of the cellulose polymer [4, 5]. The elemental composition of cellulose (carbon: 44-45%, hydrogen: 6.0-6.5%, and the rest being oxygen) was first revealed by the French chemist, Anselme Payen in 1838 [6]. Each of the AGU units consists of three free hydroxyl groups (at C-2, C-3 and C-6), which usually determine the reactivity of the cellulose polymer. The terminal hydroxyl groups at both ends of the cellulose chains (C-1 and C-4) are chemically different in nature. The C-1 at the right end of the chain is an aliphatic aldehyde group with reducing property, whereas C-4 at the left end is an alcoholic hydroxyl group with nonreducing property (see Figure 1). Cellulose exists in six different polymorphs, namely cellulose I, II, III, and IV. Among these, cellulose I occurs in two allomorphs, that is, I_α and I_β. Cellulose II or regenerated cellulose is formed by treating native cellulose I with either strong alkali solution or by precipitating cellulose I with Schweitzer's reagent (tetraamminediaquacopper dihydroxide $[Cu(NH_3)_4(H_2O)_2](OH)_2$). Cellulose III ($III_I$ and III_{II}) and cellulose IV (IV_I and IV_{II}) are obtained from either cellulose I or cellulose II with suitable modification [7-9]. Hearle et al. developed a two-phase model or fringed fibril model assuming that cellulose I contains low-ordered so-called amorphous and highly ordered, that is, crystalline regions [10]. This means that cellulose I is not perfectly crystalline. The intramolecular hydrogen bonds are formed between O-3-H and O-5' and also between O-2-H and O-6' of one AGU unit and the adjacent AGU unit of the same polymer chain. The intermolecular hydrogen bonds are formed between O-6-H and O-3' of the AGU units of one polymer chain and the neighboring chain. Eventually, the different hydrogen bonding modes are responsible for the formation of stiff and rigid linear chains, which can further assemble into microfibrils with different chain lengths and orientation. When subjected to proper combinations of mechanical, chemical, and/or enzymatic treatments, these highly ordered crystalline regions present within the microfibrils can be isolated [11]. The isolated crystalline part is referred to as cellulose nanocrystals (CNCs). Owing to its nanoscale dimension and intrinsic physicochemical properties such as excellent biodegradability, a low eco-toxicity, biocompatibility, high specific strength and modulus, high surface area, and unique optical properties, CNCs are promising renewable biomaterials that can be used as a reinforcing component in the preparation of high-performance nanocomposite scaffold materials. Many new highly porous 3D scaffold materials with attractive properties can be prepared by the physical incorporation of CNC into a natural or synthetic polymer matrix, which will be discussed in section 5.
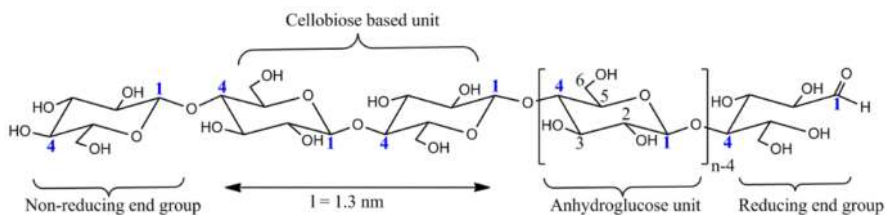
Figure 1. Molecular structure of the cellulose macromolecule.

2. Cellulose nanocrystals – Sources and methodology of preparation

Cellulose is at the forefront in scientific development in the areas of medicine, drug delivery, and sensoric applications, to name just a few, with a pronounced shift of employing cellulose substrates in nanometric dimensions, especially cellulose nanocrystals (CNCs). An array of attractive properties in combination with above-mentioned attributes are the main reason for an immense body of work, conducted with CNCs can be obtained following either of the two approaches: bottom-up by biosynthesis or top-down by disintegration of plant materials [11, 12]. Acid hydrolysis of cellulose substrates is the most widely used procedure for obtaining CNCs. The driving force of CNC formation in acidic media is the difference in solubility of structural regions in bulk cellulose, namely amorphous and crystalline entities. While amorphous regions of the cellulose substrates dissolve and hydrolyze in acidic solutions, crystalline domains readily reassemble to form nanocrystals, nanorods, or nanowhiskers (see Figure 2) [13]. Sulfuric acid (H_2SO_4) is a common hydrolyzing agent, which also imparts the resultant nanocrystals with surface sulfate groups, making their colloidal dispersions very stable due to present charges (accordingly, hydrochloric acid, for instance, yields CNC particles with a weaker charge with poor colloidal stability).

A wide variety of suitable cellulose raw materials can be employed for preparation of CNCs; consequently, different physical properties can be expected. An interesting and, so far to a certain extent, underutilized source for production of cellulose nanocrystals are fibers derived from oil palm trunks. Processed using acid hydrolysis with H_2SO_4, prepared CNCs possess an increase in crystallinity and this feature, coupled with removal of lignin and hemicelluloses contributed to enhanced thermal stability [18]. Despite its abundance, corn husks are also representatives of a class of natural materials which have not been extensively appropriated for the production of cellulose nanocrystals. Isolation of CNCs with acid hydrolysis of pretreated corn husk verified the suitability of the material as a precursor for cellulose nanocrystals, with emphasis on treatment time, which affects the degree of crystallinity, particle size, and thermal stability of the product [19]. Another environmentally sustainable approach for the production of cellulose nanocrystals is the use of wastepaper with its abundance and inexpensiveness. Acid hydrolysis with H_2SO_4 of alkali-treated and bleached cellulose particles yielded CNCs with 100-300 nm in length and 3-10 nm in diameter and
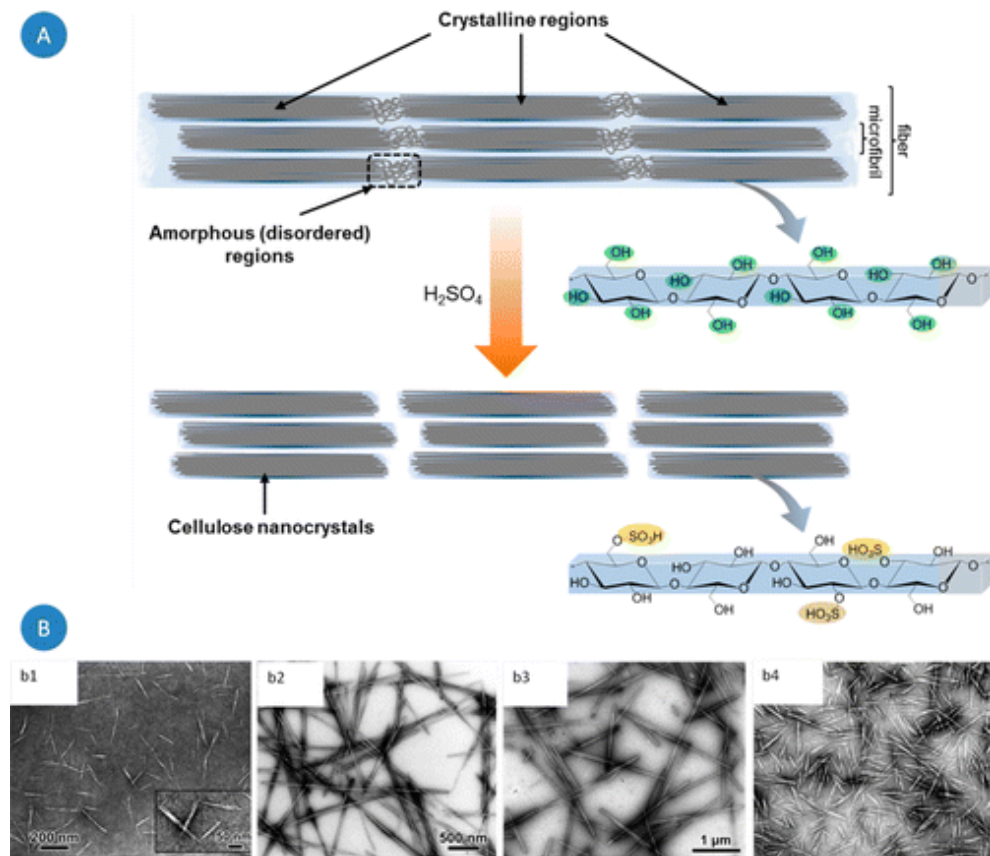
Figure 2. A) Schematics of cellulose fibers depicting crystalline and amorphous regions, and cellulose nanocrystals (with hydroxyl and sulfate groups) after sulfuric acid hydrolysis of the disordered amorphous regions. Reprinted with permission from ref [14]. Copyright 2011 Royal Society of Chemistry. (B) TEM micrographs of dispersion of cellulose nanocrystals derived from different sources: (b1) microcrystalline cellulose (Avicel), (b2) tunicate[15], (b3) green algae (*Cladophora sp*.) [16], and (b4) ramie [17]. (A) Reprinted with permission from ref [17]. Copyright 2008 Royal Society of Chemistry. (B) Reprinted with permission from refs [15] and [16]. Copyright 2012 and 2008 American Chemical Society.

