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Nanocomposites in 3D Bioprinting for Engineering Conductive and Stimuli-Responsive Constructs Mimicking Electrically Sensitive Tissue

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Rebuilding damaged or diseased tissue by means of biological additive manufacturing has recently gained lot of attention and shown very promising result. Using biofabrication techniques to mimic and replicate natural tissue as well as cell environment is a very capable way to achieve physiologically relevant conditions. Especially in electrophysiological human tissue like cardiac or neural tissue, proper signal transduction is of paramount importance for appropriate function and cell maturation as well as differentiation. Precisely, these conductive properties are challenging to engineer. However, a lot of outstanding work has been done recently. Therefore, this review focuses on additives, i.e., nanocomposites with intrinsic conductive properties, to the usually nonconductive hydrogels used in 3D-bioprinting. Recent work on exploiting the properties of these nanocomposites, such as metal nanoparticles (NPs), carbon nanotubes (CNTs), graphene, or MXenes, to alter the nanoenvironment of the manufactured construct toward conductive tissues is presented. An overview of responsiveness to external stimuli, a second intrinsic property of such nanocomposites is provided as well. Furthermore, these materials are critically analyzed concerning their electrophysiology, i.e., cell–scaffold interaction, their biocompatibility as well as their toxicological properties.

1. Introduction

Additive manufacturing (AM) using computer-assisted layer-by-layer material deposition is becoming an influential field in biological engineering and regenerative medicine. It holds the potential to regenerate or replace damaged tissue in order to help overcome organ failures and organ scarcity. In bio AM or biofabrication, biological material is deposited three dimensionally in a precise and efficient way. Custom-designed shapes, patterns, and architecture can be prepared, replicating biological tissue-level architecture. For biological engineering, a hydrogel is printed either with or without encapsulated cells. These hydrogels are a network of hydrophilic polymers, able to swell in water like the native tissue extracellular matrix (ECM),[1–3] and are the basic building blocks of defined 3D structures. Printed material composed of hydrogel with cells, is called bio-ink. But the cells do not necessarily have to be encapsulated before printing the construct. For a functional biofabricated 3D construct, cells can be seeded or grown into a previously printed design.[4]

3D bioprinting uses several technical solutions to print a defined pattern. Commonly used techniques are bioblotting (i.e., direct extrusion printing, direct dispensing), ink-jet printing, melt electro writing (MEW), solution electro writing (SEW), and electrospinning. Less commonly used or yet emerging techniques in bioprinting, are photo-curing 3D printing techniques like stereo lithography appearance (SLA), digital light processing (DLP), multijet printing (MJP),

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/anbr.202100108.

DOI: 10.1002/anbr.202100108

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continuous liquid interface production (CLIP), and two-photon 3D printing (TPP).[5] Recently, promising holographic or tomographic bioprinting technology have also emerged.[6,7] Each of those techniques has its advantages and disadvantages in terms of production time, resolution, shape fidelity, and cell viability.[8,9] While photo curing techniques are performing better in the domain of production time and resolution, the extrusion-based technologies outperform them in terms of simplicity, commercial availability, and slightly on cell viability, although later has recently been improving. Therefore, the main focus of this review lies on the blotting techniques since at present they are more used and relevant in the field of 3D bioprinting.

In recent years, a huge amount of work has been done to improve the printing system to increase printing resolution while keeping manufacturing time short and decreasing cell stress during the process. Hardware development has not been the only area of intense investigation however. Hydrogel amendment is also an important branch of biofabrication.[10–12] The ultimate goal in tissue engineering (TE) is to close the gap between hydrogels and natural tissues by exploiting hydrogel's favorable properties. Excellent formulations have to be developed which mimic the physiological cell environment of natural tissues, such as conductivity, microstructure, and mechanics. Enhancing these properties would facilitate adherence and infiltration of the printed construct with cells, its remodeling for correct tissue regeneration and full functional recovery. Hence, a number of natural as well as synthetic polymers for hydrogel preparation are used in bioprinting processes.[13]

In this review, we specifically focus on electrophysiological tissue, such as cardiac and neural tissue. Therefore, inducing electrical conductivity in mostly nonconductive hydrogels is a key feature in engineering cardiac patches or neuronal conduits. This inducement of conductivity within the hydrogel is achieved through nanosized additives, called nanocomposites. These nanocomposites, smaller than 100 nm, including metals, carbon-based materials and polymers, have shown to specifically enhance the conductivity in hydrogels, as well as playing an active role in altering the nanoenvironment of the manufactured construct. Due to their intrinsic conductivity, these nanoparticles (NPs) can make hydrogels electrically conductive and therefore susceptible to correct signal transmission. Work with conductive polymers (CP),[14,15] or inherently conductive nanocomposites, such as metal NPs (iron-oxide particles (IOP), Au-NP, or Ag-NP),[16–18] carbon nanotubes (CNTs),[19–21] graphene,[22,23] or MXenes[24,25] has already been carried out, showing exciting results for improved biofabrication approaches. Further advantages of these intrinsic conductive nanocomposite are their good biocompatibility and favorable toxicological properties. In addition, MXenes show increased water solubilization and flexibility making them excellent candidates for neural and cardiac TE (**Figure 1**). Despite these aspects, the exact mechanism of passive electric signal transduction between electroconductive engineered material and biological tissue has not yet been described in detail. Most literature take the transduction of electric to ionic current and vice versa, occurring at the cell–scaffold interface, as granted. Only recently discussions emerged about the mechanism of transduction between those two currents and their apparent nonequivalence.[26] However, mimicking the conductive system with nanocomposites, showed to promote signal transduction,[19] cell–cell interaction,[27] and cell maturation,[21,28] which are essential for a successful integration into the host tissue. Conversely, false integration can cause defects or, in the cardiac environment, arrhythmias.

Some of these nanocomposite materials have another intrinsic property which can be exploited: responsiveness to external stimuli. Stimuli-responsive hydrogels, so-called smart hydrogels, which directly respond to changes in environmental conditions,[29] are gaining more and more attention in biomanufacturing.[30–32] This remote stimulation gives additional control for even more precise construct manufacturing. The scope of those stimuli is wide and can induce several responses, such as deformation (e.g., folding, swelling, or twisting),[33] locomotion (e.g., rolling, walking, crawling)[34,35] or alignment/particle orientation.[36] Magnetic or electric fields show particularly strong potential in multiple applications. Extensive research has been done and the applied field can trigger a variety of nanocomposites, including biocompatible superparamagnetic iron oxide-based magnetic NPs or silicon NPs. Nevertheless, two main limitations still impede better implementation in printing processes: 1) long response time and 2) low control precision of stimuli-responsive smart biomaterial architecture's.

Apart from inducing electroconductivity into hydrogels by doping them with nanocomposites, other hydrogel properties can be enhanced, such as improved shape fidelity, guidance, and instruction of cells[37,38] and cell–cell interaction, e.g., in drug delivery systems.[39] Covering these topics is beyond the scope of this review however.

This review will highlight the role of nanocomposite-based hydrogels as a very promising way to rebuild natural tissues and achieve closer physiological conditions relevant for whole-heart printing or nervous system regeneration. First, we provide an insight into electrophysiological human tissue followed by an overview exclusively on nanocomposites that have been 3D bioprinted, with focus on 3D bioblotting, to induce electrical conductivity and stimuli responsiveness.

