Carbon nanotubes for cardiac tissue regeneration: state of the art and perspectives

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Abstract

Carbon nanotubes (CNTs) have appeared in recent years as innovative components for the development of the next-generation scaffolds to regenerate damaged cardiac tissue. The unique robustness, mechanical and electronic properties of CNTs, along with their ease to undergo chemical modification, make them promising candidates for the design of engineered cardiac constructs with *ad hoc* properties. The integration of CNTs with polymeric scaffolds is a promising strategy for cardiac regeneration since, as reviewed in these pages, their conductivity has a boosting effect on cardiomyocytes behaviour, including their synchronous contractility when grown on top of the nanomaterial. More recently, the conductive properties of pure CNTs are attracting interest to design innovative systems based on bare CNTs without using other fillers. Additionally, the elongated CNT morphology is a strategic asset for the mimicry of the anisotropic myocardium structure, and research has made great progress over the production of micropatterned scaffolds with nanoscale definition that aim to recapitulate the cardiac tissue.

Overall, engineered CNT constructs are revealing their great potential to develop new platforms able to interface, repair, or boost the performance of cardiac tissue.

Keywords: carbon nanotubes, cardiac regeneration, polymer matrix, 3D scaffold, 3D pattern.

1. INTRODUCTION

Ischaemic heart disease has been the leading cause of death for nearly 20 years worldwide [1]. During myocardial infarction, a coronary artery is blocked and hampers blood flow and oxygen supply to the heart, causing ischemia and eventually cell death. The extent of the resulting irreversible damage depends on a variety of factors, including infarct size and the type and timing of cardioprotective strategies put into place [2]. After the initial damage, different pathways that involve oedema, a second wave of injury caused by reperfusion, mechanical complications, and ventricular wall rupture, can lead to heart failure (HF) [3]. Chronically, the heart experiences cardiomyocyte (CM) loss, matrix degradation and fibrosis [4](doi:10.1007/s40119-020-00199-y). Chronic HF is a life-threatening condition in which cardiac output fails to meet systemic demands resulting in damage to other organs and overall reduced quality of life. Treatment may involve medical management, change of lifestyle behaviors, and use of cardiac devices [5], with the aim of reducing symptoms, slowing disease progression, and lessening mortality. However, it does not aim at repairing the heart muscle, for which new strategies are continuously being searched to restore its electrical integrity [6]. Despite research advancements, survival after a diagnosis of HF has just modestly improved [7]. The main difficulty is posed by the limited regenerative capacity of the myocardium. Organ transplantation remains the only definitive treatment for HF, being limited by availability of donors and high surgical risks [8].

The artificial regeneration of the diseased heart frameworks could be an effective alternative enabled by the implantation of cell-supportive patches [9]. Efforts in cardiac tissue engineering

(CTE) have focused on developing biomaterials that can mimic the myocardium and promote cell growth and organ repair [10-12]. To this end, a methodological process consisting of 9 steps has been proposed (Figure 1, [13]). The first three steps show the processes which led to the formation of functional heart muscle. Steps four to six are designed to identify the conditions that need to be recapitulated during in vitro culture. The next two steps (seven and eight) are designed to stablish the metabolic requirements of tissue constructs. In the final step, the functionality of the 3D cardiac patches is tested using small animal heart failure models. However, cardiac tissue structural, mechanical and electrical properties present a high level of complexity to recapitulate. Although conventional polymers have demonstrated adequate biocompatibility, their surface requires continuous optimization [14], and some of their intrinsic properties are sub-optimal. Firstly, the vast majority of them is electrically insulating at biologically relevant frequencies, thus a plethora of approaches are being developed to impart them with conductivity [15]. For reference, the myocardium displays a conductivity of ~0.5 S/m in the atria, and 0.3-0.6 S/m in the ventricula, as shown in Figure 2 [16]. It is thus not surprising that conductive polymers (*e.g.,* polypyrrole, polyaniline, see Figure 2) are attractive alternatives, yet they often present other limits in terms of the required biocompatibility, hydrophilicity, stretchability, and robustness for cardiac tissue mimicry [17]. Secondly, the anisotropic structure of the real myocardium is not trivial to reproduce, the challenge to match the native tissue mechanical and electroactive properties has not been yet achieved[16].

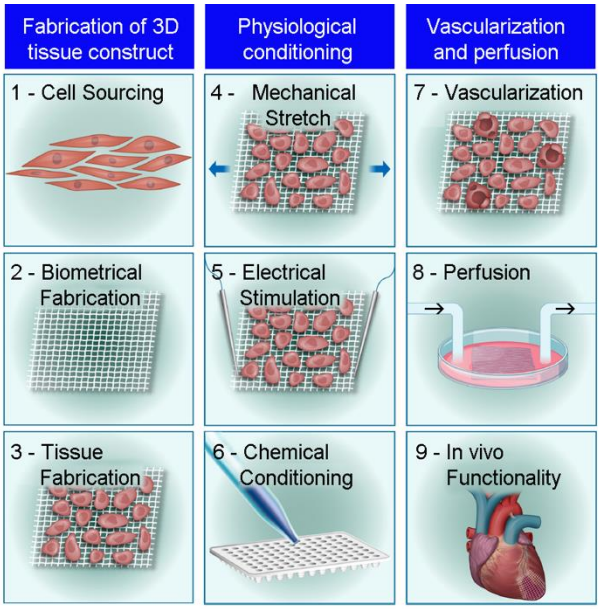

Figure 1. Methodological process to bioengineer the heart tissue in 9 steps. Adapted from [13] with permission from Elsevier.