crystallinity degree of approx. 76% [20]. An alternative source for production of cellulose nanocrystals is also bacterial cellulose, which is produced by acetic acid bacteria in either synthetic or nonsynthetic media via oxidative fermentation [21]. Preparation of bacterial cellulose-derived nanocrystals with different sulfate contents and, consequently, widely different colloidal properties was reported by varying the type of acid used as hydrolytic agent [22]. Whether hydrochloric acid (HCl), its mixture with H_2SO_4 or H_2SO_4 alone were used, prepared crystals exhibited zeta potential values of -5, -40, and -46 mV; in return, surface character of particles influenced their colloidal stability and interactions with other materials (e.g., xyloglucan). Kenaf bast fibers were also employed for the preparation of CNC colloidal

suspensions, with cellulose extraction (a combination of alkaline treatment and bleaching) followed by acid hydrolysis [23]. Sugarcane bagasse served as a source for CNCs, which were prepared with a sulfuric acid treatment [24]. Softwood and hardwood were used in addition to nonwood sources (cotton linters and cattail, i.e., Typha) and red algae as a representative of marine pulp for the production of CNCs by H_2SO_4 hydrolysis. Dimensions of the obtained CNCs can be grouped according to the material class, with wood-source-derived particles having length from approx. 170 nm to approx. 180 nm, cattail and cotton-based particles from approx. 250 nm to approx. 278 nm, while red-algae-based CNCs exhibit the longest particles, i.e., approx. 432 nm. Nonwood CNCs exhibit superior thermal stability to wood-derived ones. Behavior, performance, and interactions with host material of CNC-embedded products rely on particles' geometry, i.e., 3D size, their aspect ratio, crystallinity, amount and type of surface groups, their distribution in a host matrix, and rheological properties [25]. Besides its above-mentioned intrinsic properties, CNCs also have superior mechanical properties, and a surface covered with numerous hydroxyl groups that enables different chemical modifications like oxidation esterification, silyation, and polymer grafting. These amazing physicochemical properties and widespread application prospect of CNCs (in personal care, foods, pharma-ceuticals, as drug carriers, anticoagulant materials, and in TE) have attracted significant interest from both research scientists and industrialists.

3. Tissue engineering – Introduction to basic principles

Tissue engineering (TE) is an interdisciplinary field that applies the principles of engineering and life science toward the development of smart biological substitutes (e.g., 3D scaffolds), which potentially restore, maintain, and improve tissue functions that are malfunctioned or have been lost by different pathological conditions or as a whole organ of the human body [26]. The field of TE still remains a fast-growing area with high-potential treatments for many kinds of disease states. In TE, the cells are usually cultured/seeded onto biodegradable scaffolds that mimic the extracellular matrix (ECM), which exhibits a hierarchical architecture with structural features ranging from nano- to macrometer scale [27]. In general, cell microenvironment is very complex, and it constitutes ECM proteins, bioactive growth factors, receptors, and neighboring cells (see Figure 3). Behavior, function, and fate of cells are determined by their interaction with biochemical and biophysical cues within their surrounding microenviron-ment [28]. In particular, the interaction between biochemical cues, for example, receptor coupling to ECM proteins or cytokines, and biophysical cues such as modulus and fibrillar structure play a decisive role in cell fate decision [28, 29]. These cell–ECM interactions are very dynamic, since cells interact with and respond to ECM signals and accordingly can change their microenvironments. Therefore, the understanding and interlinking of bidirectional cross talk between the microenvironment and resident cells are highly important for developing efficient strategies to regenerate tissues [28]. Generally, ECM differs in composition and spatial assembly of their protein components such as collagen, elastin, proteoglycans, and adhesion molecules depending on the types of body tissues. It further inherently assembles to keep a specific tissue morphology and to supply specific instructive cues for the cells of the different organs [30].
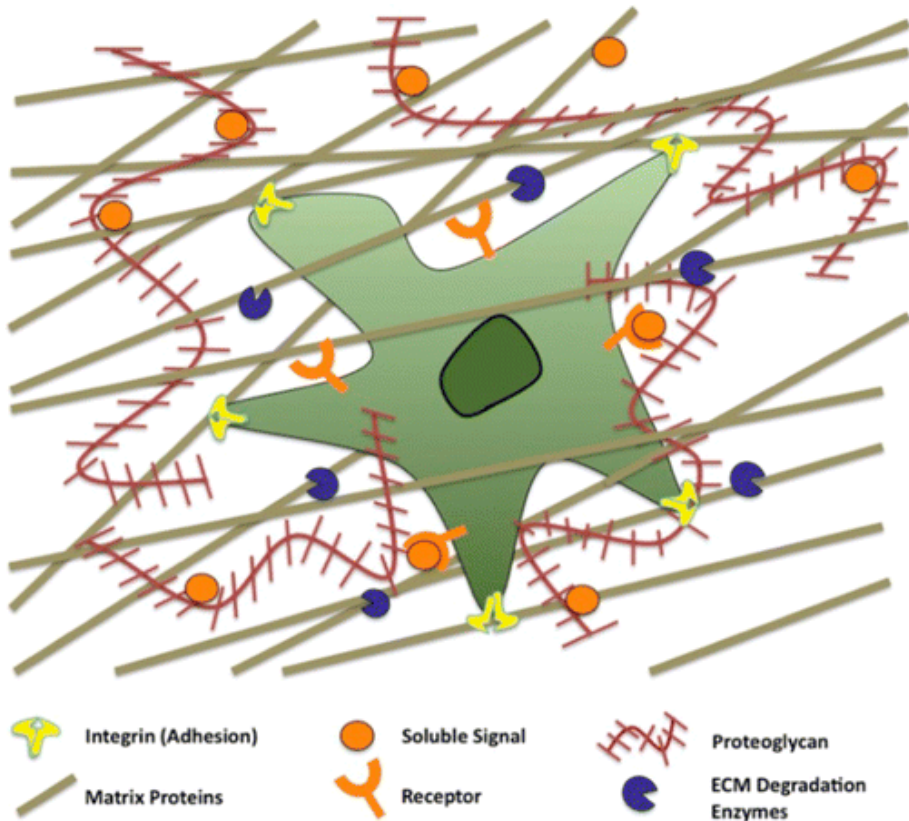
Figure 3. Schematic of the extracellular matrix. Fibrous matrix proteins (e.g., collagen, fibrin, elastin) provide structural and mechanical cues to direct cell behavior; soluble signals are sequestered by proteoglycans (proteins with polysaccharide moieties) and interact with cell surface receptors to direct cell migration, proliferation, and differentiation; integrins (transmembrane receptors) bind to matrix proteins for cell adhesion; ECM degradation enzymes (e.g., matrix metalloproteinases, serine proteinases, plasmin) cleave matrix components during cell motility and matrix remodeling. Reprinted with permission from ref [31]. Copyright 2012 Elsevier.

A unique but the simplest approach in TE and its related field regenerative medicine comprises the implantation of a scaffold that possesses inherent properties to actively promote the body's inborn capacity of healing and self-repair/growth of cells and tissue regeneration [27]. Scaffolds in general exhibit several attractive features that can influence their performance, and therefore the scaffolds should be carefully designed with all essential physicochemical properties for each targeted tissue. The scaffold materials, in general, should have the capacity to mimic the form and function of ECM in order to promote the cell–biomaterial interactions, cellular invasion, attachment, infiltration or migration, differentiation, and proliferation, thus rendering the lost tissue regeneration [27]. They should also permit sufficient transport of gases and nutrients, and allow regulatory factors to achieve cell survival [27]. Physiochemical properties of the scaffold materials, for instance, the roughness, topography, functional groups, porosity,

pore size, pore interconnectivity, and surface area to volume ratio are the key aspects that should be considered in the design of scaffolds, because they play a pivotal role in tissue growth, vascularization, and nutrient supply [28, 32-38]. Furthermore, the scaffolds should be mechanically strong and easily biodegradable at a controlled rate under the condition of interest, and provide a low degree of inflammation and cytotoxicity [39].

