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Rational Design of a Conductive Collagen Heart Patch

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Cardiovascular diseases, including myocardial infarction, is the cause of significant morbidity and mortality globally. Tissue engineering is a key emerging treatment method for supporting and repairing the cardiac scar tissue caused by myocardial infarction. Creating cell supportive scaffolds that can be directly implanted on a myocardial infarct is an attractive solution. Hydrogels made of collagen are highly biocompatible materials that can be moulded into a range of shapes suitable for cardiac patch

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applications. The addition of mechanically reinforcing materials, carbon nanotubes, at sub-toxic levels allows for the collagen hydrogels to be strengthened, up to a toughness of 30 J.m^{-1} and a 2 to 3 fold improvement in Young's modulus, thus improving their viability as cardiac patch materials. The addition of carbon nanotubes is shown to be both non-toxic to stem cells, and when using single-walled carbon nanotubes, supportive of live, beating cardiac cells, providing a pathway for the further development of a cardiac patch.

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally. 31 % of global mortality can be directly attributed to CVDs [1]. Myocardial infarction (MI), the interruption of blood supply to a part of the heart leading to cell death and fibrotic scar tissue formation, is a major cause of these deaths attributed to CVDs. The formation of fibrotic scar tissue on the myocardium leads to a weakening of the ventricular wall resulting in an increased diastolic volume caused by collagen slippage and thinning of the scar as well as new micro-infarctions affecting the cardiomyocytes residing in the myocardium adjacent to the scar [2]. This process is called ventricular remodelling and results in significant changes in the geometry and shape of the cardiac ventricles leading to further increases in diastolic volume. Thus a vicious circle ensues where increased diastolic volume leads to more ventricular remodeling which leads to further increments in diastolic volume etc. [3, 4].

Tissue engineering (TE) of materials loaded with stem cells is the next evolution of MI treatment [4]. However, TE of heart ventricular wall tissues require materials with very specific properties. These materials should transfer the electric signal from the beating heart through the TE matrix allowing for the loaded cells to become synchronous with the live tissue.[5, 6] The TE matrix also needs to be mechanically durable and flexible to provide mechanical support to the ventricle wall during tissue regrowth and to withstand the significant volume change during the heart beating [7]. Patch style TE

matrices based on both synthetic and natural polymers have gained increased research attention recently with advances in both fabrication technologies [8] and biomaterials chemistry [9]. TE matrices can serve a two-fold purpose as both a tissue regeneration matrix and vehicle for site specific delivery of drugs or growth factors [9, 10].

Hydrogels, and specifically collagen, are ideal materials for TE matrices due to their versatility of fabrication in various shape, biocompatibility, ability to deliver therapeutic agents, and flexibility [11-15]. Hydrogels are typically characterised by a relatively low toughness leading to mechanical failure in under cardiac beating conditions and poor electronic conductance. As such, mechanically strong and electronically conductive additives such as carbon nanotubes (CNTs) have been proposed for incorporation to dramatically improve the performance of these hydrogels as a TE matrix for MI [16-21]. The addition of conductive carbons to biomaterials, unlike the well studied addition of conductive polymers to biomaterials, provides the key advantage of mechanical re-inforcement and lower percolation to conduction in polymer matrices [20-22]. Herein we describe the formation and characterisation of such a CNT-collagen hydrogel using a multi-factorial design approach to fully elucidate the interaction between materials. The electrical signals of beating HL-1 cells is studied to demonstrate the CNTs are providing a matrix allowing for the desired cell function.

2. Results and Discussion

The formation of a homogenous dispersion of CNTs that is miscible through a viscous collagen matrix without significant agglomeration occurring through the 8 hour cross-linking procedure is a challenging process. In order for a homogenous CNT-collagen hydrogel to be formed, the initial

dispersion must be stabilized through both electrostatic and steric effects. The focus of the final composite as a vehicle for tissue engineering also limits dispersant choice to biocompatible and biodegradable materials only. Based on these requirements the polysaccharide chitosan was chosen as the surfactant [23, 24]. Furthermore for the application of the hydrogel composites to myocardial infarction, chitosan provides another advantage as it has been demonstrated to induce vascularization in animal models [25]. In order to study the effects of our component materials - TC, CNTs and chitosan multifactorial design (MFD) experiments were performed to gain statistically relevant data (Table 1). Multifactorial design refers to the systematic design and analysis of experiments varying specific factors between defined values (or levels). In the case of the CNT:Collagen patches fabrication the factors examined were the CNT dispersion concentration, chitosan concentration, and CNT diameter with two levels for each studied factor. This experimental design allows for ANOVA analysis of the subsequently obtained data and statistically relevant results for a minimum number of experiments [15].