2. Tissue

Looking into the two main tissues with electrophysiological properties is of paramount importance, since the main focus of this review is on electroconductive hydrogels blended with nanocomposites for fabricating implantable substitutes. New developments in this area will allow crucial mimicking of the conductive system and therefore enable correct signal transduction and maturation of cardiac and neural tissues.

2.1. Cardiac Tissue

Cardiac tissue has one of the lowest turnover rates and therefore very low regenerative potential. It is estimated that 1% of cardiomyocytes at age 25 and 0.45% at 75 are turned over per year.[40,41] This illustrates that most cardiac tissue is lost after cardiac failures and regeneration is weak. These low turnover rates may be due to the very complex composition and function of cardiac tissue. The unique architecture of the heart comprises four distinct layers. The inner-most layer is the endocardium, on top of that is the myocardium and finally the epicardium;

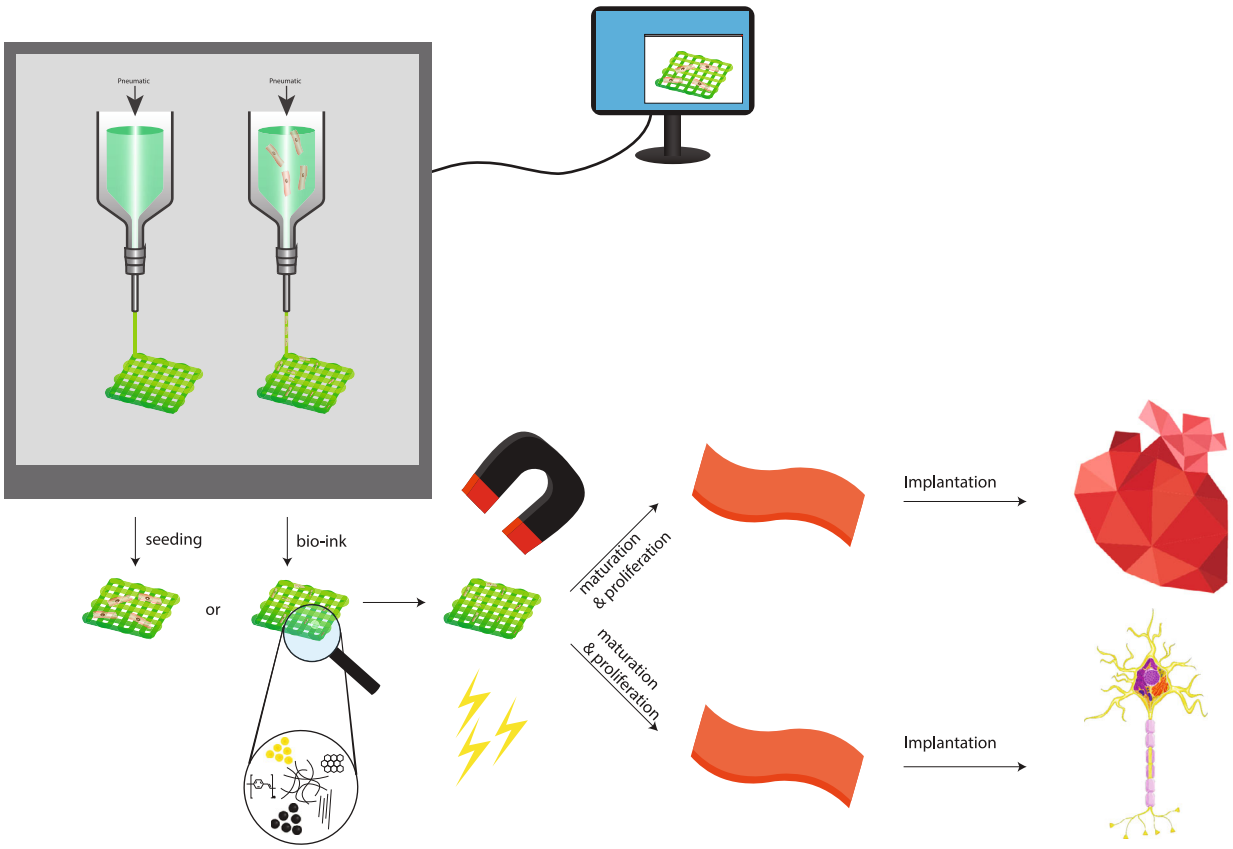

Figure 1. Schematic representation of a typical workflow of a biofabrication process to produce cardiac or neural constructs. Costum architecture is designed with a specific software and sent to the 3D-printing machine. The nanocomposite doped printed hydrogel, either already including cells or seeded on it after the printing process, is matured using electrical or magnetical cues and can be implanted into damaged or diseased cardiac or neural tissue.

together they form the heart wall. This heart wall is enclosed by a protective sac called the pericardium.[8] Different cell types are present in these layers: 1) Cardiomyocytes (CMs), the main functional building blocks of the myocardium; 2) fibroblasts, for homeostatic maintenance of the cardiac environment; and 3) endothelial cells, responsible for vascularization of the architecture. CMs are the main functional elements of the cardiac tissue which, together with other conductive cell types, such as pacemaker cells, enable the heart to propagate electrical stimuli in a synchronized pattern in order to pump blood. This functional system is called the cardiac conduction system. It connects the atria and ventricles of the heart and comprises the sinuatrial (SAN) and atrioventricular nodes (AVN), as well as the connection to the ventricles: the Purkinje fiber network (PFN).[42] The PFN consists of specialized CMs, which are physiologically closer to CMs than neural cells. Called Purkinje fibers, they receive signals from the sinuatrial node and transduce them to the CMs of the ventricles, causing tissue contraction (**Figure 2**A). Due to their higher resting potential (–60 mV) compared with normal CMs (–90 mV) they are more easily excitable, while not contracting themselves. The Purkinje fibers extend and propagate signals throughout the whole myocardium, allowing each CM to experience action potential.[43] Gap junctions between the contractile cells ensure the rapid propagation of the action potential through the whole muscle fiber network, which leads the myocardium to function as a single unit.[44]

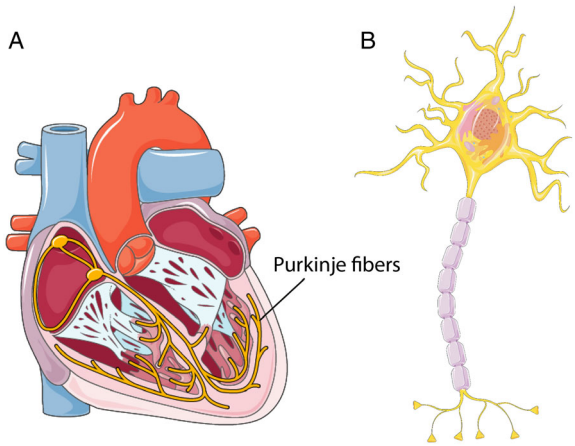

Figure 2. Schematic illustration of cardiac and neural conducting system. A) The syncytium ending in the Purkinje fibers allowes the synchronized contraction of CMs B) neurons with their axon and dendrites conduct the signal between neighbouring neurons. Illustrations adapted from Servier Medical Art, smart.servier.com.