Biodegradability of a scaffold under a given condition is an important issue in TE, and thus materials for scaffolds design should be carefully chosen. The material should be absorbable and subjectable to degradation. The rate of degradation should match the time of tissue formation, that is, until the scaffold material is mechanically stable and possesses all its intrinsic physicochemical properties and the injured tissue is completely replaced by healthy tissue and its functions are restored [35, 39]. Taking these points into consideration, the selection of the scaffold materials and the preparation methodology is highly dependent on the requirements of the desired applications and given properties of the scaffold. Further, the most difficult challenge in TE is to create a scaffold with specific physical, chemical, mechanical, and biological properties. As stated above, the mechanical (dimensional) stability of the scaffold is imperative in TE, and it should yield similar performance to the tissues to be repaired or regenerated. In this way, the scaffold would give a better mechanical integration of the construct in the biological tissue. In addition, the mechanical signals (i.e., the performance of the scaffold under a given condition) are known to create impact on the "cell fate" [40] and therefore a consideration is usually given to the role of mechanotransduction during cell expansion and differentiation [41, 42]. Overall, a precise modulation of these mentioned properties is highly challenging as biological tissues present a vast spectrum of mechanical properties, which are governed by their anatomical functions. For example, many tissues, in general, are viscoelastic with nonlinear, anisotropic, and heterogeneous mechanical properties [43].

The materials (synthetic and/or from natural origin) and fabrication methods that enable the manufacturing of scaffolds with defined micro- and nanostructure length scales for different TE applications are manifold. As natural and derivatized biopolymers starch, carrageenan, alginate, chitin, chitosan, gellan gum, hyaluronic acid, chondroitin sulfate, lingocellulose, cellulose acetate [44], carboxymethyl cellulose [45], cellulose acetate propionate [46], hydroxyproply cellulose [47] are used for creating scaffolds and cell culturing owing to their inherent bioactivity, low toxicity, high biocompatibility and in most cases biodegradability [48]. Proteins such as collagen, gelatin, elastin, silk fibroin, and fibrin are also employed for the same purpose [48-50]. In the case of the synthetically based category, aliphatic polyesters such as poly(L-lactic acid), polycaprolactone, poly(glycolide), and poly-[D,L-(lactide-co-glycolide)], polyethylene oxide, polyvinyl alcohol, poly(N-isopropylacrylamide), polyacrylic acid, and poly(2-hydroxyethylmethacrylate) are widely employed for preparing TE scaffolds since they exhibit excellent biocompatibility, biodegradability, and considerable mechanical stability [34].

While significant advances were made over the years, it is still impossible to design and engineer TE scaffolds having all desired properties from a conventional single polymeric material. In this regard, in order to better mimic the ECM hierarchical structure and for efficient growth of cells, electrospun nanocomposite fiber mats generated from multicomponent systems have gained a significant interest for different TE applications in the recent years, due

to their good mechanical properties, nanotopography, and well-defined porosity [28, 32-34]. In order to overcome some limitations such as weak mechanical properties, lack of electrical conductivity, the absence of adhesive and microenvironment-defining moieties, or the incompetence to enable cells to self-assemble to 3D tissues [51], incorporation of nanofillers as reinforcing agents have become a popular approach in the past years. Several inorganic and organic nanofillers have been used together with polymeric materials for the preparation of TE scaffolds targeting at regeneration of different tissues of interest. As nanofillers, hydroxyapatite, carbon nanotubes, metal nanoparticles (e.g., silver, gold, and iron oxide), etc. have been used for the controlled immobilization of several biologically active molecules as well as to construct complex 3D tissues of different cell types [51-53]. Despite the number of advantages of metal or carbon-based nanofillers, there are some challenges and issues that remain to be addressed, including processability, toxicity, biocompatibility, and biodegradability. In this context, CNCs in particular, are highly attractive and important. They have emerged as potential nanofillers for preparation of biobased nanostructured composite materials, due to their aforementioned intrinsic properties [54]. Moreover, as specified before, CNCs exhibit low elongation at break, high aspect ratio, high surface area, and high crystallinity, which make them excellent candidates as load-bearing components for obtaining high-strength scaffold materials [55, 56]. For fabricating 3D scaffolds with nanofibrillar structures and with nanotopography to better mimic ECM dimensions, the electrospinning technique has been used extensively during the last few years, which will be discussed in the next section.

4. Electrospinning technique – Introduction to basic principles

Electrospinning is a straightforward, cost-effective, and robust technique, which is nowadays frequently applied in engineering well-defined nanocomposites fiber mats/scaffolds that hold promise in serving as a synthetic ECM. It utilizes electrical forces to create fine polymer fibers with diameters ranging from a few micrometers down to a few nanometers using polymer solutions of both natural and synthetic polymers [57]. This technique has been known for over 60 years in the textile industry for manufacturing nonwoven fiber fabrics, but has received a great importance only in the past decade. This is not only because of its versatility in spinning a wide range of polymeric fibers with controlled pore structure, but also due to its ability to consistently produce fibers in the submicron range that is otherwise difficult to achieve by using standard mechanical fiber spinning techniques [58-62]. Among several other advantages provided by this technique, it also gives the possibility to manipulate the spun fiber composition and produce highly reproducible fiber mats with micro- or nanotopography while enabling to tailor the fiber diameter, porosity, orientation, density, and high surface-area-to-volume ratio. Owing to these advantages, electrospun nanofibers have been widely examined for their use in numerous fields including nanocatalysis, protective clothing, filtration, biomedical, pharmaceutical, optical electronics, healthcare, as well as in tissue engineering [63-68]. Techniques such as electrostatic precipitators and pesticide sprayers work similarly to the electrospinning process; however, the latter process is mainly based on the principle that the applied strong mutual electrical repulsive forces overcome weaker forces of surface tension

in the charged polymer liquid. There are, currently, two standard electrospinning setups – vertical and horizontal. In general, the electrospinning process is conducted at ambient temperature and pressure. The typical electrospinning apparatus setup is depicted in Figure 4.
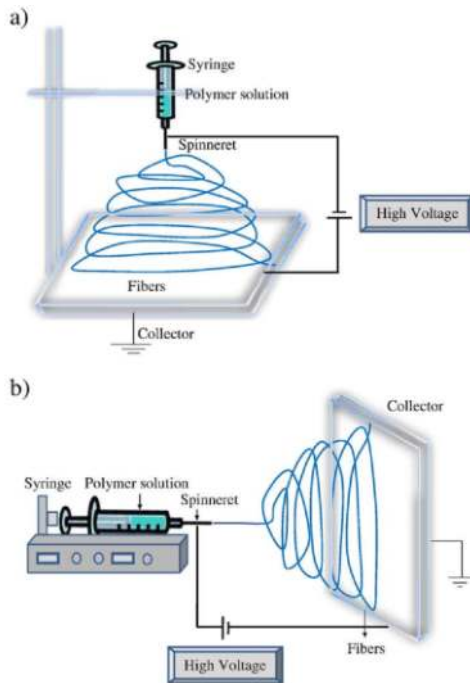
Figure 4. Schematic representation illustrating the set up of an electrospinning apparatus. A) typical vertical set up, b) horizontal setup. Reprinted with permission from ref [59]. Copyright 2010 Elsevier.

A basic electrospinning setup consists of three major components such as a high-voltage power supply; a spinneret, i.e., a pipette tip or a plastic syringe; and a grounded collecting plate that is usually a metal screen, plate, or rotating mandrel, and the technique uses electrical forces for stretching the solubilized polymer as it falls and solvent evaporates out. In this technique, prior to the spinning the dissolved polymer solution or melt of interest is introduced into the capillary tube and then a high-voltage electrical field is applied between a metallic nozzle of a syringe and a metallic collector. As a result, the polymer solution or melt, held by its surface tension at the end of the capillary tube is subjected to a high electrical field, and this high-voltage source induces charge of a certain polarity on the liquid surface of the polymer solution or melt. At end of the needle tip, the polymer solution deforms from a pendant droplet to a conical shape (Taylor cone). When the strength of the electrical force is stronger than the surface tension of the polymer solution, a jet with polymer solution is formed and ejected subsequently from the cone surface and travels to the opposite polarity, i.e., metallic collector. As soon as the polymer jet solution travels, the solvent evaporates in the air, together with stretching and

acceleration of the polymer jet, leading to the deposition of an extremely thin polymer (nano-)fiber on the collector. The polymer jet is only stable at the end of the capillary tube and after that the instability begins, and thus electrospinning process offers a simplified method for the manufacturing of ultrathin nanofibers [59-62].