The integration of CNT dispersions homogenously into the collagen hydrogels was achieved successfully resulting in clean, flexible materials with optical transmission of white light ranging between 64 % and 15 % (Figure 1). One of the crucial components of gaining cell penetration, growth, and survival into 3D tissue engineering scaffolds is control over the microstructure and porosity of the scaffolds [26].

Table 1. Multivariate Factorial Design table detailing the parameters controlled for the production of a CNT:Collagen scaffolds with the dispersion CNT concentration, chitosan concentration and CNT diameter being factors. The mass of collagen was kept constant at 500mg throughout all samples. The chitosan/C value gives a direct representation of the mass of chitosan in a given sample.

Sample No.	Dispersion	Dispersion	Average	Chitosan/C	Volume C
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	[CNT] (g/L)	[Chitosan] (wt %)	CNT diameter (nm)	(mg per 500mg TC)	(mg of 18 %v/v)
1	0.50	1.0	2	0.0125	500
2	2.00	1.0	2	0.0500	500
3	0.50	5.0	2	0.0625	500
4	2.00	5.0	2	0.2500	500
5	0.50	1.0	8	0.0125	500
6	2.00	1.0	8	0.0500	500
7	0.50	5.0	8	0.0625	500
8	2.00	5.0	8	0.2500	500
9	1.25	3.0	5	0.0940	500
Control	0.00	0.0	N/A	0.0000	500

Previous experiments using collagen only scaffolds for corneal applications have demonstrated that EDC:NHS collagen hydrogels have an excellent microstructure to foster cell growth [27-29]. The

addition of both CNTs and chitosan to the collagen matrix prior to cross-linking was shown not to have a significant impact on the microstructure of the hydrogel (Figure 1b). This result occurs due to the relatively low concentration of CNTs allowing the CNTs to be effectively wrapped within the collagen fibrils. Furthermore this results demonstrates that at the examined chitosan concentration the additional NH_2 groups from the chitosan do not adversely affect the cross-linking of collagen through EDC and NHS.

Figure 1. a) optical image of the synthesised pure collagen, and CNT:Collagen (No.1) hydrogel; b) SEM image of i) pure collagen hydrogel and; ii) CNT:Collagen (No.1) hydrogel.

This lack of change in microstructure is further validated through studying the water content of the hydrogels. Regardless of the examined composition of the hydrogels the water content ranged between 87 ± 1 and 89.9 ± 0.5 wt % water, furthermore all hydrogels could be dried and rehydrated

at least 10 times without change in the water content ($\Delta w_c < 2\%$) or apparent change in the microstructure (Table 2). This provides the potential for transport and storage of the CNT:Collagen hydrogels for longer timeframes.

Table 2. Physical properties of collagen hydrogels

Combination No.	Maximum Stress (MPa)	Maximum Strain (%)	Young's Modulus (MPa)	Toughness ($\text{J}\cdot\text{m}^{-3}$)	Water content mean (%)
Control	220 ± 30	21 ± 1	2.1 ± 0.4	18 ± 4	89.6 ± 0.4
1	380 ± 60	12.0 ± 0.5	5.6 ± 0.9	21 ± 5	89.3 ± 0.5
2	480 ± 80	16 ± 2	5.4 ± 0.5	30 ± 8	88.2 ± 0.6
3	340 ± 60	15 ± 2	4.7 ± 0.6	23 ± 5	89.4 ± 0.3
4	266 ± 30	10 ± 1	4.8 ± 0.3	10 ± 3	88.9 ± 0.6
5	178 ± 30	9 ± 1	4.1 ± 0.2	15 ± 3	89.3 ± 0.5
6	283 ± 40	13 ± 1	4.0 ± 0.4	18 ± 1	87 ± 1
7	427 ± 90	14 ± 1	5.9 ± 0.9	25 ± 7	89.2 ± 0.3
8	429 ± 60	9.2 ± 0.4	8.6 ± 0.6	18 ± 2	89.9 ± 0.5

9 342 ± 80 8 ± 2 3.5 ± 0.3 11 ± 2 88.7 ± 0.6

The addition of CNTs and chitosan to collagen hydrogels had a diverse effect on the mechanical properties of the hydrogels (Figure 2). Over all examined samples the addition of the CNT dispersion resulted in an increased tensile stress, decreased tensile strain and increased Young's modulus. Within error, toughness was found to be at worst maintained or significantly improved, dependent on the dispersion utilized. Compared to native cardiac tissue the CNT:Collagen matrix demonstrate a higher toughness, tensile stress, and tensile strain. These improved mechanical properties are advantageous to a cardiac patch as the enhanced stiffness and durability of the material is thought to prevent or inhibit vascular remodelling. This remodelling will be prevented by the patch material being providing mechanical support to the weakened cardiac wall, which will limit the damaging increase in diastolic volume.