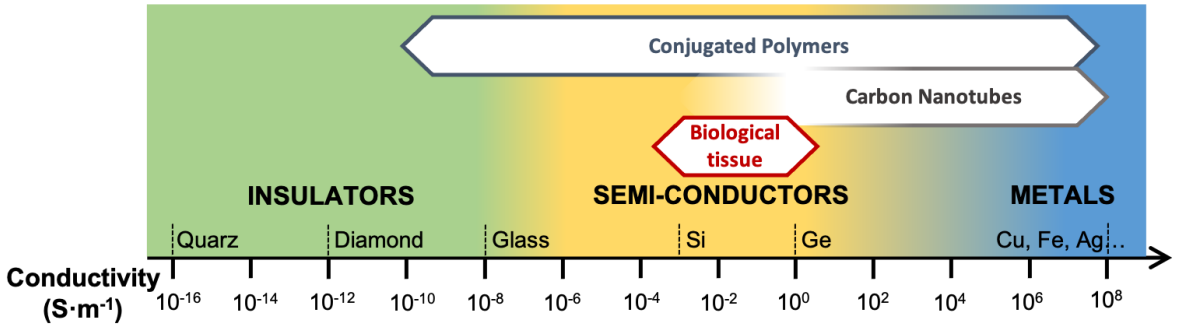

Figure 2. Schematic illustration of conductivity range of conjugated polymers, Carbon nanomaterials and biological tissue compared with most common materials. Please note that the highest value presented for Carbon Nanotubes is obtained from a single SWCNT. Also, CNT can be obtained as semiconducting and metallic materials; however, the minimum conductivity of single semiconductor CNT has not been reported, to the best of our knowledge. Si = silicon, Ge = germanium, Cu = cupper, Fe = iron, Ag = silver. Data source from ref. [18-21]

Over the years, nanomedicine has emerged for its potential to revolutionize the therapeutic success in critical areas, including cardiac repair [22]. In particular, since their discovery in 1991 [23], carbon nanotubes (CNTs) have become a strategic choice in nanotechnology, due to their unique electrical, mechanical and thermal properties [24]. Their high-strength and low-weight combined with their high-conductivity and stability make them unparalleled materials for advanced applications in the biomedical field, including nano-bioelectronics [25]. More specifically, CNTs are one of the most promising components to engineer the complex interface between biological tissue and electronics, or to reconnect conductive tissue, and to this end numerous strategies have been explored for their conjugation with biomolecules [26-29]. Interestingly, electroactive cells in contact with CNTs become "electrified", *i.e.*, electrically more active and better interconnected [30]. It is thus not surprising that they have attracted the interest of the multidisciplinary community working on the challenging development of artificial muscles [31], including bioreactors to repair the heart [32] and improved constructs for cardiac tissue engineering and pharmacological, gene and cell therapies[33, 34]. Furthermore, the ease of tuning their properties by functionalization or hybridization with other materials renders CNTs very promising components to create revolutionary biohybrids [35].

In comparison to other nanomaterial platforms, CNTs possess characteristics that make them promising materials for the future development of cardiac constructs.[36] Most of the research efforts to incorporate carbon nanomaterials into cardiac tissue engineering approaches have focused on the use of CNTs, gold nanoparticles, and graphene derivatives.[33, 37-39] However, one major advantage of CNTs in cardiac tissue engineering is their intrinsic morphology as well as anisotropic mechanical and electrical properties. CNTs are anisotropic materials that promote the alignment of the electroactive cardiac cells in one direction, mimicking the anisotropic alignment of cardiomyocytes present in the cardiac tissue.

Furthermore, the intrinsic CNT anisotropy can be combined with synthetic strategies that allow the fabrication of aligned structures to further take advantage of this property. Thus, different techniques such as electrospinning,[40] dielectrophoresis,[41] or chemical vapor deposition,[42] can be used to engineer cardiac constructs based on aligned CNTs and promote the increase in cellular alignment of cardiomyocytes. This, in turn, has demonstrated to increase the contractility of cardiac cells along the long axis, mimicking the cardiac tissue.[42] In this review, we discuss CNT application for cardiac repair, relative to their use on their own or in combination with other polymers, and either randomly oriented or aligned in the resulting scaffold (Figure 3).