5. Cellulose-based scaffold – Materials' characterization and role in cell growth

Despite several advantages mentioned above in different fields, the application of electrospinning and spun nanofibers in the field of TE is as yet not realized up to its full potential, and there are still some challenges that need proper attention. The diameters of electrospun nanofibers are usually orders of magnitude smaller when compared to the sizes of cells. As a result, the cells are able to organize over the fibers of the scaffold or spread and attach to the initially adsorbed proteins or cells at multiple focal points [62]. Regardless of its numerous attractive properties, scaffolds pose some other limitations such as nonuniform cellular distribution and lack of cellular migration/infiltration with increasing depth under normal passive seeding conditions. This is because the electrospun scaffolds or mats are often characterized by entangled fibers and a densely packed membrane structure that give obvious limitation to cellular migration/infiltration and populate in the interior of the scaffolds. These drawbacks could potentially hamper the development and potential applicability of electrospun fibers, in particular for 3D tissue or organs. In this context, the issue of cellular infiltration into the fiber architecture is gaining more and more attention owing to its potential in stagnating further applications of electrospun scaffolds materials in various TE applications. To overcome these limitations, an extensive set of fabrication techniques, such as wet electrospinning, laser or UV photolithography, salt leaching, polymer blending, solvent casting or a combination of nanofibers and microfibers, have been used to obtain scaffolds having enlarged pore size, and interconnectivity, and subsequently promote cellular migration/infiltration [69-71]. Up to now, the electrospinning technique has been increasingly exploited in the production of nanofibrous scaffolds or mats from the widest range of polymeric materials (natural and synthetic) for tissue engineering applications (see section 3). Besides the limitation to cellular migration/infiltration, another important major concern with spun 3D scaffolds, which are made from either mono or bicomponent system, is mechanical stability, since they are often not strong enough for many tissue engineering applications, as described above. One way to improve the mechanical properties of the electrospun scaffold is to integrate some reinforcing agents or fillers, and for such purpose a range of materials is used, which can be found elsewhere.

Among other materials, incorporation of stiff and rodlike CNCs with high aspect ratios as reinforcing agent into the electrospun fiber mats have been investigated as an effective and alternative method in numerous studies in fabricating a high-strength and highly porous composite scaffold with multiscaled features, which would better mimic the natural hierarchical organization of ECM [72-74]. 3D porous scaffolds consisting of naturally occurring CNCs as reinforcing agents are important and attractive for many TE applications due to their vast

number of above-mentioned advantages [73,74]. Electrospinning technique allows CNCs to orient along the fiber axis, thus yielding unidirectional fiber-reinforced composites for applications that require high-fiber stiffness, uniform morphology, and reduced fiber diameter [72-74]. Until now, numerous electrospun composite materials incorporated with CNCs have been produced from natural and synthetic polymers, and employed in the production of 3D tissues for implantation. Jia et al. investigated the potential applicability of CNCs in combination with microcrystalline cellulose (MC) as fillers in electrospun cellulose acetate (CA) nanofiber mats for vascular tissue scaffolds [75]. It has been found that the electrospun fibers are in the range of submicron and the incorporation of CNC and MC increased the porosity of the scaffolds, for example, a mean diameter of 1016 ± 572 nm and porosity of 80±9% are obtained at a ratio of 1:1 (MC:CNC, 10 wt.%). To understand the impact of CNC and MC on the viability of rat aortic vascular smooth muscle cells (VSMC) within the generated scaffolds were tested in vitro. It turned out that the cell viability, adhesion, migration, and proliferation with the scaffold integrated with CNC and MC are significantly improved compared with those of CA alone, confirming the synergistic enhancement by the micro- and nanoscaled features within the 3D fibrous mesh structure. In the recent work, He et al. fabricated the electrospun all-cellulose nanocomposite materials reinforced with 20% CNC, which are uniaxially aligned using a rotating drum as collector [76]. The obtained scaffold material showed well-dispersed and substantially orientated CNCs along the fiber axis, and, further, the inclusion of CNCs induced a positive effect in creating scaffolds with uniform morphology and ordered distribution of nanofibers with diameter ranging from 212 to 221 nm. The resulting final material with ordered microstructure demonstrated a remarkable improvement in the tensile properties; for instance, the tensile strength and the elastic modulus are increased by 102% and 172% in the fiber alignment direction, due to enhanced interfacial bonding between CNCs and the regenerated cellulose matrix employed in the production of the electrospun scaffold. The applicability of the material is tested for the growth of human dental follicle cells (cDFCs), and the results illustrated that the fiber mats are nontoxic and the cells are able to attach, infiltrate, and subsequently proliferate in the entire scaffold, thereby inducing ordered organization of cDFCs along the fiber alignment direction (Figure 5). The authors have further proposed that these microstructured electrospun scaffolds can be used in several other TE strategies, for example, in the development of blood vessel, tendon, or nerves, where the mechanical performance and cell orientation are critical issues [77].

Huang et al. manufactured mechanically highly stable electrospun scaffold composed of nanofibers from silk protein such as fibroin and CNCs (diameter: 20–40 nm, length: 400–500 nm) as nanofillers that are extracted from *Morus alba L.* branch bark [73]. Results showed that in the resulting material, the nanoparticles CNCs are well dispersed and considerably oriented along the fiber axis in the silk fibroin matrix, as reported by the author. The electrospun fiber mats reinforced with CNCs (2%) showed almost twofold increase in the mechanical properties such as the tensile strength and the Young's modulus compared to virgin (unreinforced) silk fibroin nanofiber. Interestingly, the electrospun nanofibers that were obtained using bacterial cellulose nanocrystals as nanofiller and silk fibroin showed similar increase in the mechanical properties [73, 78]. Unfortunately, the importance of electrospun scaffolds derived from fibroin/CNCs is not recognized as cell growth material in TE to date.
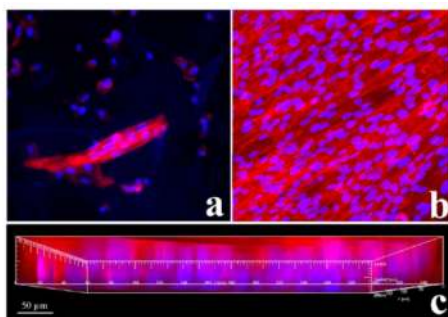
Figure 5. Confocal laser scanning microscopy images of hDFCs loaded in electrospun cellulose/CNCs nanocomposite nanofibers: (a) cultured for 3 days, (b) cultured for 7 days, and (c) the 3D view of electrospun nanofibers with cells. Scale bar: 50 µm. Reprinted with permission from ref [76]. Copyright 2014 American Chemical Society.

While the scaffolds manufactured from fully renewable cellulose are highly captivating and important for TE applications, the electrospinning of these natural origins is always demanding and needs special care in choosing the right solvent and viscosity of the polymer solution. It can be resolved, however, by blending CNCs with synthetic biopolymers to meet the right solution viscosity and consistency in electrospinning. In this regard, in the recent years, synthetic biopolymers, in particular, PLA in combination with CNCs as nanofiller, have received a vast interest in the production of electrospun nanocomposite fibers [79]. For example, in the work of Xian et al., PLA electrospun nanofiber mats incorporated with CNCs prepared from avicel (microcrystalline) cellulose are fabricated [79]. It has been found that the strength of the nanofibers is improved by 30% with loading of 1 wt.% CNCs. The latter acted as nucleating agent of PLA crystallization leading to increased crystallinity of PLA in the resulting nanocomposite fibers. In another work by Shi et al., PLA/CNCs electrospun fiber mats from a solvent mixture consisting of N,N'-dimethylformamide (DMA) and chloroform were prepared and characterized in terms of in vitro degradation properties [80]. The obtained nanocomposite mats showed 5-fold and 22-fold increase in tensile stress and Young's modulus upon the addition of 5 wt.% CNCs, and they undergo faster degradation in phosphate-buffered saline (PBS) solution than that in neat PLA mats. These types of materials exhibiting superior mechanical and fast degradation properties are particularly interesting for making TE scaffolds targeted at short-term application CNCs.