Bundles of myofibrils within the CM cells build repeating units of sarcomeres which allow the contraction of a single cell. Mechanical force transmission is guaranteed by desmosomes anchoring CMs together, so that cells are not detached from one another while contracting. Electrical signals on the other hand are transmitted from one CM to a neighboring CM through special electrical junctions (e.g., connexin 43, Cx43).[45–47] This electronic coupling of the cells within the myocardium to form a functional unit, is called syncytium, and is an important key in mimicking and biofabricating cardiac tissue.

Conductive hydrogels are therefore a powerful instrument in the toolbox of tissue engineers in order to mimic this conductive system and allow accurate, rapid signal transduction within the engineered patch/graft. Using nanocomposites in the hydrogel enables conductive inks for 3D-bioprinted constructs.

The application of cardiac patches in regenerative medicine has the potential of big impact on curing ischemic injuries. Due to the aforementioned lack of good regenerative ability of the cardiac tissue and being one of the most common causes of death in the world, engineered heart patches could be beneficial to regain healthy tissue. Implanted onto or replacing diseased tissue these biomaterials can promote normal cardiac function, i.e., cardio myogenesis, maturation, and electrophysiology, leading to fully functioning human hearts. In particular, conductive nanocomposites aim to mimic the Purkinje fiber network and thereby promote an advanced cardiac tissue maturation in vitro. Mature tissues are more prone to readily taking up the tissue function.[48,49]

2.2. Neural Tissue

Similar to cardiac tissue, the human nervous system has a limited intrinsic capacity for self-regeneration, especially the central nervous system (CNS). While the peripheral nervous system (PNS) is able to regenerate smaller injuries autonomously, CNS injuries do not regenerate in their native environment.[50–52]

The nervous system, responsible for coordinating all the actions and sensory information of the body, transmits this information via electrical and chemical signals. This synaptic transmission depends on two cell types, neurons (Figure 2B) and neuroglia. Neurons, the functional element of the nervous system, consisting of a cell body (soma) and its extensions, axons and dendrites. Axons transmit the signal between individual cells while dendrites transmit them to the cell body. This transmission among neurons depends on depolarizing the resting potential (–90 mV) of the target neuron, generating an action potential which propagates within this cell.[53]

Neuroglia or glial cells are the supporting cells of the nervous system. They provide homeostatic maintenance, protection, and insulation for the neurons. In the PNS, sheaths of Schwann cells surround the axons, while in the CNS, myelin sheaths wrap around axons. This wrapping leads to faster and more efficient signal propagation within the axons.[54,55]

Electrochemical impulses are the primary pathway of intercellular communication and information transduction. Therefore, due to their electrophysiological behavior, conductive nanocomposite hydrogels are being used more and more in neural TE approaches to support nervous system regeneration. Studies using nanocomposite doped hydrogels[56,57] demonstrate boosting of neuronal electric signaling, leading to increased natural tissue resemblance. Additionally, improved growth, differentiation, and cell adherence is also shown.[58–61]

All these advantages demonstrate the clinical impact of such printed constructs and is similar to the abovementioned clinical impact of cardiac patches. Novel ways to regenerated injured or diseased neural tissue is of great interest. Implanted onto or replacing diseased human tissue such printed bio scaffolds can promote normal neural function leading to fully working nervous system again. Similar to cardiac tissue the conductivity introduced by the nanocomposites may promote an advanced tissue maturation in vitro and thus be beneficial for rapidly taking up the function after implantation.

3. Nanocomposites

Adding nanocomposites is a very promising way to address the challenges of next-generation hydrogels. Their good biocompatibility and intrinsic properties make them a preferred application for 3D printing. Nanocomposites bring several advantages, especially for engineering and replacing electrophysiological tissue.

Structures such as particles or fibers are considered to be nanometer scale if their size is smaller than 100 nm.[62] In 3D bioprinting, the range of nanocomposites used is wide. They range from particle structures such as iron[63] (**Figure 3**A), gold[28] (Figure 3B), or silicon[64] NPs, to tubular structures, e.g., CNTs[65] (Figure 3C), sheet structures such as graphene[66] or even wire/fiber structures.[67] CNTs are some of the most commonly used NPs in 3D-bioprinted hydrogels, either in a single-walled (SWCNT) or multiwalled (MWCNT) configuration.[68] CNTs display high biocompatibility, which can be increased by CNT surface functionalization and, due to their high aspect ratio, high cell interactivity. With their outstanding electrical capabilities, they are used in both neural[60] and cardiac[65] TE. Graphene, considered the strongest, thinnest material ever reported,[69] also shows favorable electrical, toxicological, and mechanical properties. Furthermore, graphene enhances the differentiation of embryonic stem cells toward cardiomyogenic lineages,[70] maturation into adult CMs and the cellular response between the construct and the cells.[71]

MXenes, first described in 2011 by Barsoum and coworkers,[24,72] is a material consisting of few atoms thick layers of transition metal carbides, nitrides, or carbonitrides with the following formula: Mn + 1Xn, where M is an early transition metal, and X is C or N.[24,25,73,74] Titanium carbide (Ti_3C_2) is the most commonly investigated type. MXenes have several advantages over other metallic meshes, including biocompatibility, good water solubilization, and high flexibility. Nevertheless, research into these materials is preliminary and further studies have to be done.[75] Not only particles, etc., are considered to be nanocomposites in hydrogels however. CPs are also used in hydrogel enhancement, especially for cardiac TE[76] although 3D bioprinting including CPs are very rare up till now. Mostly CPs are used in 2D hydrogels[77,78] and electrospinning processes[79] with subsequent cell seeding. Therefore, only a small focus lies on these CPs later in this review. For further reading into CPs, Distler et al.[80] and Solazzo et al.[76] are