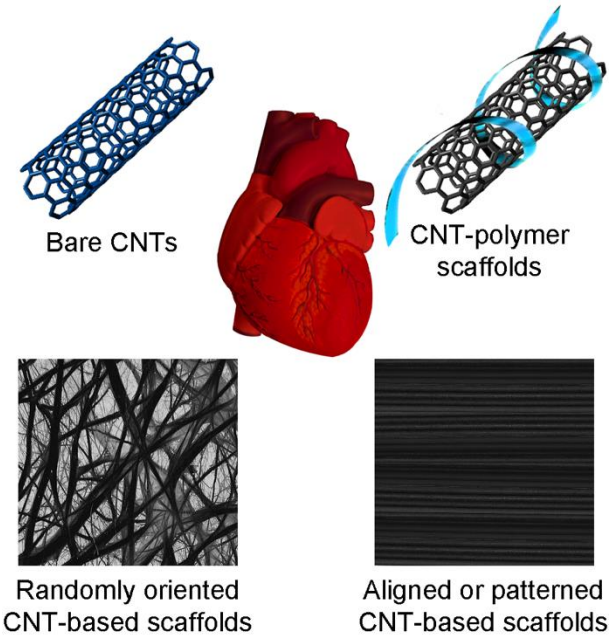

Figure 3. CNTs for cardiac repair can be envisaged to be used as bare, wrapped in polymers, randomly oriented or aligned in the resulting scaffolds.

2. PURE CNT SCAFFOLDS

The effects of CNTs on the electrophysiological behavior of cardiomyocytes was first characterized by Martinelli *et al.* when they cultured neonatal rat ventricular myocytes

(NRVMs) on multi-walled CNT (MWCNT) substrates [43]. Transmission electron microscopy imaging revealed a tight interaction between CNTs and the cell membrane that promoted CM proliferation and enhanced NRVM electrophysiological properties, when compared with CNT-free controls (Figure 4A and 4B). Subsequently, Martinelli *et al.* found that pure-CNT substrates increased the expression of connexin 43 (Cx43) (Figure 4C), which is associated with gap junctions, and promoted a gene expression profile characteristic of terminal differentiation and physiological growth on NRVMs, relative to gelatin controls [44]. In addition, such CNT platforms presented protective activity against CM hypertrophy, induced by the G-protein-coupled receptor ligand phenylephrine. These first studies attracted the use of CNTs to improve biomaterials for CTE.

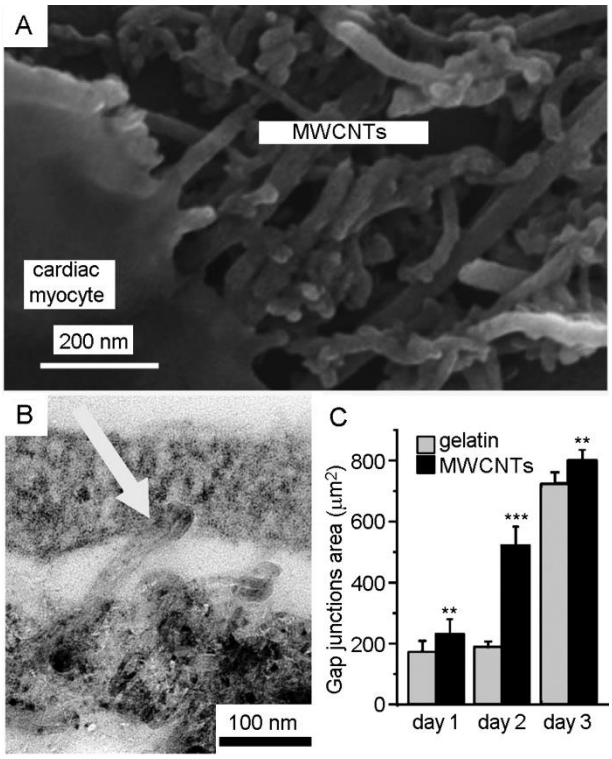

Figure 4. A-B) Scanning (A) and transmission (B) electron microscopy images reaveal intimate contact between CNTs and CM membranes (arrow). C) Gap-junction areas are increased for CMs grown on CNTs relative to gelatin controls. Adapted with permission from [43] and [44]. Copyright © 2012 and 2013 American Chemical Society.

Pure CNTs can induce stem cell differentiation to CMs, as demonstrated on mouse embryoid bodies, which showed electrical coupling between neighbouring cells, and enhancement of cardiac differentiation and beating activity upon electrical stimulation [45]. The fabrication of cardiac constructs based on pure CNT aligned arrays has not been extensively studied. Super-aligned CNT sheets obtained from a spinnable CNT forest, cultured with CMs, induced elongated and aligned cell morphology, resulting in improved intercellular coupling, reduced repolarization dispersion, and providing signal-transmission pathways to synchronize cells and potentially decrease risks of arrhythmias [46]. The excellent control and enhancement of the cardiac conduction achieved with CNTs was also demonstrated *in vivo* [47]. In this case, CNT fibers were sewn across epicardial scars in sheep, following radiofrequency ablation to create a conduction delay. CNT fibers not only improved conduction across the scar, but also maintained conduction for one month post atrioventricular nodal ablation. These findings suggested a promising use of freestanding aligned CNT arrays for future applications in CTE. However, just a few attempts were made in this direction because of existing barriers: (i) the fabrication of 3D architectures composed merely of CNTs and absence of an agglutinant material yields scaffolds that are difficult to manipulate [48]; (ii) several data revealed that pristine CNTs within the body behave as nano-needles and aggregate leading to adverse effects comparable to inhaled pathogenic silica and asbestos [49, 50]. It should be noted, though, that CNT modification with chemical groups to avoid aggregation and ensure their dissolution in biological media can alleviate their toxicity for biomedical applications [51, 52]. Nevertheless, in CTE, the combination of CNTs with polymeric matrices is a far more popular option than using bare CNTs to offer scaffolds that are more stable, easy-to-handle and localized within the area of interest [53].

