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Cell electrospinning cardiac patches for tissue engineering the heart

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Cell electrospinning has tremendous applicability to a wide range of uses within both the laboratory and clinic. This has directly resulted from the technology's unique ability to immobilize multiple cell types with a wide range of molecules simultaneously within a fiber during the scaffold generation process. The technology has been shown to generate many cell laden complex architectures from true three-dimensional sheets to those multi-core vessels. Although those studies have demonstrated the versatility of this platform biotechnology, we show here for the first time the ability to immobilize primary cardiac myocytes within these fibers in our quest to develop this technology for creating three-dimensional cardiac patches which could be used for repairing, replacing and rejuvenating damaged, diseased and/or ageing cardiac tissues. These advances are unrivalled by any other technology currently available in the regenerative medicine toolbox, and have many interesting ramifications to repairing a damaged heart.

Electrospinning is an electric field driven fiber and scaffold generation technology, which has been around for over a century. [1] The technology has been used for many applications, however in the context of tissue engineering the technology has been explored as a method for forming scaffold substrates on which cells are manually seeded and later explored for engineering tissues. Although some success has been achieved, there are major roadblocks, which have inhibited electrospinning truly making a mark on tissue engineering. This results from the generated scaffold significantly limiting cell infiltration throughout the thickness of the scaffold, the poor accessibility of nutrients into the scaffold to initiating foreign body reactions. These obstacles have not been overcome and therefore has seen electrospinning standing still in this respect. In 2005 Jayasinghe *et al.,* [2, 3] demonstrated the ability to directly electrospin living cells for forming living scaffolds. The cells were assessed for their viability in comparison to controls and shown to be indistinguishable. These studies put electrospinning back into the tissue engineering enterprise and was coined "cell electrospinning (CE)". Since then Jayasinghe *et al.,* [4, 5] have demonstrated the complete inertness of this technology by carrying out biological interrogation from the molecular level upwards to mouse model-based in-vivo investigations.

The uniqueness of this platform technology [5] over all other cell scaffolding technologies is that a) it is able to process both small and large quantities of cells (in a wide range of permutations and combinations) with a raft of molecules for generating cell-laden fibers and scaffolds, b) the ability to generate architectures having true three-dimensional features in particular in the z-axes (which could be varied with ease) to c) the ability to handle practical quantities for cells while also tuning the system to not only align the cell-bearing fibers as required over a functional of depth but also to cross stitch the architecture during the forming stage. Interestingly cross-stitching allows the ability to incorporate and control the stiffness of a generated cell-bearing fibrous scaffold for withstanding internal and external pressures

within the anatomy post-transfer, in cases of vessels accommodating flow to the anchoring of a patch containing a therapeutic payload or in the context of this article for repairing the heart where the architecture is found to undergo a twisting like deformation during the pumping of blood and nutrients around the vasculature. These unique features can only be replicated by our other pioneered technologies, which are referred to as aerodynamically assisted bio-threading, pressure driven/assisted cell spinning. [6, 7] That being said in this communication we will only focus on cell electrospinning.

At present there is an unmet demand for repair tissues for replacing damaged, diseased and/or ageing tissues. This need is most evident in the field of cardiac medicine where architectures of the kind generated by cell electrospinning could revolutionize cardiac medicine and surgery. Therefore in this article we demonstrate for the first time the ability to directly handle primary neonatal cardiac myocytes as concentrated cellular suspensions for cell electrospinning a functional cardiac scaffold. In these studies we have assessed the viability of post-cell electrospun cells using a range of proteins (MyBP-C, sarc. alpha-actinin, F-actin, connexin-43, and myomesin) in comparison to controls. These investigative studies demonstrate the possibility of generating fully functional and thick cardiac tissues for repair, replacement and rejuvenation (with the incorporation of a therapeutic payload) on demand.

Detailed equipment set-up and methods explored in these cell electrospinning studies could be found in the associated supplementary file. In brief primary cardiac cells were isolated from rats as previously described [8], and primary cell suspensions were prepared as described. The equipment arrangement explored in these studies is similar to those used in our previous studies. [2-5] Several cell samples were assessed post-treatment in comparison to control cell samples using microscopy and flow cytometry. These studies also explored transfected cells for imaging purposes for identifying cells within the fibers and scaffolds. Finally

immunofluorescence coupled with confocal microscopy was explored as previously described [8, 9] for assessing a range of proteins (MyBP-C, sarc. alpha-actinin, F-actin, connexin-43, and myomesin) for assessing the functionality of post-cell-electropun cells in comparison to control cells.

The equipment explored in these studies was a basic cell electrospinning equipment set-up using a concentric needle arrangement as those explored in our previous studies, [10] with the addition of an ammeter for measuring the current between the electrodes in real time. In these investigations the equipment arrangement consisted of a coaxial needle system having a high voltage power supply connected to it and capable of supplying a voltage of upto ±30kV (FP-30, Glassman Europe Ltd, Tadley, UK). The inlet of the charged coaxial needles were connected to two syringes placed on two individual syringe pumps (PHD 4400, HARVARD Apparatus Ltd., Edenbridge, UK), having the capacity of varying the flow rate from ~10^{-15}-10^{-5} m^3s^{-1}. Below the coaxial charged needle system a sterile petri dish was placed on a grounded mesh target electrode. The grounded electrode was placed on a controlled heated surface capable of maintaining the fluid in the petri dish at approximately 37^0C. This entire cell electrospinning set-up was arranged within a class II laminar flow safety hood (**Figure 1A**). A wide range of parametric studies were carried out to identify which operational conditions would be suitable for generating cell-laden composite living fibers containing the primary cardio myocytes. These studies enabled us to identify and generate cell-bearing fibers and scaffolds at an applied voltage of ~8kV for an electric field strength of ~0.4kV/mm (**Figures 1B and 1C**).