Owing to the fact that CNCs are highly polar and show high tendency for the formation of hydrogen bonding promoting agglomeration, dispersing aqueous or nonpolar-based CNCs in a PLA matrix is highly tedious and time consuming. To overcome this hurdle, Li et al. proposed an alternative approach based on a water-in-oil (W/O) emulsion system consisting of a dispersed phase of CNCs aqueous suspension and an immiscible continuous phase of PLA solution [81]. The electrospun fiber mats prepared from these system showed either a core-shell or hollow structure depending on the emulsion droplet size, and the CNCs are aligned along the core or on the wall of the hollow cylinder (Figure 6). Upon the addition of 5 wt.% CNCs, a drastic increase in stiffness (Young's modulus), i.e., up to 549% and maximum increase

of tensile strength of 90% are obtained for the electrospun composite fiber mats. As described by the authors, besides the reinforcement offered by the CNCs, these hollow structures, as multifunctional system, can be potentially employed in several applications such as for the attachment of biologically active molecules, controlled drug delivery, and as scaffolds.
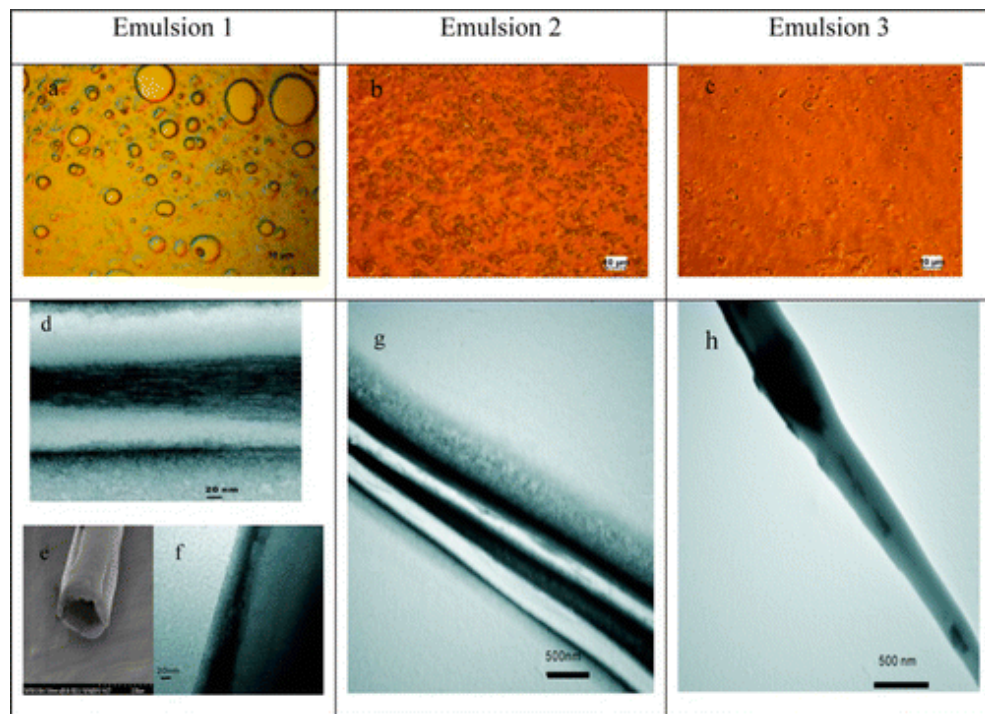
Figure 6. Correlations between emulsion droplet size (a ~6 µm, b ~3 µm, and c <1 µm) and electrospun fiber structure (d and g represent core-shell; e and f, hollow cylinder; and h, CNC aggregation). Reprinted with permission from ref [81]. Copyright 2013 American Chemical Society.

Zhou et al. used PLA grafted with maleic anhydride (MPLA) in order to advance the interfacial tension between the hydrophobic PLA matrix and the hydrophilic CNCs [82]. The fiber mats electrospun from MPLA and CNCs (5 wt.%) showed reduced diameter and polydispersity with increased CNCs content, and improved thermal and mechanical properties (e.g., tensile strength increased up to more than 10 MPa). The suitability of scaffolds for in vitro degradation and cytocompatability are tested using human adult adipose (derived from mesenchymal stem cells, hASCs; see Figure 7). Furthermore, it has been demonstrated that the cells could attach and proliferate in the entire scaffold (not only on the surface but also deep inside the fiber mats) and induce ordered cellular organization in fiber alignment direction. Results showed that the scaffolds are highly biocompatible, biodegradable, and cytocompatible, demonstrating that they have high potential to be used in bone tissue engineering [82].
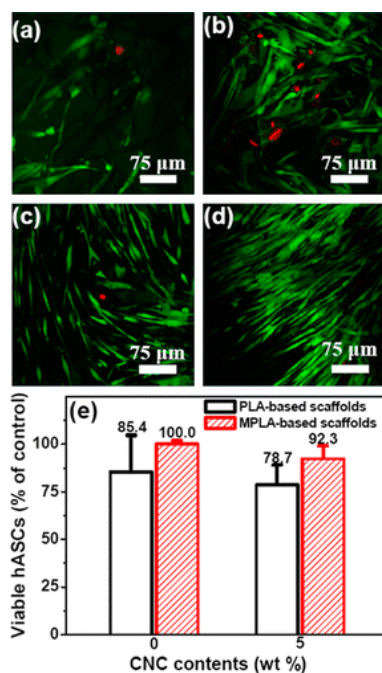
Figure 7. Response of hASCss to PLA/CNC and MPLA/CNC nanofibrous scaffolds after 7 days of culture. Fluorescence micrographs of stained cells consisting of live (green) and dead (red) cells for PLA (a); PLA/CNC-5 (b); MPLA (c); and MPLA/CNC-5 (d) scaffolds, scale bar represents 75 μm; proliferation viability of cells (e). Reproduced with permission from ref [82]. Copyright 2013 American Chemical Society.

Nanocomposite fiber mats based on biodegradable poly(ε-caprolactone), PCL, widely used in TE, is also prepared by means of electrospinning by incorporating with CNCs [83]. The inclusion of CNCs with increasing concentrations into the PCL matrix induced considerably positive effects on the mechanical, thermal, and surface properties. The tensile strength and modulus are increased to 36% (with 10 wt.% CNCs) and 167% (with 15 wt.% CNCs). In another study, electrospun PCL nanofibers are reinforced with CNCs, which are obtained from ramie cellulose fibers. In this case, in order to improve the interfacial tension with the PCL matrix, the surface of CNCs is chemically modified with low molecular weight PCL diol [84]. Although this approach yielded unexpected results on the morphology of the nonwoven mats in which the individual nanofibers became annealed during the electrospinning process, the mechanical properties of the nanofibers are significantly improved after reinforcing with 2.5 wt.% CNCs. For example, the Young's modulus and the tensile strength are increased up to 1.5-fold and many folds compared to those of pure PCL mats.

As CNCs are dispersible in water, a direct mixing of CNCs with several water soluble polymers is feasible. As a consequence, several research groups have attempted to develop multifunctional electrospun scaffolds based on water-soluble polymer matrices applicable for TE applications, such as polyethylene oxide (PEO) [85, 86] and polyvinyl alcohol (PVA) [74, 84].

In the case of nanofiber mats electrospun from PVA and CNCs aqueous dispersion, the incorporation of CNCs displayed a considerable reinforcement effect, in particular, on the aligned electrospun PVA fiber mats compared with the isotropic ones. For instance, the aligned mats demonstrated a 35% and 45% increase in Young's modulus and tensile strength compared to the isotropic mats. Moreover, aligned PVA/CNCs fibers yielded a lower diameter compared to the fibers that are produced from PVA alone. With addition of 15 wt.% CNCs the tensile strength and Young's modulus are further increased to 95% and 118% for the aligned PVA/CNCs, clearly demonstrating that the CNCs with high aspect ratio can align inside the polymer matrix during electrospinning because of the higher shear force of the polymer jet, thereby causing tremendous increase in the physical properties of the material [74]. In a similar work carried out by Peresin et al., the reinforcement effect of CNCs on electrospun PVA with different concentrations of acetyl groups and CNCs is studied, and the results showed a significant improvement in the mechanical properties [84]. It is suggested that the higher hydrolysis degree of PVA caused a strong interaction between PVA and CNCs, led to effective reinforcement of the fiber mats, which can be related to the reinforcing effect of the dispersed phase of CNCs, via the percolation network held by hydrogen bonds. The PEO/CNCs electrospun nanocomposite fiber mats are also prepared by using different concentration of wood-based CNCs (up to 20 wt.%). In this case, upon increasing the CNCs concentration in electrospinning solution, more uniform and finer nanofibers are formed [85], due to an enhanced electrical conductivity of the spinning solution. Furthermore, the tensile strength and Young's modulus are substantially improved 152% and 180% upon the addition of nanofiller CNCs. This enhanced mechanical property is due to the efficient stress transfer from PEO to CNCs stemming from their strong hydrogen bonding interactions and uniform dispersion, as well as high alignment of CNCs in the electrospun nanocomposite fibers.