Figure 2. Mechanical testing graphs visualising the data for CNT:Collagen hydrogels; a) Tensile Stress; b) Tensile Strain; c) Young's Modulus; and d) Toughness.

Over the examined range of CNT concentrations an increasing CNT concentration resulted in an increasing tensile stress and a decreasing strain, both of these results are directly explained by the exceptionally high Young's modulus of the CNTs themselves, along with the mismatch in Young's modulus of the pure collagen hydrogel 2.1 MPa and pure CNTs (1 TPa). Applying a strain to the CNT:Collagen hydrogel results in good load transfer between the CNTs and the collagen allowing a higher stress to be achieved, however, once the threshold for this load transfer is reached the collagen will begin to take more of the load deforming at a much greater rate than the CNTs, leading to the formation of cracks at the interface of the CNTs and collagen. These regions of poor load transfer will act as stress concentration points and result in rapid failure, as demonstrated by the

lower observed strains. The consistent higher performance of SWNTs over the equivalent MWNT hydrogels can be directly attributed to more CNTs being dispersed per carbon mass for SWNTs, this results in a higher CNT concentration (at equivalent carbon concentrations), assuming the structures behave according to the law of mixtures.

The effect of Chitosan concentration can be explained by two competing mechanisms, firstly the dispersion quality of CNTs and secondly the ability of chitosan to participate in the EDC cross-linking reaction of collagen due to the amine group within the chitosan structure. In terms of the maximum observed tensile stress for the samples for SWNT dispersions stress decreases with increasing chitosan concentration; whereas for MWNT dispersions the stress increases with increasing chitosan concentration. This occurs as for SWNTs the lower concentration of chitosan is optimal for the dispersion formation resulting in minimal excess chitosan being present within the structure. This results in any secondary cross-linking that occurs between the Chitosan and Collagen binding the collagen tighter to the CNTs. With increasing chitosan concentration this beneficial effect is lost as excess chitosan is free to react with EDC without any bond to CNTs weakening the structure. For MWNTs the lower concentration of chitosan is not sufficient to fully disperse the CNTs at this concentration, due to the difference in radius of curvature for the SWNTs and MWNTs resulting in poorer load transfer between the CNTs and the collagen for these samples. At higher collagen concentrations the synergistic effect between chitosan, EDC and collagen is again observed. The combination of these factors, load transfer, CNT diameter and dispersion quality, and chitosan – collagen cross-linking, lead to the identification of combination number two having the best mechanical properties, highlighted by a 167 % improvement in toughness compared to the base collagen hydrogel. It is important to note that these values attained for mechanical properties are

for the composites material under tension. In the cardiac system the expansion of the myocardium wall would lead to significant bending and torsional stresses on the material. Furthermore, the flexibility of the casting technique for the formation of the hydrogels allows for the tuning of physical dimensions to match the required flexural properties via controlling the mould structures. The key result is the dramatic increase in mechanical properties attainable even at low CNT loadings, which can be tailored through a range of factors. The formation of hydrogels with a homogenous CNT distribution through the collagen matrix leads to the observed increase in mechanical properties. These mechanical reinforcement results provide a platform for future development of tough hydrogel materials, and opens avenues for using other 1D or 2D nanomaterials as reinforcing agents targeting specific applications [30, 31].

Equally as important as mechanical strength for tissue engineering is the ability of the scaffold to degrade, and ideally, degrade in a controllable manner. For thermal degradation, no change was observed in any sample prior to one week, after one month the collagen only samples were observed to have experienced a mass loss of between 20 and 100 %, a significant variation; whereas no CNT containing hydrogel had observed a mass loss greater than 5 %. This result indicates that the addition of CNTs increases the thermal stability of the collagen hydrogels. A likely mechanism for this is the entanglement of un-crosslinked collagen chains around the CNTs resulting in an entangled network preventing the slow disintegration of the structure through diffusion.

The addition of collagenase for enzymatic degradation experiments reversed the result obtained for thermal degradation. In this case all samples were observed to fully degrade over the course of a week. In absence of *in vivo* experiments these two degradation results indicate the potential to tune the degradation rate of the samples through the addition of collagenase.

Table 3. Enzymatic degradation experiments for hydrogels in 37 °C PBS solution with 100 µL/mL collagenase added.