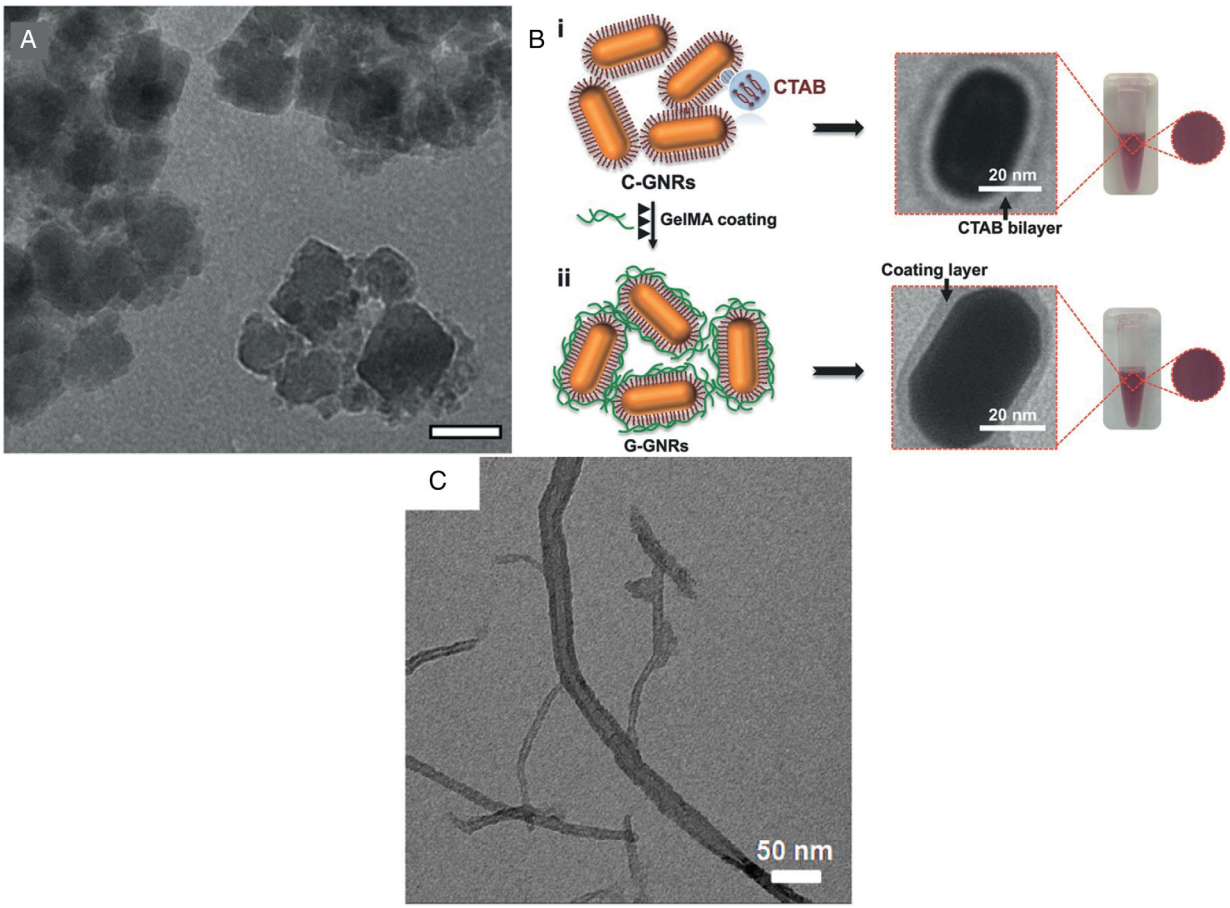

Figure 3. A) TEM image of square-shaped IOPs. Scale bar = [20] nm. Reproduced with permission.[63] Copyright 2019, Wiley-VCH B) Schematic and TEM image of gold-nano-rods (GNRs) coated with GelMA. Reproduced with permission.[28] Copyright 2017, Wiley-VCH C) HRTEM images of bare CNTs used for 3D-bioprinting. Reproduced with permission.[88] Copyright 2012, American Chemical Society.

recommended since they provide very good in-depth reviews on CP based nanocomposites.

While working with nano-structures an important issue has to be taken into account: they should not negatively interfere with the hydrogel's biocompatibility. Furthermore, pharmacokinetics/toxicokinetics as well as metabolism or biodegradation of the nanocomposites are not yet clear but are of paramount importance in TE applications. Many materials show excellent biocompatibility and cell viability and several NPs are already being approved by the Food and Drug Administration (FDA). It is known that particles are either removed by the liver[81,82] or the kidney,[83] depending on the particle size. Particles larger than [5] μm are cleared by the lymphatic system.[84] Between [5.5] nm and [200] nm they are sequestered by phagocytes[85] and below this value they are quickly discharged by the kidney.[83] Nonetheless, long-term fate studies are lacking and these issues have to be taken into consideration when working with nanocomposites in regenerative medicine and TE.

Among a very broad range of available nanocomposites, the following sections focus merely on composites, which are nanometer sized, 3D bioprinted and targeted for applications in cardiac and neuronal TE. Additionally, the selected nanocomposites should also have a high intrinsic conductivity and biocompatibility.

3.1. Inducing Conductivity in 3D-Bioprinted Hydrogels with Nanocomposites

As mentioned previously, human tissues with electrophysiological properties such as cardiac or neural tissue lack substantial regenerative potential. This is especially the case for cardiac cells. Consequently, the engineering of patches for cardiac ischemia or neural conduits for neural damage is an important branch of regenerative engineering. Since, in the aforementioned tissues, signal transduction and electrical conductivity is fundamental, ways of creating electric conductive hydrogels are very important in order to mimic native tissue more closely. Researchers are using electrical conductivity nanocomposites such as fibers, rods, sheets, tubes and wires to expand the scope of hydrogel applications.

3.1.1. CNTs

CNTs are among the most commonly used nanocomposites in 3D-bioprinting, either in a single walled (SWCNT) or multi walled (MWCNT) configuration.[68] They have excellent electrical capabilities,[86,87] good biocompatibility (which can even be

increased by CNT surface functionalization), and high cell interaction due to their high aspect ratio, all of which make them interesting candidates for enhancing hydrogels.[60] Shin et al. are using CNTs as an addition to their hydrogel. They show that extracellular matrix (ECM) mimicking gelatin methacrylate (GelMA) hydrogels doped with CNTs exhibit better proliferation of human mesenchymal stem cells (hMSCs) compared to a control and good spreading of these cells in the construct. Cell viability was in all tested conditions (up to [0.5] mg/mL) higher than 90% after 48 h in culture.[65] In a further study, GelMA-CNT constructs enhance electrophysiological functions, proliferation and maturation of seeded myocardial tissue. Seeded neonatal rat CMs showed higher spontaneous synchronous beating rates (3 times higher than control) and lower excitation thresholds (85% lower than control), leading to the assumption that GelMA-CNT hydrogels are enhancing cardiac cell adhesion, cell-cell electrical coupling and organization[88] (**Figure 4**). Alongside these findings, higher mechanical strength than pristine GelMA is reported as well, leading to the assumption that tuning the mechanical properties of the hybrid material is possible, making it suitable for adjustments in TE applications. This could lead to higher shape fidelity and resolution, which ultimately leads to more complex 3D-bioprinted constructs.

Kelly et al. biofabricated a reinforced cardiac patch with CNTs. Over the course of the study the hydrogel presented improved viscoelastic and electrical behaviour significantly. Human coronary artery endothelial cells (HCAECs) showed enhanced cellular migration and proliferation after ten days in culture.[19]