Generally, the incorporation of CNCs can decrease the fiber diameter of the electrospun nanocomposite fiber mats, and this can impose some limitations of these system in TE applications. One such example is cellular infiltration which can be hindered by decreasing the scaffolds pore size/fiber diameter. Zhou et al. reported that 24% decrease in fiber diameter for the electrospun nancomposite PEO/CNCs when incorporated with 20 wt.% [85]. Whereas only a 19% reduction in fiber diameter is observed for the nanocomposite fiber mats electro-spun from MPLA/CNCs upon incorporation of 5 wt.% CNCs. This limitation can be alleviated by adopting the electrospinning technique to increase the pore size of the scaffolds [82]. It is known that the mechanical stability of the electrospun scaffolds is weakened by the increased porosity and pore size. In this case, incorporation of nanofiller CNCs to reinforce or impart higher mechanical strength of the nanofibrous scaffolds is highly useful strategy, while offering effective and appropriate 3D scaffolds for the accommodation and sufficient migration or infiltration of cells.

6. Summary and outlook

During the past few years, the need to utilize biomaterials such as cellulose nanocrystals for tissue engineering application has increased dramatically. CNCs as a potential nanofiller and

reinforcement material have created a significant interest especially in the design and fabrication of three-dimensional porous scaffold materials for the growth of 3D tissue. Scaffolds prepared from the CNC-based nanocomposites using electrospinning technique have demonstrated to be highly suitable to better mimic the extracellular matrix. Regardless of the considerable numbers of different polymeric (natural and synthetic) materials used for scaffold preparation, incorporation of CNCs has significantly improved the mechanical properties, porosity, and thus the adhesion, migration, and proliferation of the cells. Even though only a very few and no in vivo biological tests have been carried out in order to access the performance of scaffolds for practical applications, several studies are in progress to develop novel multifunctional biomaterials with tailored properties for tissue engineering applications.

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References

[1]   Klemm D, Heublein B, Fink H-PBohn A: Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angewandte Chemie Int Ed*. 2005;44:3358-3393. DOI:10.1002/anie.200460587.

[2]   D. Klemm, B. Philipp, T. Heinze, U. HeinzeWagenknecht W: In Comprehensive Cellulose Chemsitry: Fundamental and Analytical Methods. Vol. 1. 1998: Wiley-VCH.

[3]   Lin NDufresne A: Nanocellulose in biomedicine: Current status and future prospect. *Eur Polym J*. 2014;59:302-325. DOI:http://dx.doi.org/10.1016/j.eurpolymj.2014.07.025.

[4]   Parthasarathi R, Bellesia G, Chundawat S P S, Dale B E, Langan P, Gnanakaran S: Insights into Hydrogen Bonding and Stacking Interactions in Cellulose. *J Physic Chem* A. 2011;115:14191-14202. DOI:10.1021/jp203620x.

[5]   Krassig H A: *Cellulose: Structure, Accessibility and Reactivity*. Amsterdam [u.a.]: Gordon and Breach. 1996.

[6]   Anselme P: Troisieme memire sur le development vegetaux. *Comptes rendus*. 1838;7:1052-1125.

[7]   Stipanovic A J, Sarko A: Packing Analysis of Carbohydrates and Polysaccharides. 6. Molecular and Crystal Structure of Regenerated Cellulose II. *Macromolecules*. 1976;9:851-857. DOI:10.1021/ma60053a027.

[8]   Hess K, Kissig H: Zur kenntnis der hochtemperatur-Modifikation der Cellulose (Cellulose IV). *Zietschrift Physikalische Chemie* B. 1941;49:235-239.

[9]   Marrinan H J, Mann J: Infrared Spectra of the Crystalline Modifications of Cellulose. *J Polym Sci*. 1956;21:301-311. DOI:10.1002/pol.1956.120219812.

[10] Hearle J W S: A Fringed Fibril Theory of Structure in Crystalline Polymers. *J Polym Sci*. 1958;28:432-435. DOI:10.1002/pol.1958.1202811722.

[11] Habibi Y, Lucia L A, Rojas O J: Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. *Chem Rev*. 2010;110:3479-3500. DOI:10.1021/cr900339w.

[12] Lee K-Y, Aitomäki Y, Berglund L A, Oksman K, Bismarck A: On the Use of Nanocellulose as Reinforcement in Polymer Matrix Composites. *Composites Sci Technol*. 2014;105:15-27. DOI:http://dx.doi.org/10.1016/j.compscitech.2014.08.032.

[13] Lu P, Hsieh Y-L: Preparation and Properties of Cellulose Nanocrystals: Rods, Spheres, and Network. *Carbohydrate Polym*. 2010;82:329-336. DOI:http://dx.doi.org/10.1016/j.carbpol.2010.04.073.

[14] Moon R J, Martini A, Nairn J, Simonsen J, Youngblood J: Cellulose Nanomaterials Review: Structure, Properties and Nanocomposites. *Chem Soc Rev*. 2011;40:3941-3994. DOI:10.1039/C0CS00108B.

[15] Elazzouzi-Hafraoui S, Nishiyama Y, Putaux J-L, Heux L, Dubreuil F, Rochas C: The Shape and Size Distribution of Crystalline Nanoparticles Prepared by Acid Hydrolysis of Native Cellulose. *Biomacromolecules*. 2008;9:57-65. DOI:10.1021/bm700769p.

[16] Osorio-Madrazo A, Eder M, Rueggeberg M, Pandey J K, Harrington M J, Nishiyama Y, Putaux J-L, Rochas C, Burgert I: Reorientation of Cellulose Nanowhiskers in Agarose Hydrogels under Tensile Loading. *Biomacromolecules*. 2012;13:850-856. DOI:10.1021/bm201764y.

[17] Habibi Y, Goffin A-L, Schiltz N, Duquesne E, Dubois P, Dufresne A: Bionanocomposites Based on Poly([varepsilon]-caprolactone)-Grafted Cellulose Nanocrystals by Ring-Opening Polymerization. *J Mater Chem*. 2008;18:5002-5010. DOI:10.1039/B809212E.

[18] Lamaming J, Hashim R, Sulaiman O, Leh C P, Sugimoto T, Nordin N A: Cellulose Nanocrystals Isolated from Oil Palm Trunk. *Carbohydrate Polym*. 2015;127:202-208. DOI:http://dx.doi.org/10.1016/j.carbpol.2015.03.043.

[19] Mendes C A d C, Ferreira N M S, Furtado C R Gde Sousa A M F: Isolation and Characterization of Nanocrystalline Cellulose from Corn Husk. *Mater Lett*. 2015;148:26-29. DOI:http://dx.doi.org/10.1016/j.matlet.2015.02.047.

[20] Danial W H, Abdul Majid Z, Mohd Muhid M N, Triwahyono S, Bakar M BRamli Z: The Reuse of Wastepaper for the Extraction of Cellulose Nanocrystals. *Carbohydrate Polym*. 2015;118:165-169. DOI:http://dx.doi.org/10.1016/j.carbpol.2014.10.072.

[21] Esa F, Tasirin S MRahman N A: Overview of Bacterial Cellulose Production and Application. *Agriculture Agricul Sci Proced*. 2014;2:113-119. DOI:http://dx.doi.org/10.1016/j.aaspro.2014.11.017.

[22] Pirich C, de Freitas R, Woehl M, Picheth G, Petri D S, Sierakowski M: Bacterial Cellulose Nanocrystals: Impact of the Sulfate Content on the Interaction with Xyloglucan. *Cellulose*. 2015;22:1773-1787. DOI:10.1007/s10570-015-0626-y.

[23] Kargarzadeh H, M. Sheltami R, Ahmad I, Abdullah I, Dufresne A: Cellulose Nanocrystal: A Promising Toughening Agent for Unsaturated Polyester Nanocomposite. *Polymer*. 2015;56:346-357. DOI:http://dx.doi.org/10.1016/j.polymer.2014.11.054.

[24] Van Hai L, Son H, Seo Y: Physical and Bio-composite Properties of Nanocrystalline Cellulose from Wood, Cotton Linters, Cattail, and Red Algae. *Cellulose*. 2015;22:1789-1798. DOI:10.1007/s10570-015-0633-z.

[25] Li M-C, Wu Q, Song K, Lee S, Qing Y, Wu Y: Cellulose Nanoparticles: Structure–Morphology–Rheology Relationships. *ACS Sustain Chem Engin*. 2015;3:821-832. DOI:10.1021/acssuschemeng.5b00144.

[26] Brahatheeswaran Dhandayuthapani, Yasuhiko Yoshida, Toru MaekawaKumar D S: Polymeric Scaffolds in Tissue Engineering Application: A Review. *Int J Polym Sci*. 2011;2011. DOI:10.1155/2011/290602.