Time (days)	% Mass Loss									
	Control	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
0	0	0	0	0	0	0	0	0	0	0
1	33	20	31	21	15	12	16	23	2	7
2	57	43	51	44	40	42	43	59	17	20
3	67	59	76	52	47	60	51	80	25	39
4	74	100	100	100	100	100	100	100	60	61
6	100	-	-	-	-	-	-	-	100	100
Thermal Only 30 days	60±30	99±1	97±2	98±1	98±1	99±1	97±1	96±3	98±2	99±1

The ability of the matrices to sustain a viable cell population over a longer period of time was evaluated using iPS cells' grown on CNT:Collagen scaffolds. This study demonstrated a clear

differentiation between scaffolds containing SWNT ($\phi_{ave} = 1.5$ nm) and those containing MWNT ($\phi_{ave} = 8.0$ nm), with live cells represented in green and dead cells represented in red (Figure 3). The iPS cells on the SWNT containing scaffolds are confluent, adherent and formed a dense coating over the visible surface at all examined concentrations. In contrast the iPS cells cultures on MWNT containing hydrogels formed only sparse coatings with many areas remaining uncoated by live cells. Hydrogel composition No.9 consisting of both SWNT and MWNT combined was shown to be an exceptionally poor surface for iPS cell growth and adhesion, due to the surface heterogeneity induced by the mixing of individual MWNT and SWNT dispersions.

This suggests that iPS cell viability on CNT:Collagen hydrogels is dependant on the type of CNT incorporated as opposed to the CNT concentration within the hydrogel or the chitosan loading. This result is in opposition to that of Watari *et. al.* who observed improved adhesion, growth, and differentiation of mouse iPS cells on MWNT sheets compared to SWNT sheets (however both performed worse than a collagen sheet) [32]. We propose that this contradiction arises due to the fact that the MWNTs are changing the surface chemistry and morphology of the collagen hydrogel to a greater extent than the SWNTs. The synthesis methods for the initial dispersion play a role in the cause of this difference. As the dispersion composition is constant for SWNT and MWNT in terms of surfactant added per mass of carbon, there is excess surfactant per CNT surface for the MWNT compared to the SWNT (as only the outer shell of the MWNT can be wrapped in the surfactant). This difference in free surfactant concentration means that the distribution of both the surfactant and CNTs within the collagen hydrogel will vary between SWNT and MWNT batches even given equivalent synthesis conditions.

Figure 3. iPS cells seeded on CNT collagen – hydrogels; a) false colour SEM images of iPS cells on hydrogel combination No.1 (i) scale bar = 1 μ m, ii) scale bar = 2 μ m); Live dead stains of iPS cells cultured on different hydrogels; b) tissue culture plastic; c) collagen only hydrogel; hydrogel combination d) No.1; e) No.2; f) No.3; g) No.4; h) No.5; i) No.6 j) No.7; k) No.8; and l) No.9 (scale bar = 100 μ m)

In order to probe the behaviour of cardiac cells on our CNT collagen scaffolds we studied the effect of different material compositions on beating atrial HL-1 cells using the patch clamp technique.

Action potentials (APs) recorded from confluent atrial HL-1 cells commonly have two different shapes. One “*pacemaker*” with a clear diastolic depolarization phase, located at or close to the pacemaking site. One “*atrial*” were the maximal diastolic potential occur near threshold of the following action potential (Figure 4). This is a propagated action potential that indicates electrical coupling between the confluent cells. Single atrial HL-1 cells on the other hand do not consistently demonstrate spontaneous activity. This provides a model to study the spontaneous beating, the action potentials electrical properties and the coupling between the cardiomyocytes. We examined the effect on HL-1 APs when cultured on different CNT:Chitosan:Collagen hydrogels and the results are summarized in Figure 4.

Figure 4. Action potentials (APs) recorded from atrial HL-1 cells cultured on different CNT:Collagen hydrogels (No.1-8). To the left; Summary of spontaneous activity and APs shapes recorded from atrial HL-1 cells on various hydrogels. The shape of the APs were characterized as described in the Methods and the composition of the hydrogels are found in Table 1. To the right; Example of APs shapes, pacemaker (top), atrial (middle) and double peaks (early after depolarizations (EADs)) (bottom).