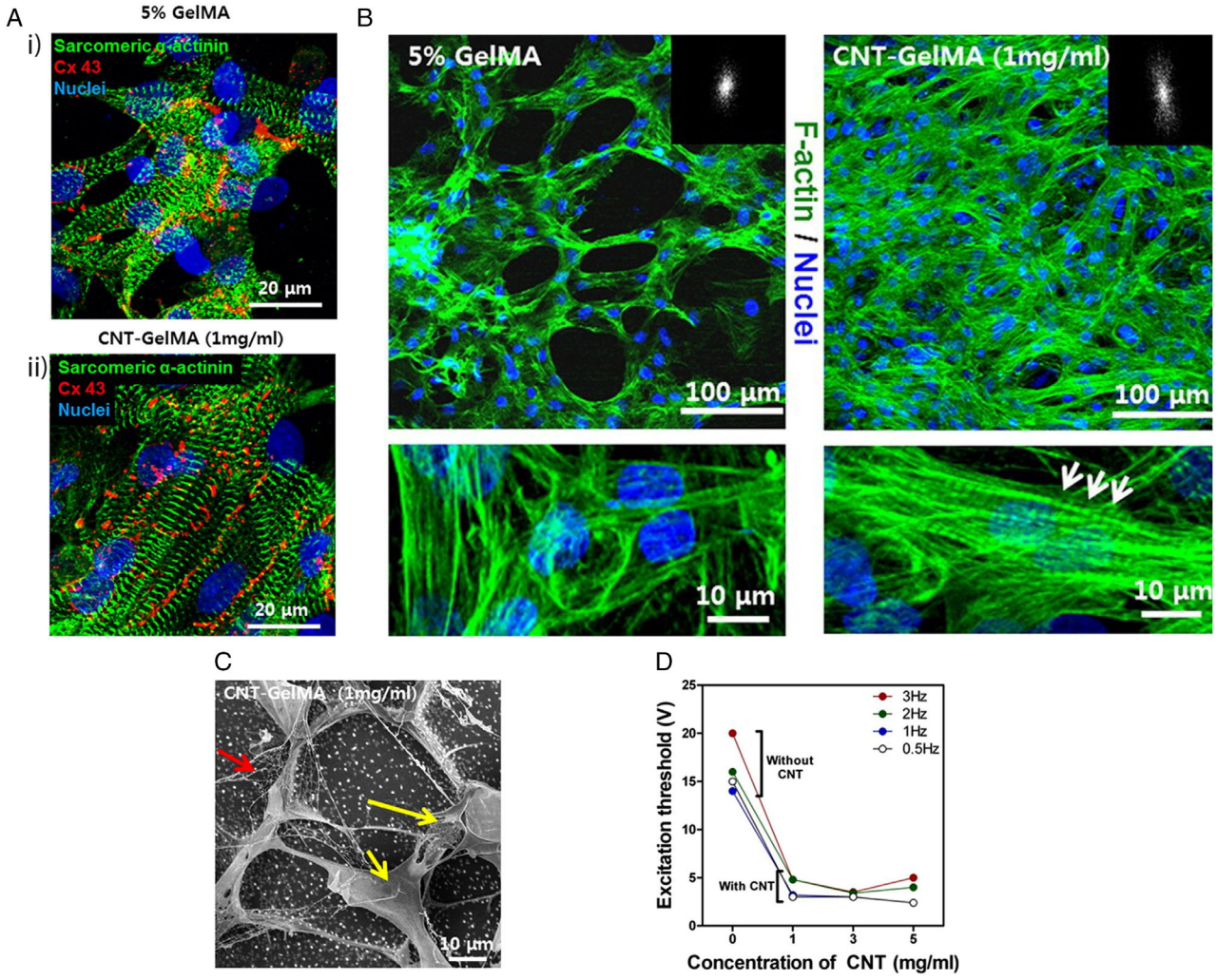

Figure 4. A) CM phenotype cultured for 8 days on GelMA alone (i) or CNT-GelMA (ii) hydrogels. More robust intercellular junctions and uniaxial sarcomere alignment reveal phenotypical differences in favour of CNT-GelMA hydrogels. Reproduced with permission.[88] Copyright 2013, American Chemical Society. B) Confocal images of CMs after culturing for 5 days on pristine GelMA and CNT-GelMA reveal improved CM cell adhesion, maturation and alignment on CNT-GelMA. FFT insets show higher alignment. Higher magnification images display elongated CMs and F-actin cross-striations (bottom right, white arrows) on CNT-GelMA but not on GelMa alone (bottom left). Reproduced with permission.[88] Copyright 2013, American Chemical Society. C) SEM images showing morphology of cardiac cells cultured on CNT-GelMA. Yellow arrows indicate cell bodies. Red arrows indicate cytoplasmic prolongations adhering to CNT fibers. Reproduced with permission.[88] Copyright 2013, American Chemical Society D) Excitation threshold of CMs on CNT-GelMA displays 85% lower threshold compared to CMs on pristine GelMA. Reproduced with permission.[88] Copyright 2013, American Chemical Society.

Wu et al. used CNTs to mimic the anisotropic cardiac structure more closely. Use of CNTs in an interwoven yarn within a hydrogel with seeded CMs promoted alignment and elongation of CMs. The nucleus aspect ratio, a quantitative analysis of cell elongation, was increased from 1.2 to 2.3 between the control and the CNT yarn 3D construct. The alignment index (cells within ±10°) increased from 13% to 82%.[27] This process ultimately led to better CM maturation and cell-cell coupling within this artificial 3D-bioprinted construct, since Cx43 and sarcomeric α-actinin are expressed significantly higher than in the control group.

Other studies, by Koppes et al. and Zhang et al., using SWCNT/MWCNT as additional composite in hydrogels indicate neurite outgrowth and neural stem cell (NSC) differentiation into cortical neurons after electrical stimulation of the conductive hydrogel. Koppes et al. came up with a 1.7 fold higher bulk conductivity and a 3.3 fold bigger neural outgrowth in SWCNT hydrogels compared to nanocomposite free hydrogels. With electrical stimulation this difference could be increased up to a 7.0 fold greater outgrowth.[89] Zhang et al. reported an increase of average neural outgrowth of 28% compared to neurons in nanocomposite free hydrogels. Moreover, electrical stimulation of NCS in MWCNT hydrogel constructs shows promotion of their neuronal differentiation, which was measured by the quantitative analysis of the upregulation of several neuromarkers.[22]

Overall, the favorable properties of CNTs, such as exceptional electrical conductivity paired with good biocompatibility make them fitting candidates for 3D-bioprinting. The addition of CNTs to cell-laden bioinks is reported to be beneficial for cellular maturation of both cardiac and neural cells. Therefore, inducing electroconductivity using CNTs opens new paths for regenerative applications in medicine, by attempting to mimic native tissue more closely. A further benefit, in addition to the altered electrophysiology, is the reported advantageous modification of a hydrogel's mechanical properties, i.e., increasing shear thinning and shape fidelity. Nevertheless, open questions remain regarding the low control of dispersing these particles in viscous polymer solutions,[90] shelf life, suspension stability or the long term-toxicity.[91]

3.1.2. Graphene

Like CNTs, graphene possesses inherently high conductivity and favorable toxicological properties.[80] Considered to be the strongest and thinnest material ever reported,[69] it has further advantageous qualities. Using graphene as a substrate is reported to stimulate differentiation of stem cell cultures and several studies have shown that this substrate exhibits stimulatory effects on multipotent adult stem cell lineage specification. Human embryonic stem cell (hESC) differentiation into CMs,[70] human neural stem cell (hNSC) differentiation into neural cells[92] and mesenchymal stem cell (MSC) differentiation into osteogenic cells[93] are all documented as being at least partially enhanced by graphene. These results were used in several 3D-bioprinting applications for cardiac regeneration. O'Brien et al. blended collagen hydrogels with graphene. In the 32 wt% mixture they showed improved electrical conductivity by 6.2 fold to [0.65] S/m and higher mechanical stability, mimicking native tissue better. Electrical stimulation of the seeded cells improved ESC-CMs alignment and maturation within the construct. Moreover, growth of seeded human cardiac fibroblasts was enhanced while metabolic activity (45% higher) and sarcomeric development of ESC-CMs seems to be boosted[71] (**Figure 5**).