3. CNT-INTEGRATED POLYMERIC SCAFFOLDS

Ideally, cardiac scaffolds should be able to mimic the myocardium extracellular matrix (ECM) for a successful integration with the tissue. More specifically, they should be (i) electrically conductive to reduce the abnormalities in the electrical signaling through the CMs of the injured heart, (ii) mechanically robust to withstand the applied forces during heartbeating, (iii) stretchable to accompany the movements of heart contraction, and (iv) biocompatible and non-immunogenic. In most of the reported scaffolds, the function of CMs is restricted because of mismatches in the mechanics, conductivity, and sub-micrometric structure of the matrix [54]. Several CTE strategies have been focused on the development of 'sheets' or two-dimensional (2D) patches, using a wide array of natural and synthetic polymer composites and blends. However, those approaches have not been able to recapitulate the extremely complex structure and functionality of the native myocardium. By contrast, several investigations have shown that three-dimensional (3D) scaffolds constitute the most effective tools in CTE as they have large surface areas for cell or biomaterial attachment, proliferation, sensing, etc. [55]. Additionally, cell behavior is more similar to the native tissue in 3D cell cultures that may provide a more realistic model for biological tissue mimicry.

The introduction of CNTs has allowed to overcome many limitations, providing patches with enhanced conductive and mechanical properties [36]. CNTs promoted CM maturation and increased cardiac tissue functionality relative to other conductive carbon nanomaterials, such as graphene oxide and reduced graphene oxide [56, 57]. Furthermore, CNTs embedded into, or bound to, polymeric matrices behave more like particulate materials instead of nanometric materials, thus without interaction with the cellular cytoskeleton, with no cytotoxicity, inflammation or any notable negative effect when tested in animal models or *in vitro* for CTE applications [58]. To date, CNTs have been incorporated into various synthetic and natural polymers to yield conductive hydrogels and solid/fibrous scaffolds, with a large variety of structures and dimensions and providing excellent electrical and mechanical properties [59].

3.1 Randomly-distributed CNTs

3.1.1 Hydrogels

Hydrogels are outstanding biomimetic materials for soft tissue applications as they can be integrated with living cells with good viability. Their highly hydrated nature and mechanical properties can be adjusted to create microenvironments ideally suited for cell growth [60]. There is a large variety of gelling polymers that can match the stiffness of biological tissues, which is key to guide cell response [61], although they typically don't display the complex viscoelastic properties of the ECM, such as non-linear elasticity and mechanical plasticity [62]. Supramolecular hydrogels are emerging as attractive alternatives, with new approaches being devised to control their shape and structure for tissue engineering [63]. Moreover, CNTs can easily be dispersed within either type of hydrogel matrix before gelation, providing electrical cues and higher mechanical strength to the system [64], and even self-healing properties to the resulting CNT-containing hydrogel [65]. Overall, these new features allow the preparation of promising cardiac constructs, as described in detail below.

One class of hydrogelators that has gained wide popularity comprises natural biomolecules. In particular, collagen is appealing as it is the major protein component in the native cardiac ECM and, thus, exhibits good biocompatibility [66]. CNT-dispersion into collagen type I-based substrates promoted the assembly and formation of intercalated discs within CMs by activating the β_1-integrin-mediated signalling pathway [67]. Gelatin, which can be derived from the hydrolysis of collagen, is a gelator that has been extensively used in pharmaceutical and medical applications and addition of CNTs led to improved performance *in vitro* and *in vivo* for use in CTE [68]. CNTs provided electrical cues to gelatin hydrogels cross-linked with glutaraldehyde [69]. The 3D scaffolds promoted CM contractile and electrical activity *in vitro*. These findings led to the first *in vivo* study of the implanted patch into rat infarcted hearts,

observing its successful biohybridization with the host myocardium and the subsequent regeneration of the cardiac tissue [69]. Furthermore, CNT-gelatin methacrylate hydrogels successfully recapitulated the hierarchical anisotropic structure of the cardiac tissue, yielding stronger, spontaneous, and synchronous beating rates and lower excitation threshold of NRVMs (Figure 5), relative to those cultured on CNT-free gel controls [70]. The underlying mechanism confirmed involvement of β_1-integrin mediated FAK and RhoA signalling pathways. Also in the case of chitosan-based hydrogels, CNT addition yielded higher mechanical strength, with CNTs acting as electrical nanobridges between CMs for enhanced electrical coupling, synchronous beating, and thus overall improved CM function [71].