Confluent atrial HL-1 cells cultured on coverslips coated with fibronectin/gelatin mostly displayed atrial APs (n=14) and HL-1 cells cultured directly on coverslips without coating had an equal distribution of pacemaker/atrial APs (n=9). When HL-1 cells were cultured on CNT:collagen hydrogels with SWNTs, 0.5 mg/L or 2 mg/L CNT together with 1 % Chitosan surfactant, APs were comparable to fibronectin/gelatin coated control, No.1 (n=12) and No.2 (n=13). When the amount of chitosan increased to 5 % only APs with pacemaker shape were seen and spontaneous beating decreased, No.3 (n=9) and No.4 (n=4). The results on SWNT scaffolds demonstrate that at higher chitosan concentrations there is a significant loss of electrophysiological performance of the HL-1 cells. This is in contrast to the IPS cell growth results which show excellent cell growth over all SWNT matrices, and emphasises the need to study the electrophysiological performance of a matrix in addition to simple biocompatibility for cardiac applications. HL-1 cells were also cultured on the MWNT CNT:collagen hydrogels. In accordance with a lower growth/confluency, as for the human iPS cells, the HL-1 cells had less spontaneous activity and more pacemaker APs were recorded compared to propagated atrial APs. The APs on MWCNTs also showed a third action potential shape, similar to early after depolarizations (EADs), which can arise due to blockage of potassium channels, No 5 (n=9) and No.7 (n=8). Due to difficulties in studying a stable whole cell from matrix No.6 and No.8 with 2.0 mg/mL MWNTs we only focused on No.8 (n=7), showing that most of the cells lack spontaneous activity. Previous studies have shown that MWCNTs decrease potassium channel activity in PC12 cells [33], also in cardiac cells, whereas MWCNTs prolong cardiac action potential duration and induce bradycardia in vivo [34], and in hippocampal CA1 neurons, were the excitability increase due to blockage of potassium channels [35].

These electrophysiological results correlate well with the iPS cell growth results whilst allowing us to study the interaction of the live cells on the hydrogels. SWNTs were shown to have a beneficial effect on cell beating, with matrix No.2 appearing an optimal combination of cell growth and electrophysiology, while poor adhesion and potassium channel blocking disrupted cell beating on MWNT scaffolds.

The successful performance of SWNT based CNT:Collagen scaffolds supports the findings of Pok *et al.* who demonstrate that SWNTs at low, subtoxic, concentrations can act as electrical bridges between cardiomyocytes and hence improve the performance of resistive polymer tissue engineering scaffolds as cardiac patch materials [21]. Furthermore, the significant variance in cell response between hydrogels containing SWNTs vs MWNTs demonstrates the need for application specific studies moving forward with a specific type and functionality of carbon nanotube. The SWNT:Collagen matrix provides a key development in conductive, mechanically reinforced TE scaffolds for a cardiac patch.

3. Conclusion

Herein we successfully demonstrated the synthesis of a hybrid CNT:Collagen hydrogel and preliminarily assessed its relevance for the treatment of myocardial infarction. The addition of the CNTs did not lead to a significant change in microstructure of the collagen scaffold yet led to an increased tensile stress, Young's modulus and toughness whilst adding electrical conductivity to the hydrogels. Thermally the CNT loaded hydrogels were observed to be stable over the course of one month and enzymatically degrade over one week. The hydrogels loaded with SWNTs provided

excellent surfaces for iPS cell growth and differentiation, however the MWNT loaded hydrogels showed significantly poorer cellular adhesion. The electrophysiology properties of cardiomyocytes cells grown on these substrates demonstrated that the free chitosan present in the hydrogels used as a surfactant for CNT dispersion attenuated the spontaneous action potentials of the cells significantly providing an explanation for the poor MWNT:Collagen iPS growth. Electrophysiology of HL-1 cells studied by the patch clamp technique demonstrated the SWNT and chitosan concentrations were sub-toxic for matrix combinations No.1 and No.2 whereas combinations No.3 through No.8 resulted in a significant number of non-beating cells. We demonstrated that the optimal studied concentration CNT:Collagen hydrogels for treating myocardial infarction is made from a CNT:Chitosan dispersion containing 2 g/L CNTs, 1.0 % w/v chitosan dispersant using SWNTs. Overall this cardiac patch material provides improvement in two key areas for addressing myocardial infarction; it provides mechanical support for the weakened ventricular wall/prevention of ventricular remodeling; and the promotion of cell survival, proliferation and differentiation/maintenance of cardiomyocyte phenotype as shown by the preservation of the ability to continue to generate action potentials. Overall, these scaffolds provide great promise for cardiac tissue engineering fulfilling the requirements of mechanical durability, electrical conductivity, biocompatibility and degradation.

4. Experimental Section

4.1. Cardiac Patch Synthesis

4.1.1. Carbon Nanotube Dispersion

Carbon nanotube dispersions were formed in milli-q water with the addition of CNTs equating to 0.5 g/L or 2.0 g/L with 2 % wt/v of Chitosan solution added with a volume proportional to the added CNTs. This mixture was sonicated for 2 hrs at a 40 % intensity of a 400 W sonic horn.