In various recent studies graphene-oxide (GO) was used because of its oxygen rich surface, resulting in higher water solubilization.[66] Results from CM seeding, hMSCs or endothelial cells showed improved cardiac cell maturation, cell-cell electrical coupling and organization. This was indicated by synchronized cell beating and upregulated cardiac markers, like sarcomeric α-actinin. Again, it was shown that hydrogels exhibit better mechanical stability (higher elastic modulus, resistance to rotational stress and improved electrical conductivity) leading to higher printing resolution.[94,95]

Shah et al., using graphene in neural TE approaches, report significantly enhanced cell adhesion, proliferation and neurogenic differentiation of hMSCs. Proliferation of these cells on hydrogels without graphene was 2 to 3 times lower than on graphene dotted hydrogels and after 14 days of culture expression of neurogenic relevant genes was upregulated between 2 to 6 times compared the expression levels of unseeded hMSC at day 0[96] (Figure 5). Additionally, Mallapragada et al. report increased nerve growth factor secretion in gelatine and graphene based 3D-printing applications.[97]

Overall, graphene-based engineered materials have the potential to mimic cardiac and neural tissue more closely. The aforementioned findings of these graphene biohybrid platforms are also promising for a plethora of other applications, particularly in electrically sensitive tissues. Additionally, they broaden the scope of regenerative medical applications, improving the therapeutic approach in cardiac ischemia or neural degeneration. The same open questions as for CNTs can be stated about the limitations of using graphene. Follow up studies on the long-term toxicity have to be done as well as the exact mechanism of interfacial interactions between cells and scaffold.[98]

3.1.3. Conductive Polymers

CPs have attracted a lot of attention since their discovery some 40 years ago, including a Nobel Prize in Chemistry, in 2000. The basis for their intrinsic electrical properties lies in the formation of a π-system created by unoccupied *p*-orbital electrons.[99] In addition to excellent electric conductivity, they possess good biocompatibility, making them available for bioengineering purposes. Compared with structure-like nanocomposites (nanoparticulate phases) however, CPs need additional steps if they are to be integrated into printable hydrogels. Depending on the CP used, synthesis or polymerization steps within the hydrogel are required after blending. Furthermore, washing steps to eliminate unreacted monomers or oxidizers are necessary, unlike with structures such as nanocomposites, which only have to be blended uniformly into the hydrogel without any further post-blending steps.[80]

Regardless of their beneficial electrical properties, CPs in 3D-bioprinting are not yet frequently used. Richter-Dahlfors et al.[100] reported improved cell adhesion on poly(3,4-ethylenedioxythiophene) (PEDOT)-doped substrate and Redenti et al.[101] showed

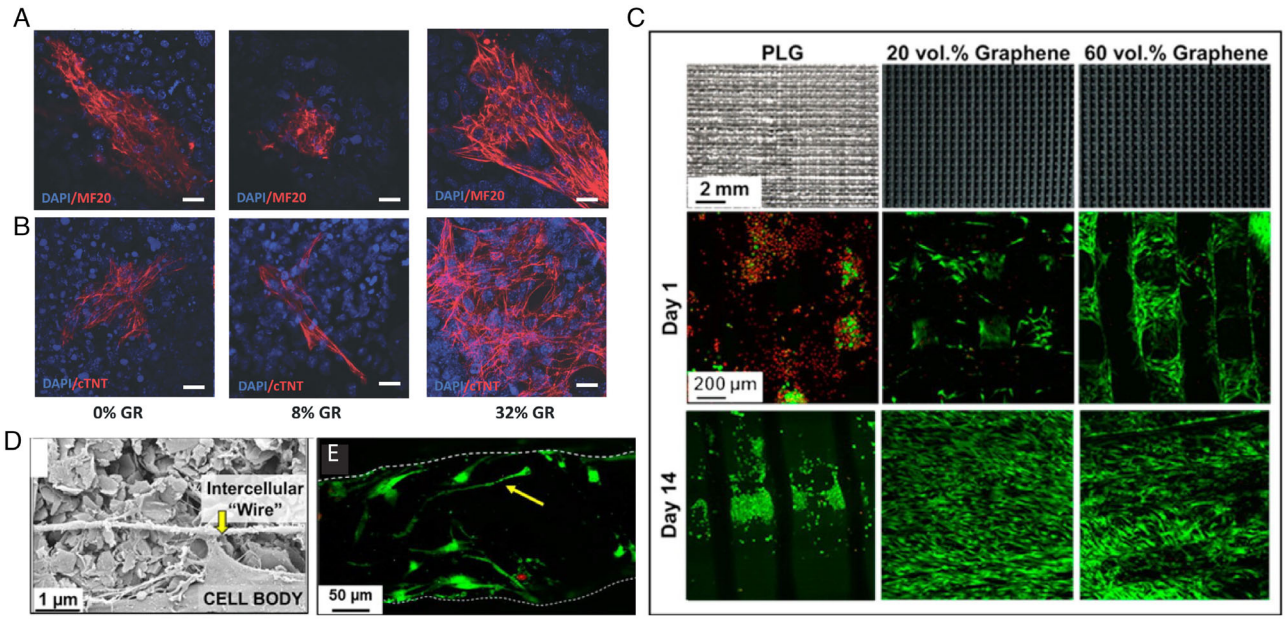

Figure 5. A) and B) Immunofluorescent staining of CM troponin and sarcomeric myosin. While in PLG hydrogels doped with 8% graphene poorly formed and randomly aligned striations were found, in 32% significantly enhanced cross-striated sarcomeric structures and alignment was present. Reproduced with permission.[71] Copyright 2018, Wiley-VCH. C) Photographs (upper row) and scanning laser confocal 3D reconstruction (lower rows) of live stained (green) and dead stained (red) hMSCs on pristine PLG or PLC with various graphene doping at day 1, 7 and 14 after seeding on construct. Reproduced with permission.[96] Copyright 2015, American Chemical Society. D) High magnification SEM micrograph of cells at day 7 on 60% graphene scaffolds. Reproduced with permission.[96] Copyright 2015, American Chemical Society. E) Scanning laser confocal 3D reconstruction of CMs at day 14 on 60% graphene scaffolds. Reproduced with permission.[96] Copyright 2015, American Chemical Society.

an influence on cell differentiation on polypyrrole substrates. These findings may be useful for future applications in electrophysiological TE using 3D-bioprinting approaches.

For additional information on the working principle of CPs and their use in bioengineering applications, which are not limited only to 3D-bioprinting, the recent reviews of Distler et al.[80] and Solazzo et al.[76] are recommended.

3.2. Inducing Conductivity and Stimuli Responsiveness in 3D-Bioprinted Hydrogel with Nanocomposites

3.2.1. NPs, Nanowires, and Nanorods

Using NPs and nanowires (NWs) in 3D-bioprinting applications is still in its infancy. For biological implementation, NPs and NWs made of silicon or iron are the current state-of-the-art. Their intrinsic conductivity is accompanied by an intrinsic magnetism, making them available for stimuli-responsive 3D-printing approaches. This means that the printed constructs are able to react to externally applied stimuli and are consequently able to change functionality or shape.[102] Being flexible and nonstatic brings the construct closer to the native environment where dynamics and biological turnover play a key role. Furthermore, these intrinsic conductive capabilities and magnetic properties can be exploited simultaneously. For silicon and iron NPs and NWs, their magnetism is exploited most frequently but, depending on the nanocomposite used, the printed hydrogels can be responsive to light, charge, pH, stress, or temperature.[103–108] In other words, using "time" as the fourth dimension expands the tool set in biofabrication.