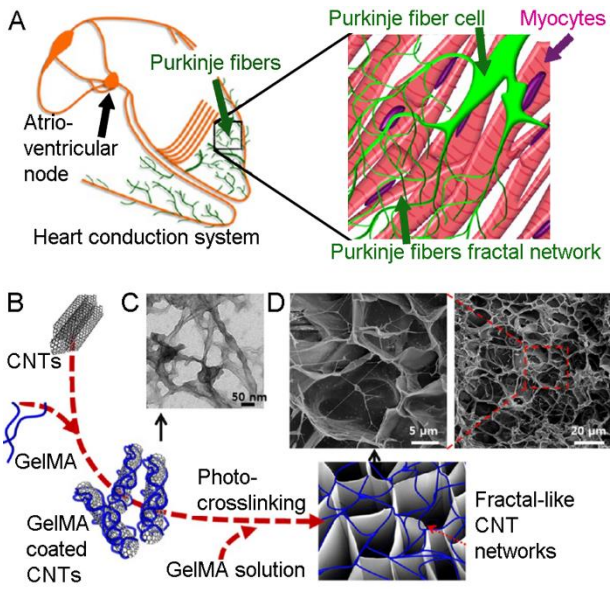

Figure 5. CNT-Gelatin methacrylate (GelMA) hydrogels. (A) Schematic diagram of the heart conduction system showing the Purkinje fibers, which are located in the inner ventricular walls (left) and enlargement (right) showing the structure of the heart muscle with Purkinje fiber networks on the surface of myocytes. (B) Preparation of fractal-like CNT networks embedded in GelMA hydrogel. (C) Transmission electron microscopy image of GelMA-coated CNTs. (D) Scanning electron microscopy images show porous surfaces of CNT-GelMA thin film. Adapted with permission from [70] Copyright © 2013, American Chemical Society.

Ideally, biomaterials should be implanted with a minimally invasive procedure to prevent any additional tissue damage [14]. For this reason, injectable hydrogels are particularly desirable [72, 73]. They can be designed to have specific physical, chemical and electrical properties to provide structural and electrical support to the damaged cardiac tissue [74]. Additionally, they can also serve as a delivery vehicle for cells and biomolecules to further promote cardiac repair [75, 76]. Injectable acellular alginate hydrogels have already been tested in clinical trials in HF patients, with favorable tolerability [77, 78]. However, due to the complexity of heart tissue, the ideal injectable hydrogel that mimics the cardiac electrical cues has not yet been developed [59].

Among the natural injectable polymers, ECM-derived hydrogels are increasingly used as scaffolds for cardiac repair [79]. In particular, porcine myocardial ECM-derived hydrogels improved cardiac function in both small and large animal infarct models, with obvious advantages of availability and ease of processability over the human-derived analogs [80]. However, ECM-derived hydrogels lack electrical cues which are needed for efficient cardiac function. To overcome this limitation, Roshanbinfar *et al.* proposed the dispersion of MWCNTs into sheep-derived decellularized pericardial tissue [81]. CM cultures displayed enhanced proliferation and cell alignment, increased expression of cardiac specific gap junction protein Cx43, and improved synchronous contraction [39]. Although ECM-derived hydrogels have shown promise both *in vitro* and *in vivo*, a more cost-effective extraction method to reduce the denaturation of ECM proteins is still needed [82]. To this end, several investigators have developed and studied other natural injectable hydrogels for cardiac repair, confirming once again collagen as one of the preferred options [54, 83]. Although the incorporation of CNTs into collagen hydrogels improved the mechanical strength and electrical performance, thus leading to better cell alignment and function, there are still concerns regarding the batch-to-

batch variability of natural hydrogels [72], and the lack of chemically-bound CNTs that can increase the risk of CNT release and consequent toxicity.

Synthetic polymer-based scaffolds are attractive alternatives, as they can be constructed with improved, easily-tunable, and reproducible properties, through their chemical functionalization, also to achieve controlled degradation and gelation kinetics [84, 85]. As an example, poly(N-isopropylacrylamide) (PNIPAAm)-based hydrogels are common thermosensitive materials broadly used in the biomedical field, as they remain in solution at room temperature and turn into physical gels at body temperature [86]. Thus, once injected, (PNIPAAm)-based hydrogels can conform to the irregularities of the injury site *via in situ* gelation. Moreover, they can be covalently bound to CNTs, for instance through conjugation of COOH-functionalized CNTs with the primary amine groups of PNIPAAm-lysine hydrogels [73]. 3D NRVM cultures displayed promoted proliferation, stronger and more homogeneous spontaneous calcium transients, and an increased and more organized localization of the Cx43 protein. Li *et al.* used CNT-containing PNIPAAm hydrogels to inject and localize brown-adipose-derived stem cells into the myocardium of a rat infarct model [87]. They observed an improved cell engraftment, cardiac repair and function, *i.e.*, pumping the blood circuit. The outcome of these studies is that the incorporation of CNTs not only enhanced function and promoted early CM proliferation *in vitro*, but also improved cell integration and therapeutic benefit *in vivo*, thus representing an important step forward in the quest to use CNTs and stem cells to promote cardiac tissue repair.