4.1.2. Collagen Cross-Linking

Collagen-CNT hydrogels were synthesized by mixing the CNT dispersion with an 18% v/v collagen solution and 2M NaOH. (or MES only for the control experiments); These materials were mixed thoroughly. EDC and NHS solutions were added to the above mixture at a 1:1 molar ratio and mixed rapidly to initiate cross-linking. The samples were then dispensed into the desired molds and cured at room temperature for 16 hours followed by 8hrs of curing in a 37 °C incubator. The samples were removed from the molds by immersing the samples in PBS for 5 minutes before gently separating the mold sections. The final samples were stored in PBS at 4 °C.

4.2. Characterisation

4.2.1. Physical Characterisation

Water Content Analysis

Water content analysis was performed by examining the difference in mass of hydrated hydrogels stored in PBS at 4 °C and the same sample after drying in a dessicator for two days. The samples were then re-immersed in PBS at 4 °C for two days, this cycle was repeated up to ten times to determine the cycle-ability of the hydration process.

Tensile Testing

Mechanical testing of the hydrogels was performed on samples immersed in milli-q water heated to 37 °C using a Instron 3343 equipped with a Biopuls bath to best mimic the mechanical performance of these materials *in vivo*. The dogbone shaped hydrogels were subjected to constant strain experiments with elongation set to 5.0 mm/min.

4.2.2. Biomaterial Characterisation

HL-1 cells and electrophysiology

HL-1 cardiomyocytes, derived from the from the AT-1 mouse *atrial cardiomyocyte* tumor lineage, were a kind gift from Claycomb (Claycomb et al, 1998). Cell cultures were grown in 5 % CO₂ at 37 °C and maintained in Claycomb medium, supplemented with batch-specific 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM L-glutamine and 100 U/ml and 100 µg/ml penicillin-streptomycin. Cells were enzymatically dissociated with 0.05 % trypsin-EDTA and culturing flasks were coated with 0.02 % gelatin; 0.5 % fibronectin overnight.

HL-1 cells (20.000 cells/ml) were cultured direct on hydrogels (or for control on 13 mm coverslips, with or without gelatin; fibronectin coating). Spontaneously action potentials were recorded after 1-2 days with an Axopatch 200B amplifier (Axon Instrument) in whole-cell configuration, current clamp mode ($I=0$), of the patch clamp technique. Signals were acquired at 10 kHz, low pass filtered at 5 kHz and digitized by a Digidata 1440A converter (Molecular Devices). pClamp 10.3 software (Molecular Devices) was used to obtain data. The extracellular solution contained (in mM): 5.4 KCl, 140 NaCl, 1 MgCl₂, 1.8 CaCl₂, 10 Glucose and 10 HEPES (pH 7.4 adjusted with NaOH). The intracellular (pipette) solution contained

(in mM): 130 K-gluconate, 9 KCl, 8 NaCl, 10 EGTA, 1 MgCl₂, 10 HEPES and 3 Na₂ATP (pH 7.3, adjusted with KOH). All chemical products used were purchased from Sigma Aldrich. Pipettes were made from borosilicate glass capillaries (Harvard apparatus Ltd) and had a resistance between 4-6 MΩ when filled with intracellular solution. For all measurements the temperature was maintained at 35 °C. Liquid junction potential were not adjusted for (14.6 mV). Characterization of the action potentials (APs) were done based on the shape of the AP, the RR interval (time between peaks) and action potential duration at 90 % of repolarization (APD₉₀) accordingly; Pacemakers, APs with a clear diastolic depolarization phase. Atrial, APs were the maximal diastolic potential occurred near the threshold of following AP. Double peaks (EADs), occurrence of APs with double peaks similar to early after depolarizations (EADs). Non-beating, when a stable whole cell could be achieved but no APs were seen, or the RR-interval were over 3 seconds, or the APD₉₀ were over 1.5 seconds.

Degradation Testing

The degradation rate of scaffolds was investigated by monitoring the mass loss of the samples immersed in PBS and in collagenase solutions as a function of exposure time.

Thermal degradation was performed by immersing 6 repeats per CNT:Collagen hydrogel in PBS in an incubator at 37 °C. The samples were weighed at 0, 1, 2, 3, 4, and 30 days with the calculated weight loss assigned as the observed degradation.