[27] Place E S, Evans N D, Stevens M M: Complexity in Biomaterials for Tissue Engineering. *Nat Mater*. 2009;8:457-470. DOI:http://www.nature.com/nmat/journal/v8/n6/suppinfo/nmat2441_S1.html.

[28] Keung A J, Kumar S, Schaffer D V: Presentation Counts: Microenvironmental Regulation of Stem Cells by Biophysical and Material Cues. *Ann Rev Cell Develop Biol*. 2010;26:533-556. DOI:doi:10.1146/annurev-cellbio-100109-104042.

[29] Lutolf M P, Hubbell J A: Synthetic Biomaterials as Instructive Extracellular Microenvironments for Morphogenesis in Tissue Engineering. *Nat Biotech*. 2005;23:47-55.

[30] Kim H N, Jiao A, Hwang N S, Kim M S, Kang D H, Kim D-H, Suh K-Y: Nanotopography-Guided Tissue Engineering and Regenerative Medicine. *Adv Drug Deliv Rev*. 2013;65:536-558. DOI:http://dx.doi.org/10.1016/j.addr.2012.07.014.

[31] Wade R J, Burdick J A: Engineering ECM Signals into Biomaterials. *Mater Today*. 2012;15:454-459. DOI:http://dx.doi.org/10.1016/S1369-7021(12)70197-9.

[32] Bauer A L, Jackson T L, Jiang Y: Topography of Extracellular Matrix Mediates Vascular Morphogenesis and Migration Speeds in Angiogenesis. *PLoS Comput Biol*. 2009;5:e1000445. DOI:10.1371/journal.pcbi.1000445.

[33] Edalat F, Bae H, Manoucheri S, Cha J, Khademhosseini A: Engineering Approaches Toward Deconstructing and Controlling the Stem Cell Environment. *Annals Biomed Engin*. 2012;40:1301-1315. DOI:10.1007/s10439-011-0452-9.

[34] Place E S, George J H, Williams C K, Stevens M M: Synthetic Polymer Scaffolds for Tissue Engineering. *Chem Soc Rev*. 2009;38:1139-1151. DOI:10.1039/B811392K.

[35] Hutmacher D W: Scaffolds in Tissue Engineering Bone and Cartilage. *Biomaterials*. 2000;21:2529-2543. DOI:http://dx.doi.org/10.1016/S0142-9612(00)00121-6.

[36] Hutmacher D W, Schantz J T, Lam C X F, Tan K CLim T C: State of the Art and Future Directions of Scaffold-Based Bone Engineering from a Biomaterials Perspective. *J Tissue Engin Regen Med*. 2007;1:245-260. DOI:10.1002/term.24.

[37] Karageorgiou V, Kaplan D: Porosity of 3D Biomaterial Scaffolds and Osteogenesis. *Biomaterials*. 2005;26:5474-5491. DOI:http://dx.doi.org/10.1016/j.biomaterials. 2005.02.002.

[38] Rezwan K, Chen Q Z, Blaker J J, Boccaccini A R: Biodegradable and Bioactive Porous Polymer/Inorganic Composite Scaffolds for Bone Tissue Engineering. *Biomaterials*. 2006;27:3413-3431. DOI:http://dx.doi.org/10.1016/j.biomaterials.2006.01.039.

[39] Holzapfel B M, Reichert J C, Schantz J-T, Gbureck U, Rackwitz L, Nöth U, Jakob F, Rudert M, Groll J, Hutmacher D W: How Smart Do Biomaterials Need to Be? A Translational Science and Clinical Point of View. *Adv Drug Deliv Rev*. 2013;65:581-603. DOI:http://dx.doi.org/10.1016/j.addr.2012.07.009.

[40] Discher D E, Janmey P, Wang Y L: Tissue Cells Feel and Respond to the Stiffness of their Substrate. *Science*. 2005;18:1139-1143. DOI:10.1126/science.1116995.

[41] Chen C S: Mechanotransduction – A Field Pulling Together? *J Cell Sci*. 2008;121:3285-3292. DOI:10.1242/jcs.023507.

[42] DeForest C AAnseth K S: Advances in Bioactive Hydrogels to Probe and Direct Cell Fate. *Ann Rev Chem Biomol Engin*. 2012;3:421-444. DOI:10.1146/annurev-chem-bioeng-062011-080945.

[43] Hollister S J: Scaffold Design and Manufacturing: From Concept to Clinic. *Adv Mater*. 2009;21:3330-3342. DOI:10.1002/adma.200802977.

[44] Entcheva E, Bien H, Yin L, Chung C-Y, Farrell M, Kostov Y: Functional Cardiac Cell Constructs on Cellulose-Based Scaffolding. *Biomaterials*. 2004;25:5753-5762. DOI:http://dx.doi.org/10.1016/j.biomaterials.2004.01.024.

[45] Barbieri D, Yuan H, de Groot F, Walsh W Rde Bruijn J D: Influence of Different Polymeric Gels on the Ectopic Bone Forming Ability of an Osteoinductive Biphasic Calcium Phosphate Ceramic. *Acta Biomaterialia*. 2011;7:2007-2014. DOI:http://dx.doi.org/10.1016/j.actbio.2011.01.017.

[46] Gomes G d S, Almeida A T d, Kosaka P M, Rogero S O, Cruz Á S, Ikeda T I, Petri D F S: Cellulose Acetate Propionate Coated Titanium: Characterization and Biotechnological Application. *Mater Res*. 2007;10:469-474.

[47] Kitamura M, Ohtsuki C, Iwasaki H, Ogata S-i, Tanihara M, Miyazaki T: The Controlled Resorption of Porous α-Tricalcium Phosphate Using a Hydroxypropylcellulose Coating. *J Mater Sci: Mater Med*. 2004;15:1153-1158. DOI:10.1023/B:JMSM.0000046399.40310.47.

[48] Mano J F, Silva G A, Azevedo H S, Malafaya P B, Sousa R A, Silva S S, Boesel L F, Oliveira J M, Santos T C, Marques A P, Neves N M, Reis R L: Natural Origin Biodegradable Systems in Tissue Engineering and Regenerative Medicine: Present Status and Some Moving Trends. *J. R. Soc. Interface*. 2007;4:999-1030.

[49] Fernandes E M, Pires R A, Mano J F, Reis R L: Bionanocomposites from Lignocellulosic Resources: Properties, Applications and Future Trends for their Use in the Biomedical Field. *Progr Polym Sci*. 2013;38:1415-1441. DOI:http://dx.doi.org/10.1016/j.progpolymsci.2013.05.013.

[50] Gomes S, Leonor I B, Mano J F, Reis R L, Kaplan D L: Natural and Genetically Engineered Proteins for Tissue Engineering. *Progr Polym Sci*. 2012;37:1-17. DOI:http://dx.doi.org/10.1016/j.progpolymsci.2011.07.003.

[51] Dvir T, Timko B P, Kohane D S, Langer R: Nanotechnological Strategies for Engineering Complex Tissues. *Nat Nano*. 2011;6:13-22.

[52] Armentano I, Dottori M, Fortunati E, Mattioli S, Kenny J M: Biodegradable Polymer Matrix Nanocomposites for Tissue Engineering: A Review. *Polym Degrad Stability*. 2010;95:2126-2146. DOI:http://dx.doi.org/10.1016/j.polymdegradstab.2010.06.007.

[53] Okamoto M, John B: Synthetic Biopolymer Nanocomposites for Tissue Engineering Scaffolds. *Progr Polym Sci*. 2013;38:1487-1503. DOI:http://dx.doi.org/10.1016/j.progpolymsci.2013.06.001.

[54] Dugan J M, Gough J E, Eichhorn S J: Bacterial Cellulose Scaffolds and Cellulose Nanowhiskers for Tissue Engineering. *Nanomedicine*. 2013;8:287-298. DOI:10.2217/nnm.12.211.

[55] Dreher K L: Health and Environmental Impact of Nanotechnology: Toxicological Assessment of Manufactured Nanoparticles. *Toxicol Sci*. 2004;77:3-5. DOI:10.1093/toxsci/kfh041.

[56] El-Ansary A, Al-Daihan S, Bacha A, Kotb M:, *Toxicity of Novel Nanosized Formulations Used in Medicine*, in *Oxidative Stress and Nanotechnology*, D. Armstrong and D. J. Bharali, (Eds.). 2013, Humana Press. p. 47-74.

[57] Agarwal S, Wendorff J H, Greiner A: Use of Electrospinning Technique for Biomedical Applications. *Polymer*. 2008;49:5603-5621. DOI:http://dx.doi.org/10.1016/j.polymer.2008.09.014.