In a recent study using iron oxide nanoparticles (IOPs), Zwi-Dantsis et al. reported the controlled orientation of CMs within a 3D collagen hydrogel construct by applying an external magnetic field. These patterned constructs are viable and functional, i.e., CMs exhibit normal cardiac functions after implantation onto rat hearts. Moreover, it is a relatively simple approach for the reproduction of cellular organization[16] (**Figure 6**). Tognato et al. showed the same alignment of encapsulated C2C12 skeletal myoblasts to aligned IOPs. Furthermore, measuring higher expression of myosin heavy chain (MyHC)and immunofluorescence analysis of C2C12 cells in this IOP constructs, indicates an enhanced myotube organization. This influence on cell morphology could be very important to induce maturation of well-organized muscle tissue.[63]

Recent development in non-3D-printing electroconductive biomaterials engineering using NPs and NWs pave the way for applications in bioprinting. Tan et al. showed improved electrical conductivity from 0.001–0.1 to 150–500 $\mu S\ \mu m^{-1}$ and accelerated structural and functional human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC)-CMs development in spheroids using silicon NWs. These NWs can therefore be used to improve the therapeutic importance of tissue-engineered patches.[109]

Au NPs show similar hydrogel enhancements. Hosoyama et al. report better conductivity (320-fold increase compared with the control) of Au NP incorporated collagen hydrogel and increased Cx43 expression in cardiac patches.[110] Meanwhile,

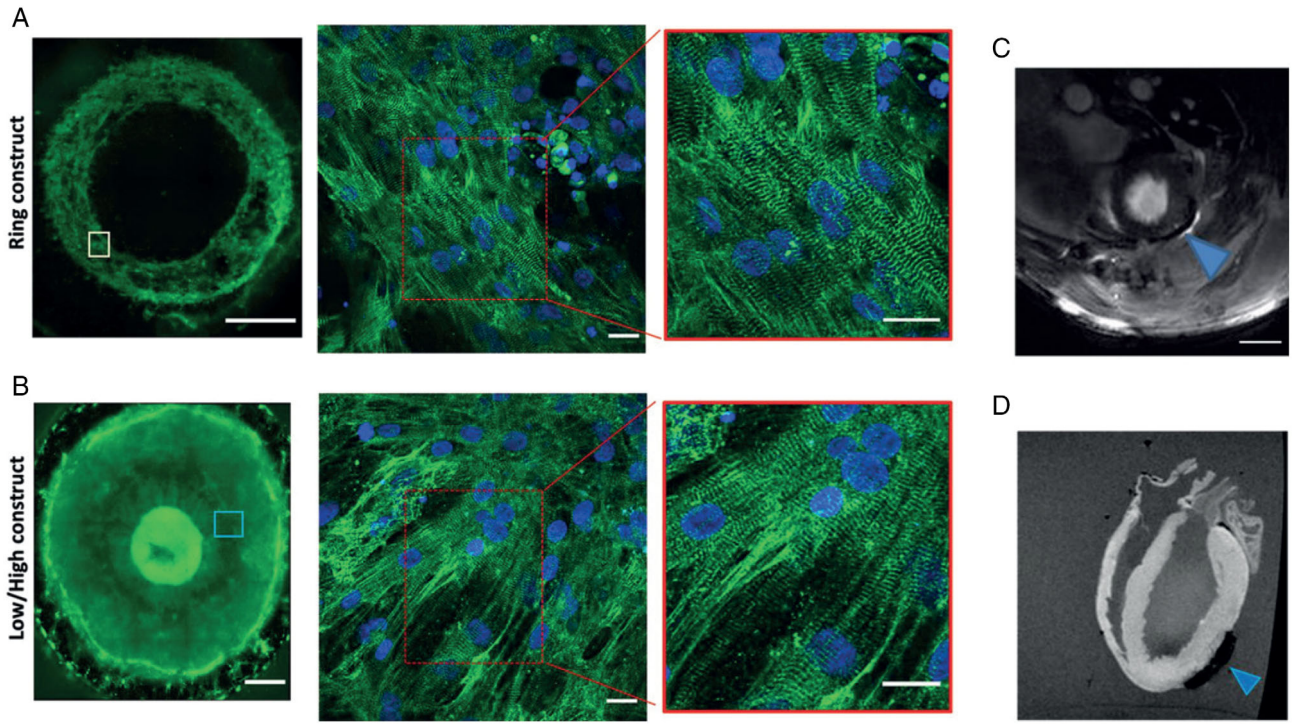

Figure 6. Structural characterization of two different construct created by external magnetic stimuli. A) showing the immunostaining of α-actinin (green) of a ring-shaped CM hydrogel and B) showing a low/high density CM hydrogel. DAPI (blue) staining of nuclei. D) In vivo MRI image of magnetic NP-labeled cardiac hydrogels attached to rat heart epicardium 2 days after implantation and E) ex vivo 8 days after implantation respectively. The arrowheads indicate the cardiac hydrogel location. Reproduced with permission.[16] Copyright 2020, Wiley-VCH.

You et al. characterized tunable hydroxyethyl methacrylate (HEMA) hydrogels with doped Au NPs. Likewise, they reported increased expression of cardiac markers in neonatal rat CMs. Cx43 was observed to be 2-fold higher in stimulated scaffolds compared with nonstimulated nonconductive ones. And even without electrical stimulation the Cx43 expression of cells seeded on electroconductive hydrogels was 60% higher than on nonconductive hydrogels.[18] In another study, gold nanorods in a GelMA bioink improved cell–cell electronic coupling and promoted synchronized contraction of the printed construct seeded with CMs. Cardiac marker expression like Cx43 and troponin I was both increased.[28]

Dvir et al. state the improved electrical conductivity of alginate constructs by incorporating gold NWs, enhancing electrical cell-cell interaction between adjacent seeded cardiac cells. These cells show better alignment and a higher level of cardiac markers such as α-actinin and Cx43.[17]

Generally speaking, it can be said that NPs and NWs are already widely used in nonprinted 3D hydrogel constructs despite their relatively short history. Conversely, in 3D-printing applications, their use is still very limited. The abovementioned studies indicate however, that these nanocomposites add favorable properties to hydrogels, making them available for printing applications in electroconductive TE. These favorable properties range from higher electroconductivity, to better alignment and increased expression of molecular markers. Additionally, the intrinsic magnetic properties of IOPs make them available for external stimuli, surmounting the static nature of classic 3D-bioprinted constructs. This brings the constructs closer to natural tissue and hence, widens the potential range of applications. Nonetheless, in particular for NWs and nanorods more work has to be carried out to precisely assess the following challenges for 3D bioprinting: 1) influence of these additives on cell viability during printing, 2) difficulty to disperse them uniformly in the hydrogel, 3) limited knowledge on the long-term toxicological effects on living tissue.