3.1.2 Fibrous patches

Nanofibers are also promising materials for tissue regeneration, as their shape and distribution can be modified to mimic the ECM more effectively than other biomaterials [88, 89]. However, as with hydrogels, natural or synthetic polymer fibers are non-conductive and the challenge

remains to introduce electrical properties without influencing the scaffold biocompatibility. As previously mentioned, CNTs are ideal to provide electrical cues to non-conductive materials along with enhanced mechanical properties and reduction in degradability. For example, Wickham *et al.* demonstrated that the inclusion of CNTs into 3D poly-caprolactone fibrous meshes increased their mechanical strength without altering their morphology, resulting in enhanced proliferation of stem/progenitor cells from murine hearts [90]. The authors observed that the 3D electrospun meshes supported cell adhesion, proliferation, and differentiation, while solvent casted sheets were unable to support cell attachment and spreading.

Conductive fibers are typically produced by electrospinning a solution containing a polymer and CNTs to yield a fibrous mesh with CNTs embedded within the matrix. Thus, a variety of polyurethane/chitosan/CNTs composite nanofibrous scaffolds with random and aligned orientation were fabricated and H9C2 cells incubation revealed that the aligned fibrous pattern could be very promising for myocardium regeneration purposes.[91] However, the synthesis of a polymer solution that results in posterior electrospun fibers with homogeneous distribution of nanomaterial can sometimes be unattainable. Therefore, in materials such as spider silk, coating techniques, *e.g.* solution casting, solvent evaporation or electrospraying, are needed to attach CNTs onto the fibrous surface [88, 92].

The addition of conductive properties to porous scaffolds has become key for electric stimulation and to provide a new kind of interaction with biological systems. Several studies demonstrated that local electrical stimulation favored and accelerated the regeneration and repair of electroactive tissues, enhancing cell-cell and cell-substrate interaction [93]. Similarly, even though there is no clear observation of the impact of CNTs on cellular differentiation, the combination of a CNT-based conductive scaffold with electrical stimulation promoted differentiation of cardioprogenitor cells and, therefore, cardiomyogenesis [94]. To this end, 3D nanofiber scaffolds composed of polyvinyl alcohol, chitosan, and CNTs were produced to

promote differentiation of rat mesenchymal and unrestricted somatic stem cells with electrical stimulation [95, 96]. In those reports, it was shown that the substrates containing 1-2% CNTs had optimal conditions for this purpose and concluded that the electronic properties of CNTs could be used to achieve the manipulation of the stem cell differentiation pathway. Finally, CNTs have also been introduced into patches and showed improved cardiac conduction across surgically impaired epicardium [97]. Overall, these studies confirmed the promising application of CNTs for cardiac resynchronization therapy in patients at risk of arrhythmias following myocardial scarring, *i.e.,* fibrosis after heart failure.

3.2 Aligned or patterned CNT-incorporation

Despite the fact that inclusion of CNTs with random distribution improved the physical and electrical properties of the resulting scaffolds, the road is still long to achieve the desired toughness, architectural properties and electrical conductivity for CTE. In addition, randomly dispersed CNTs are more likely to aggregate and penetrate into cells, leading to inefficient electrical transfer and toxicity [98].

The native myocardium ECM consists of a large variety of electrically conductive and aligned nanofibrous-like proteins, mainly collagen and laminin, that enable electrical signal propagation along the cardiac cells to eventually facilitate the synchronous contraction of the cardiac tissue. Therefore, nanofibrous matrices with aligned morphologies were prepared for biomimicry and shown to induce the formation of oriented engineered cardiac tissue with enhanced functionality and mature phenotype [99]. Such alignment can guide the oriented organization of cardiac cells, sarcomeres and gap junctions, to favor the electric propagation and subsequent synchronized contraction. Furthermore, aligned-CNT-based microelectrodes can allow pacing in close contact with cells and at lower voltages, thus controlling the electrical pulses between cells and tissue constructs, and regulating their behavior and function. This

kind of platform, which possesses high similarity to the nanofibrous microstructure of native ECM, enables localized stimulation and, hence, guidance of cardiac cells.

3.2.1 Aligned-CNT hydrogel scaffolds

Although micropatterned hydrogels with aligned CNTs are not easy to manufacture, scientists have succeeded in their production. For example, Shin *et al.* developed an innovative approach to prepare biohybrid actuators consisting of layered constructs of vertically-aligned CNT arrays and hydrogels [100]. First, chemical vapor deposition was used to produce a vertically-aligned CNT forest, which was then encapsulated within gelatin methacrylate and polyethylene glycol hydrogel layers, into constructs with excellent mechanical integrity. CMs cultured in the anisotropic structure showed homogeneous cell organization with good cell-to-cell coupling associated with maturation and continuous beating. In addition, the beating frequency of these 3D biohybrid actuators could be precisely controlled by applying an electrical signal along the parallel and perpendicular directions, relative to the long axis of alignment of the CNT forest. In another study, Ahadian *et al.* also used gelatin methacrylate to encapsulate CNTs, which were aligned using dielectrophoresis. The CNT-hydrogel was tested on mouse embryoid bodies and revealed a superior cardiac differentiation upon electrical stimulation, relative to non-conductive or randomly-dispersed-CNT hydrogel controls [101].