[58]   Kai D, Liow S S, Loh X J: Biodegradable Polymers for Electrospinning: Towards Bio-
       medical Applications. *Mater Sci Engin: C*. 2014;45:659-670. DOI:http://dx.doi.org/
       10.1016/j.msec.2014.04.051.

[59]   Bhardwaj N, Kundu S C: Electrospinning: A Fascinating Fiber Fabrication Technique.
       Biotechnol   Adv.   2010;28:325-347.   DOI:http://dx.doi.org/10.1016/j.biotechadv.
       2010.01.004.

[60]   Reneker D H, Yarin A L, Fong H, Koombhongse S: Bending Instability of Electrically
       Charged Liquid Jets of Polymer Solutions in Electrospinning. *J Appl Phys*.
       2000;87:4531-4547. DOI:doi:http://dx.doi.org/10.1063/1.373532.

[61]   Schreuder-Gibson H, Gibson P, Senecal K, Sennett M, Walker J, Yeomans W, Ziegler
       D, Tsai P P: Protective Textile Materials Based on Electrospun Nanofibers. *J Adv Ma-
       ter*. 2002;34:44-55.

[62]   Pham Q P, Sharma U, Mikos A G: Electrospinning of Polymeric Nanofibers for Tis-
       sue Engineering Applications: A Review. *Tissue Engin*. 2006;12:1197-1211. DOI:
       10.1089/ten.2006.12.1197.

[63]   Luu Y K, Kim K, Hsiao B S, Chu B, Hadjiargyrou M: Development of a Nanostruc-
       tured DNA Delivery Scaffold via Electrospinning of PLGA and PLA–PEG Block Co-
       polymers. *J Controlled Release*. 2003;89:341-353. DOI:http://dx.doi.org/10.1016/
       S0168-3659(03)00097-X.

[64]   Subbiah T, Bhat G S, Tock R W, Parameswaran S, Ramkumar S S: Electrospinning of
       nanofibers. *J Appl Polym Sci*. 2005;96:557-569. DOI:10.1002/app.21481.

[65]   Ramakrishna S, Fujihara K, Teo W-E, Yong T, Ma Z, Ramaseshan R: Electrospun
       nanofibers: solving global issues. *Mater Today*. 2006;9:40-50. DOI:http://dx.doi.org/
       10.1016/S1369-7021(06)71389-X.

[66]   Cui W, Zhou S, Li X, Weng J: Drug-loaded biodegradable polymeric nanofibers pre-
       pared by electrospinning. *Tissue Engin*. 2006;12:1070-1070.

[67]   Barnes C P, Sell S A, Knapp D, C, Walpoth B H, Brand D, Bowlin G L: Preliminary
       Investigation of Electrospun Collagen and Polydioxanone for Vascular Tissue Engi-
       neering Applications. *Int J Electrospun Nanofibers Applic*. 2007;1:73-87.

[68]   Welle A, Kröger M, Döring M, Niederer K, Pindel E, Chronakis I S: Electrospun Ali-
       phatic Polycarbonates as Tailored Tissue Scaffold Materials. *Biomaterials*.
       2007;28:2211-2219. DOI:http://dx.doi.org/10.1016/j.biomaterials.2007.01.024.

[69]   Gomes M, Malafaya P, Reis R: Methodologies for Processing Biodegradable and Nat-
       ural Origin Scaffolds for Bone and Cartilage Tissue-Engineering Applications. In *Bio-
       polymer Methods in Tissue Engineering*, A. Hollander and P. Hatton, (eds.). 2004,
       Humana Press. p. 65-75.

[70]   Ma P X: Biomimetic Materials for Tissue Engineering. *Adv Drug Delivery Rev*. 2008;60:184-198. DOI:http://dx.doi.org/10.1016/j.addr.2007.08.041.

[71]   Ma P X: Scaffolds for Tissue Fabrication. *Mater Today*. 2004;7:30-40. DOI:http://dx.doi.org/10.1016/S1369-7021(04)00233-0.

[72]   Changsarn S, Mendez J D, Shanmuganathan K, Foster E J, Weder C, Supaphol P: Biologically Inspired Hierarchical Design of Nanocomposites Based on Poly(ethylene oxide) and Cellulose Nanofibers. *Macromol Rapid Commun*. 2011;32:1367-1372. DOI: 10.1002/marc.201100183.

[73]   Huang J, Liu L, Yao J: Electrospinning of Bombyx mori Silk Fibroin Nanofiber Mats Reinforced by Cellulose Nanowhiskers. *Fibers Polym*. 2011;12:1002-1006. DOI:10.1007/s12221-011-1002-7.

[74]   Lee J, Deng Y: Increased Mechanical Properties of Aligned and Isotropic Electrospun PVA Nanofiber Webs by Cellulose Nanowhisker Reinforcement. *Macromol Res*. 2012;20:76-83. DOI:10.1007/s13233-012-0008-3.

[75]   Jia B, Li Y, Yang B, Xiao D, Zhang S, Rajulu A V, Kondo T, Zhang L, Zhou J: Effect of Microcrystal Cellulose and Cellulose Whisker on Biocompatibility of Cellulose-Based Electrospun Scaffolds. *Cellulose*. 2013;20:1911-1923. DOI:10.1007/s10570-013-9952-0.

[76]   He X, Xiao Q, Lu C, Wang Y, Zhang X, Zhao J, Zhang W, Zhang X, Deng Y: Uniaxially Aligned Electrospun All-Cellulose Nanocomposite Nanofibers Reinforced with Cellulose Nanocrystals: Scaffold for Tissue Engineering. *Biomacromolecules*. 2014;15:618-627. DOI:10.1021/bm401656a.

[77]   Dugan J M, Collins R F, Gough J EEichhorn S J: Oriented Surfaces of Adsorbed Cellulose Nanowhiskers Promote Skeletal Muscle Myogenesis. *Acta Biomater*. 2013;9:4707-4715. DOI:http://dx.doi.org/10.1016/j.actbio.2012.08.050.

[78]   Park D J, Choi Y, Heo S, Cho S Y, Jin H-J: Bacterial Cellulose Nanocrystals-Embedded Silk Nanofibers. *J Nanosci Nanotechnol*. 2012;12:6139-6144. DOI:10.1166/jnn.2012.6371.

[79]   Xiang C, Joo Y L, Frey M W: Nanocomposite Fibers Electrospun from Poly(lactic acid)/Cellulose Nanocrystals. *J Biobased Mater Bioenergy*. 2009;3:147-155. DOI:10.1166/jbmb.2009.1016.

[80]   Shi Q, Zhou C, Yue Y, Guo W, Wu Y, Wu Q: Mechanical Properties and In Vitro Degradation of Electrospun Bio-nanocomposite Mats from PLA and Cellulose Nanocrystals. *Carbohyd Polym*. 2012;90:301-308. DOI:http://dx.doi.org/10.1016/j.carbpol.2012.05.042.

[81]   Li Y, Ko F KHamad W Y: Effects of Emulsion Droplet Size on the Structure of Electrospun Ultrafine Biocomposite Fibers with Cellulose Nanocrystals. *Biomacromolecules*. 2013;14:3801-3807. DOI:10.1021/bm400540v.

[82] Zhou C, Shi Q, Guo W, Terrell L, Qureshi A T, Hayes D J, Wu Q: Electrospun Bio-Nanocomposite Scaffolds for Bone Tissue Engineering by Cellulose Nanocrystals Reinforcing Maleic Anhydride Grafted PLA. *ACS Appl Mater Interf*. 2013;5:3847-3854. DOI:10.1021/am4005072.

[83] Sheng L, Jiang R, Zhu Y, Ji Y: Electrospun Cellulose Nanocrystals/Polycaprolactone Nanocomposite Fiber Mats. *J Macromol Sci*, Part B. 2013;53:820-828. DOI: 10.1080/00222348.2013.861311.

[84] Zoppe J O, Peresin M S, Habibi Y, Venditti R A, Rojas O J: Reinforcing Poly(ε-caprolactone) Nanofibers with Cellulose Nanocrystals. *ACS Appl MaterInterf*. 2009;1:1996-2004. DOI:10.1021/am9003705.

[85] Zhou C, Chu R, Wu R, Wu Q: Electrospun Polyethylene Oxide/Cellulose Nanocrystal Composite Nanofibrous Mats with Homogeneous and Heterogeneous Microstructures. Biomacromolecules. 2011;12:2617-2625. DOI:10.1021/bm200401p.

[86] Park W-I, Kang M, Kim H-SJin H-J: Electrospinning of Poly(ethylene oxide) with Bacterial Cellulose Whiskers. Macromol Symp. 2007;249-250:289-294. DOI:10.1002/masy.200750347.