4. Conclusion

In the past few years, tremendous progress has been made in the field of 3D bioprinting in order to create biologically engineered 3D constructs which can be used in therapeutic applications, e.g., as a replacement for damaged native human tissue. Recently, the blending of hydrogels or cell-laden bioinks with nanocomposites has shown favorable impact on the desired final construct and increased the resemblance to native tissue. These nanocomposites are manifold and can be particle structures, tubular, or sheet structures or wired/fibrous structures. Improving conformity with natural tissue can be achieved in many ways but in the context of this review, mimicking the electroconductive physiology of neural or cardiac tissue is of primary interest. Since hydrogels are not intrinsically conductive, this property can be integrated by doping with nanocomposites. It is increasingly evident that electroconductive 3D-bioprinted constructs open new therapeutic pathways to tackle heart diseases and neural degeneration, i.e.,

dealing with damaged electrophysiological human tissue. For this purpose, electrical properties have to resemble the physiological conductivity of the human heart, which lies between 0.02 and 0.06 S cm^{-1}. As one can see in **Figure 7**, nanocomposite-doped hydrogels generally do not achieve the desired conductivity window of the human heart. Hence, there is still a huge potential for hydrogel and nanocomposite optimization in order to come closer to the desired window.

Apart from advantageous electroconductivity and the consequent possibility of electrical stimulation for better cell maturation, nanocomposites add further beneficial properties to the hydrogels toolset. They improve mechanical properties, such as shear thinning, shape fidelity, and increased printing resolution. As seen in the previous nanocomposite section, CNTs as an additive in hydrogels are reported to increase mechanical strength of the bioink and enable better cell attachment while increasing proliferation. Graphene seems to have very favorable electroconductivity and influence on the guided differentiation on cell types with electrophysiological properties. Additionally, the mentioned IOPs, NW, and NP increase the mechanical properties of hydrogels, alignment of cells within the construct and alter the cellular expression.

According to this manifold amount of nanocomposites with its abundance of amelioration to hydrogel characteristics, this review should help the readers to find the nanocomposite most suitable for their respective application.

In addition, nanocomposites which exhibit magnetic capabilities are accessible for 4D-printing processes. In 4D printing, functionalities and shapes of the given construct can be altered via external stimuli over time. Since tissues of the human body are situated in a very plastic, nonstatic environment, this dynamic altering of printed constructs resembles biological functionality to a very high degree. However, it has to be said that 4D bioprinting is still in its early days and suffers from several limitations. Among the biggest of these is the lack of reliable computational models able to predict accurately the evolution (i.e., deformation over time) of a printed construct exposed to external stimuli.

Despite the advantages of using nanocomposites in printing applications, only a few composites are as yet allowed for in vivo applications. Long-term studies on cytotoxicity and biodegradability are lacking, which currently makes it difficult for therapeutic utilization. No engineered graft or patch with biological relevance should neither induce a toxic response in the host environment, nor should it interfere with the normal physiological function of said tissue. Engineered conductive biomaterial has to assimilate into the host tissue properly in order not to cause any disturbance in signal transduction, such as arrhythmia. But this is not only a problem of nanocomposites in 3D printing. In all branches of engineered biomaterials mixed with NPs, no universal proof of their safe clearance out of the human body has yet been confirmed.[111,112] Since it has been observed that NPs with a diameter smaller than[40] nm penetrate cell membranes and even nuclei and CNTs,[91] graphene,[98] and NPs[113] have negative effects, systematic toxicological studies are of paramount importance. Especially, the invasion of cell nuclei can lead to severe adverse events such as alteration of DNA methylation.[112] Nevertheless, in vitro studies of the nanocomposites covered in this review suggest a good acceptance of these substances in cardiac and neural cell experiments.

Recently, questions have been raised on the mechanism of the electroconductive transduction between biological tissue and electroconductive composite scaffolds.[26] The complexity of the cell–scaffold interface is often underestimated and not yet well understood. Most cited studies assume that ionic conductance (biological) and electronic conductance (artificial) is equivalent.[17,28,71,88] However, electron propagation differs fundamentally from action potentials in cells.[114] In particular, it is not yet clear how the electroconductive scaffolds interact and propagate the signals toward biological tissue. The concept of equivalency could be valid for externally stimulated scaffolds where the transduction of the signal at the interface can occur due to redox reaction or capacitive coupling.[115] Without these stimuli, the coupling between both systems is not as straight forward.[76,116,117] Therefore, favorable outcomes in the here reviewed literature could have occurred due to the improved

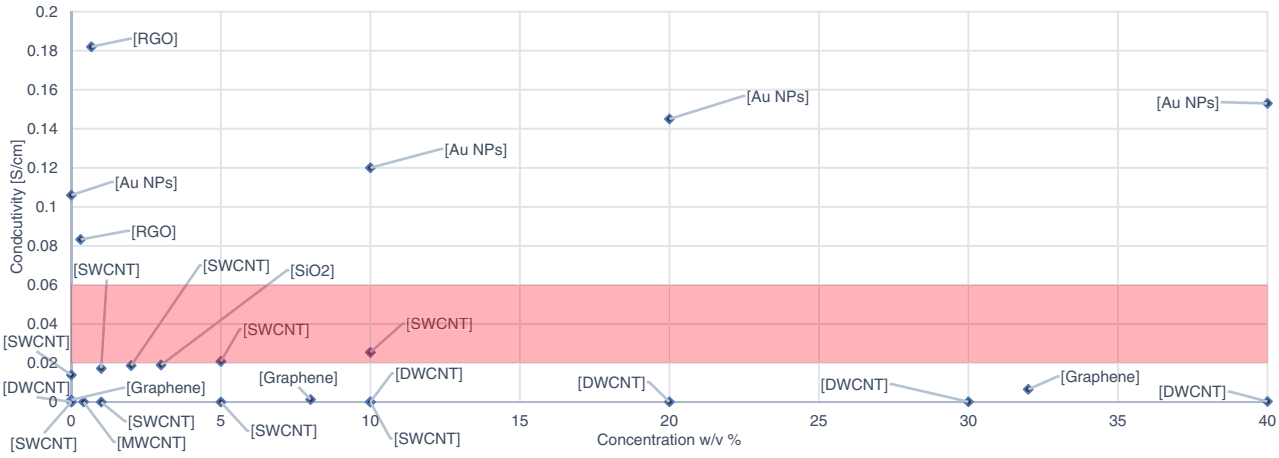

Figure 7. Graphical illustration of the electroconductivity of hydrogels doped with different nanomaterials. The red window depicts the physiological conductivity of the human heart.

electronic conductance of the material as well as other processes, e.g., mechanical changes of the scaffold.[26] However, in most literature, it was not discussed in more depth, i.e., the concept of equivalence is rarely questioned. More investigations and better understanding of this significant cell–scaffold interface would facilitate the development of new biomaterials, nanocomposites, and better regenerative strategies.[118,119]

As elaborated in this review and since 3D bioprinting is an interdisciplinary scientific field, it can be concluded that a variety of research still needs to be carried out for successfully printing functional patches, conduits for damaged native human tissues and ultimately, whole organs. Recent advances in material science, developmental biology, hardware and software printing technology, and translational technology might pave the way for a seminal technology usable in regenerative medicine.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors acknowledge financial support from the Swiss Nanoscience Institute (SNI) at the University of Basel. The authors thank Andrew Brown for his writing support.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

biofabrication, cardiomyocytes, electroconductive, nanocomposites, neurons, scaffolds, tissue engineering

Received: August 26, 2021
Revised: November 1, 2021
Published online: December 4, 2021

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