Enzymatic degradation was performed by immersing the hydrogel samples in PBS containing 100 $\mu\text{L}/\text{mL}$ collagenase in a 37 °C incubator for 0, 1, 2, 3, 4 and 7 days. All samples were weighed at the above time intervals to determine their biodegradation rate.

iPS Cell Compatibility Testing

Induced pluripotent stem cells (iPS) were generated from human fibroblasts by retroviral induction of four transcription factors and cultured as described previously [36]. Same number of iPS cells (~ 5.000) was seeded on scaffolds and cultured for 72 h. LIVE/DEAD® Viability/Cytotoxicity kit (Life Technologies) was used to investigate cell adhesion, viability, and proliferation rate. Briefly, prior to the assay, cells on scaffolds were washed in PBS to remove serum esterase activity present in serum-supplemented growth media. Next, solution of 2 μM calcein AM and 4 μM EthD-1 in PBS was prepared and added to cells. Cells were incubated with dyes at room temperature for 20 minutes and then analyzed using Axio Vert.A1 (Carl Zeiss AG) inverted fluorescence microscope. Live cells emitted green fluorescence, whereas dead cells emitted red signal.

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Conductivity and mechanical strength are two core requirements for a tissue engineering patch targeting myocardial infarction. Combining carbon nanotubes with collagen hydrogels creates an electrically conductive, mechanically strong, biocompatible materials for the support of both stem and myocardial cells. Patch clamping shows a demonstrable effect of the carbon nanotube concentration, and type on the cellular response.

Keywords: Carbon Nanotube, Hydrogel, Collagen, Myocardial Infarction, Stem Cell

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References

1. *Cardiovascular Diseases (CVDs)*. 2015, World Health Organisation - Fact Sheet No 317.
2. Blom, A.S., et al., *Cardiac Support Device Modifies Left Ventricular Geometry and Myocardial Structure After Myocardial Infarction*. *Circulation*, 2005. **112**(9): p. 1274-1283.
3. Shah, P.K., *Preservation of Cardiac Extracellular Matrix by Passive Myocardial Restraint: An Emerging New Therapeutic Paradigm in the Prevention of Adverse Remodeling and Progressive Heart Failure*. *Circulation*, 2005. **112**(9): p. 1245-1247.
4. Locke Davenport, H., et al., *Biomaterial based cardiac tissue engineering and its applications*. *Biomedical Materials*, 2015. **10**(3): p. 034004.
5. Qazi, T.H., R. Rai, and A.R. Boccaccini, *Tissue engineering of electrically responsive tissues using polyaniline based polymers: A review*. *Biomaterials*, 2014. **35**(33): p. 9068-9086.
6. Gelmi, A., et al., *Influence of conductive polymer doping on the viability of cardiac progenitor cells*. *Journal of Materials Chemistry B*, 2014. **2**(24): p. 3860-3867.
7. Haraguchi, Y., et al., *Concise Review: Cell Therapy and Tissue Engineering for Cardiovascular Disease*. *Stem Cells Translational Medicine*, 2012. **1**(2): p. 136-141.
8. Mehta, P., et al., *Pharmaceutical and biomaterial engineering via electrohydrodynamic atomization technologies*. *Drug Discovery Today*, 2017. **22**(1): p. 157-165.
9. Rasekh, M., et al., *Electrospun PVP–indomethacin constituents for transdermal dressings and drug delivery devices*. *International Journal of Pharmaceutics*, 2014. **473**(1–2): p. 95-104.
10. Ahmad, Z., E. Stride, and M. Edirisinghe, *Novel preparation of transdermal drug-delivery patches and functional wound healing materials*. *Journal of Drug Targeting*, 2009. **17**(9): p. 724-729.
11. Hardy, J.G., P. Lin, and C.E. Schmidt, *Biodegradable hydrogels composed of oxime crosslinked poly(ethylene glycol), hyaluronic acid and collagen: a tunable platform for soft tissue engineering*. *Journal of Biomaterials Science, Polymer Edition*, 2015. **26**(3): p. 143-161.
12. Zhang, X., et al., *Application of hydrogels in heart valve tissue engineering*. *J Long Term Eff Med Implants*, 2015. **25**(1-2): p. 105-34.