3.2.2 Aligned-CNT fibrous scaffolds

As aforementioned, in the native ECM, cardiomyocytes are embedded in a compliant network based on collagen and laminin fibers that are aligned in parallel to cardiomyocytes, providing structural stability and tensile strength (Figure 6A). These ECM proteins provide alignment cues, biochemical signals, and mechanical support to cardiomyocytes,[102] which has a

profound impact on the electrical and mechanical properties of cardiac tissue, yielding anisotropy in both, cardiac cell action potential and contractile forces.[103]

Aligned polymeric nanofibers prepared by electrospinning have been increasingly investigated in CTE to mimic the anisotropic structural organization of the native ECM [104, 105]. CNTs have also been shown to improve the fibrous alignment of electrospun poly(glycerol sebacate)-gelatin polymeric matrices. Together with the enhanced electrical conductivity and toughness, the resulting flexible and aligned scaffolds promoted the spontaneous and synchronous beating of NRVMs [40]. Similar effects were noted when different concentrations of CNTs were electrospun into fibres of poly(ethylene glycol)-poly(D,L-lactide) copolymers [106]. The as-prepared constructs facilitated CM growth with enhanced production of contractile proteins and synchronous beating.

However, the typical dense nanofibrous structure of nanofibrous materials can hamper cell migration and CM orientation within the scaffold. This has led the scientific community to pursue the challenging objective of designing promising patterned CNT-polymer hybrid materials.

3.2.3 Patterned and hybrid patches

Micropatterned electrospun mats, constructed by combination of lithography techniques and electrospinning, were used to achieve fibrous multi-patterned scaffolds that reproduced the anisotropic structure of myocardium and provided conductivities at the same level of cardiac muscles. CMs were co-cultured with cardiac fibroblasts and endothelial cells to provide an alternative strategy for the *in vitro* construction of engineered cardiac patches [107]. The authors concluded that honeycomb-patterned scaffolds presented higher CM viabilities, cell better elongation, more ECM synthesis, higher production of organized contractile proteins, and a pulsation frequency close to those of adult and neonatal rat hearts. Similarly, the co-

culture on electrospun fibrous mats inoculated with CNTs and patterned with strip-, oval- and wave-shaped arrays resulted in different responses in terms of cell viability, cell elongation ratios, expression of different proteins, and beating rates [108]. These micropatterned models demonstrated the promise of co-cultured CMs with other myocardial cells, as fibroblasts, as a more real-to-in vivo platform for screening cardiac side-effects of drugs. Patterned 3D structures were also obtained from hybrid patterns of polycaprolactone, silk fibroin, and CNTs. This is prepared from polycaprolactone, silk fibroin, and CNTs through weaving in a hydrogel shell, which enables the engineering anisotropic and endothelialized cardiac constructs [109]. The incubation of CMs in these scaffolds demonstrated their good biocompatibility and ability to guide cellular alignment and elongation, enhancing CM maturation and function (Figure 6).

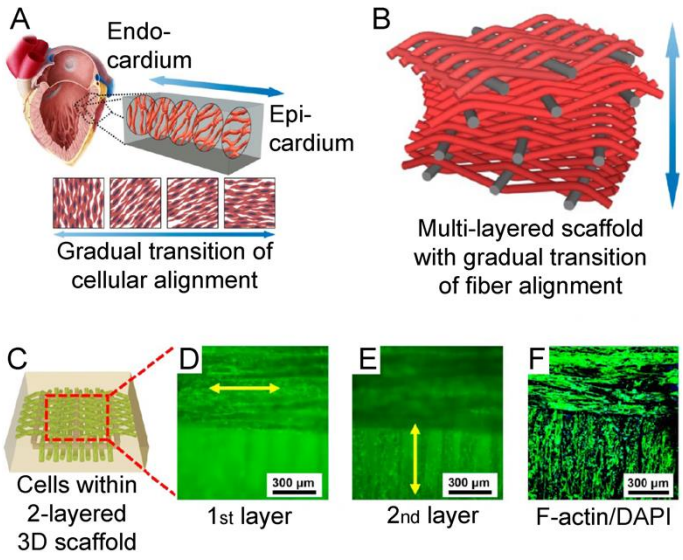

Figure 6. A) Schematic representation of the anisotropic layered structure of the heart walls. B) Engineered multi-layered woven scaffold for heart wall mimicry. Red fibers: PCL/silk fibroin/CNT; grey rods: suturing threads for the scaffold's network construction. C) Schematic diagram of cells cultured within a 2-layered woven scaffold. D-F) Fluorescent microscopy of aligned cells with different directions in each layer (D-E) and top-view of the two layered cells

(F). Actin is stained green and nuclei blue with DAPI. Adapted with permission from [109]. Copyright © 2017 American Chemical Society.

3D printing strategies have recently emerged as a promising industrial manufacturing method for patterned and super-aligned structures for tissue engineering applications. Integration of UV-lighting with a 3D bioprinter provides a new tool to obtain photo-crosslinkable hydrogels with a predefined complex structure, curing the gel while it is printed. Furthermore, conductive inks incorporating CNTs have been prepared to print 3D cardiac patches [110]. For example, Izadifar *et al.* generated hybrid hydrogels with frames composed of alginate, which incorporated CNTs, and human coronary artery endothelial cells-laden methacrylated collagen [111]. Also in this case, CNTs improved not only the electrical and mechanical properties of the hydrogel, but also cellular attachment and elongation. Vaithilingam *et al.* 3D-printed biomimetic substrates of an acrylate matrix containing CNTs with a linear pattern [112]. CMs were cultured in the patterned substrates, and the authors demonstrated that cellular behavior could be modulated thanks to the scaffold architecture and electric properties provided by the CNTs.

There are some more methods to fabricate the scaffolds for complex cardiac-constructs. Polymeric scaffolds consisting of CNTs integrated with poly(octamethylene maleate (anhydride) 1,2,4-butanetricarboxylate) showed the ability to be molded and cross-linked to give elastomeric scaffolds that improved tissue maturity [113]. Similar dimensions and a comparable level of complexity of the designed patterns have also been achieved using photolithography techniques. For example, Wang *et al.* developed star-like and stingray-like micropatterns as robotic systems to mimic living organisms using engineered muscle tissue and biomaterials [114]. The authors used CNT-gelatin methacrylate hydrogels to engineer cardiac muscle tissue from NRVMs. The hydrogel was previously attached to poly(ethylene

glycol) diacrylate, which provided structural cartilage-like support, and to flexible gold microelectrodes, for electrical stimulation control. They concluded that including electrically conductive nanomaterials, such as CNTs, not only improved the electrical coupling of the cardiac tissue, but also induced an excellent tissue architecture and spatial organization *in vitro*. In summary, artificial tissue constructs that use micro-patterned hydrogels represent a promising tool to implement robotic systems and obtain bioactuators that mimic well the complex behavior of organs such as the heart.

4. CONCLUSION

Nowadays, CNTs are one of the most promising nanomaterials with great potential for the successful engineering of constructs to promote the growth of healthy cardiac tissue. Pure CNT constructs allow the increment of the electrical activity along the direction of the nanotubes favoring the cardiac differentiation and beating activity. However, a major concern when using pure CNT scaffolds is the issue of toxicity and the number of studies involving pure CNTs is still limited. The incorporation of CNTs into polymeric scaffolds for the development of constructs for CTE have demonstrated to enhance the electrophysiological performance, improved mechanical integrity, better cell adhesion, viability, uniformity and increased beating rate of cardiac tissue. Natural scaffolds showed high potential due to their high biocompatibility, although they have major drawbacks such as the high-cost purification methods and their batch-to-batch variability. Therefore, synthetic polymer scaffolds have gained popularity in light of the ease to tune their properties in a reproducible manner, such as increased mechanical strength, improved porosity, and controlled degradation and gelation kinetics. CNT orientation in the scaffold is also important. Randomly distributed CNTs tend to aggregate, thus limiting their structural and electrical properties required for CTE. By contrast, aligned CNTs yielded excellent matrices to induce the formation of oriented engineered cardiac

tissue with enhanced functionality and mature phenotype. Furthermore, these platforms display high similarity to the nanofibrous microstructure of native ECM, enable localized cell stimulation and, hence, guidance of cardiac cells.

The number of studies for the fabrication of cardiac constructs employing CNTs has been growing in the past years. Although the definite scaffolds that fulfill all the requirements for successful CTE has not yet been reached, the results shown and discussed so far are encouraging and confirm that the usage of CNTs for such purpose is the correct path to achieve it. Over the years, fine control has been achieved in the production of nanostructured and micropatterned constructs. 3D bioprinting is a promising avenue for their production, although challenges remain such as the correct integration of cardiac constructs with the patient vascular system [115]. A holy grail is the control of neovascularization, which is essential to restore the blood flow to the injured myocardium, since the endogenous processes to induce the growth of new vessels are still poorly understood [116]. To this end, new strategies are continuously being developed, such as the recruitment of regulatory T cells [117], the use of artificial cell-free cardiac patches [118], the development of mechano-induced cell remodelling [119], and the use of stem cell-derived CMs to promote heart repair [120], also derived from placentas [121], and guided by multi-omics approaches [122]. As we learn how to recapitulate the complexity of the cardiac tissue, the possibility emerges to include self-healing chemistry into soft electronics to push the frontiers of conductive tissue regeneration [123]. All possible avenues ae being explored, with revolutionary approaches making big progress in the development of new tools to understand and engineer cardiac tissue. As an example, 2020 marks a decade that optogenetics, which combines optical stimulation with molecular biology, has been translated from the neural to the cardiac tissue for light-enabled sensing and bioactuation of electrical activity [124]. Clearly, as the number of mutually-interacting variables in CTE increases exponentially, modern approaches must be embraced for their

mastering, first of all, systems theories, which include network analysis and machine learning [125], and secondly artificial-intelligence-aided fabrication of constructs [126]. Overall, the final goal to recapitulate the natural heart tissue is getting nearer, and we anticipate a bright future where hearts will be repaired in new ways, with organ transplants being just a memory from the past.

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