13. Van Vlierberghe, S., P. Dubruel, and E. Schacht, *Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review*. *Biomacromolecules*, 2011. **12**(5): p. 1387-408.
14. Rafat, M., et al., *Composite core-and-skirt collagen hydrogels with differential degradation for corneal therapeutic applications*. *Biomaterials*, 2016. **83**: p. 142-155.
15. Sherrell, P.C., et al., *Cardiac and stem cell-cocooned hybrid microspheres: A multi factorial design approach*. *Sensors and Actuators B: Chemical*, 2016. **236**: p. 480-489.
16. Sridharan, I., T. Kim, and R. Wang, *Adapting collagen/CNT matrix in directing hESC differentiation*. *Biochemical and Biophysical Research Communications*, 2009. **381**(4): p. 508-512.
17. Cho, Y. and R.B. Borgens, *The effect of an electrically conductive carbon nanotube/collagen composite on neurite outgrowth of PC12 cells*. *Journal of Biomedical Materials Research Part A*, 2010. **95A**(2): p. 510-517.
18. MacDonald, R.A., et al., *Collagen-carbon nanotube composite materials as scaffolds in tissue engineering*. *Journal of Biomedical Materials Research Part A*, 2005. **74A**(3): p. 489-496.
19. Gopalakrishnan, R. and V. Subramanian, *Interaction of Collagen with Carbon Nanotube: A Molecular Dynamics Investigation*. *Journal of Biomedical Nanotechnology*, 2011. **7**(1): p. 186-187.
20. Shin, S.R., et al., *Carbon-Nanotube-Embedded Hydrogel Sheets for Engineering Cardiac Constructs and Bioactuators*. *ACS Nano*, 2013. **7**(3): p. 2369-2380.
21. Pok, S., et al., *Biocompatible Carbon Nanotube-Chitosan Scaffold Matching the Electrical Conductivity of the Heart*. *ACS Nano*, 2014. **8**(10): p. 9822-9832.
22. Balint, R., N.J. Cassidy, and S.H. Cartmell, *Conductive polymers: Towards a smart biomaterial for tissue engineering*. *Acta Biomaterialia*, 2014. **10**(6): p. 2341-2353.
23. Silvestri, A., et al., *Biomimetic Materials and Scaffolds for Myocardial Tissue Regeneration*. *Macromolecular Bioscience*, 2013. **13**(8): p. 984-1019.
24. Muzzarelli, R.A.A., *Human enzymatic activities related to the therapeutic administration of chitin derivatives*. *Cellular and Molecular Life Sciences CMLS*, 1997. **53**(2): p. 131-140.
25. Lovett, M., et al., *Vascularization Strategies for Tissue Engineering*. *Tissue Engineering Part B: Reviews*, 2009. **15**(3): p. 353-370.

26. Hirt, M.N., A. Hansen, and T. Eschenhagen, *Cardiac Tissue Engineering: State of the Art*. Circulation Research, 2014. **114**(2): p. 354-367.
27. Rafat, M., et al., *PEG-stabilized carbodiimide crosslinked collagen–chitosan hydrogels for corneal tissue engineering*. Biomaterials, 2008. **29**(29): p. 3960-3972.
28. McLaughlin, C.R., et al., *Regeneration of Corneal Cells and Nerves in an Implanted Collagen Corneal Substitute*. Cornea, 2008. **27**(5): p. 580-589.
29. Rafat, M., et al., *Surface modification of collagen-based artificial cornea for reduced endothelialization*. Journal of Biomedical Materials Research Part A, 2009. **88A**(3): p. 755-768.
30. Li, X., et al., *Recent Advances in Synthesis and Biomedical Applications of Two-Dimensional Transition Metal Dichalcogenide Nanosheets*. Small, 2016: p. 1602660-n/a.
31. Liu, J., et al., *Synthesis of Graphene Peroxide and Its Application in Fabricating Super Extensible and Highly Resilient Nanocomposite Hydrogels*. ACS Nano, 2012. **6**(9): p. 8194-8202.
32. Akasaka, T., et al., *Adhesion of human osteoblast-like cells (Saos-2) to carbon nanotube sheets*. Bio-medical materials and engineering, 2009. **19**(2-3): p. 147-153.
33. Haifei, X., et al., *Multi-walled carbon nanotubes suppress potassium channel activities in PC12 cells*. Nanotechnology, 2009. **20**(28): p. 285102.
34. Tan, X.-Q., et al., *Multi-Walled Carbon Nanotubes Impair Kv4.2/4.3 Channel Activities, Delay Membrane Repolarization and Induce Bradyarrhythmias in the Rat*. PLoS ONE, 2014. **9**(7): p. e101545.
35. Chen, T., et al., *Multi-walled carbon nanotube increases the excitability of hippocampal CA1 neurons through inhibition of potassium channels in rat's brain slices*. Toxicol Lett, 2013. **217**(2): p. 121-8.
36. Cieślak-Pobuda, A., et al., *The expression pattern of PFKFB3 enzyme distinguishes between induced-pluripotent stem cells and cancer stem cells*. Oncotarget, 2015. **6**(30): p. 29753-29770.

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