It should be noted that cells used for flow cytometry and those studies on proteins were not labeled with GFP as this would have interfered with the analysis. Hence only for the purposes of demonstrating the intact cardiac cells within fibers, a combination of brightfield and

fluorescent microscopy was used in conjunction with those cells transfected with a GFP adenovirus. This enabled the clear identification of cells within fibers and scaffolds (**Figure 1C**).

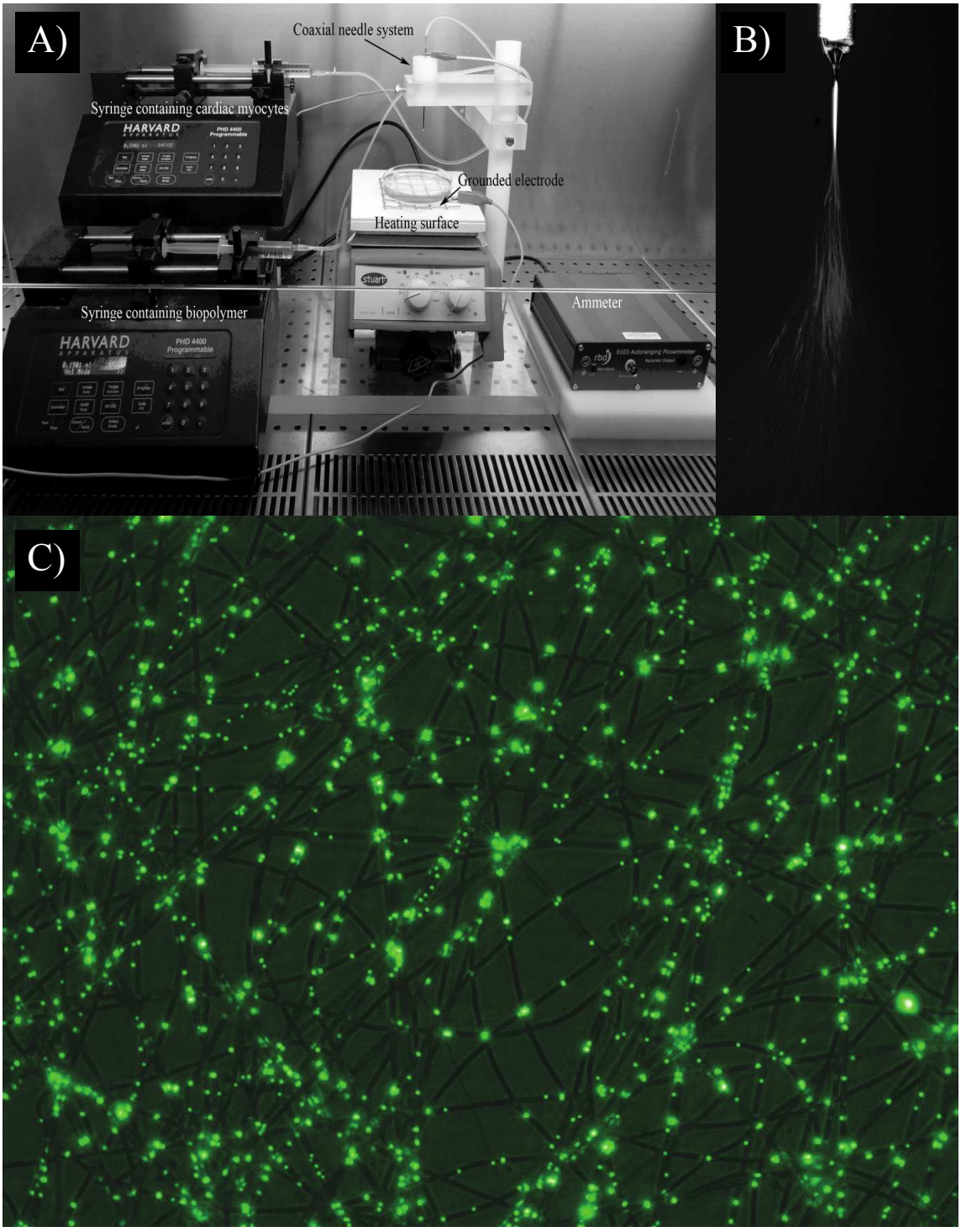

Figure 1. Characteristic digital micrographs elucidating A) the cell electrospinning set-up within a class II laminar flow safety hood, B) the cell electrospinning process in action in the stable spinning mode. Panel C) depicts a combined brightfield and fluorescent image of the cardiac cell bearing fibers/scaffold collected over a function of time. The cardiac cells were labeled as our previous work using a GFP adenovirus.

In these experiments we assessed two groups, namely 1) which contained those samples which were subjected to cell electrospinning (CE) and 2) which were those control cells (CC) that were not subjected to any form of physical treatment. Several samples of cell electrospun cardiac myocyte scaffolds were generated and were subjected to the cell recovery process as previously mentioned in our studies. [5] Recovered cells from these samples were individually aliquoted alongside those control cells, which were later labeled as per manufactures protocols (BD Bioscience). Following cellular labeling the cells were analyzed using flow cytometry for 10000 events. The cellular samples referred to as control samples are those samples that have been prepared into a cell suspension as those that were for cell electrospinning but these cells were not subjected to any other post-processing. Repeated flow analysis on those cell electrospun and control samples demonstrated that those cells subjected to the cell electrospinning process were indistinguishable from control cells (**Figure 2**).

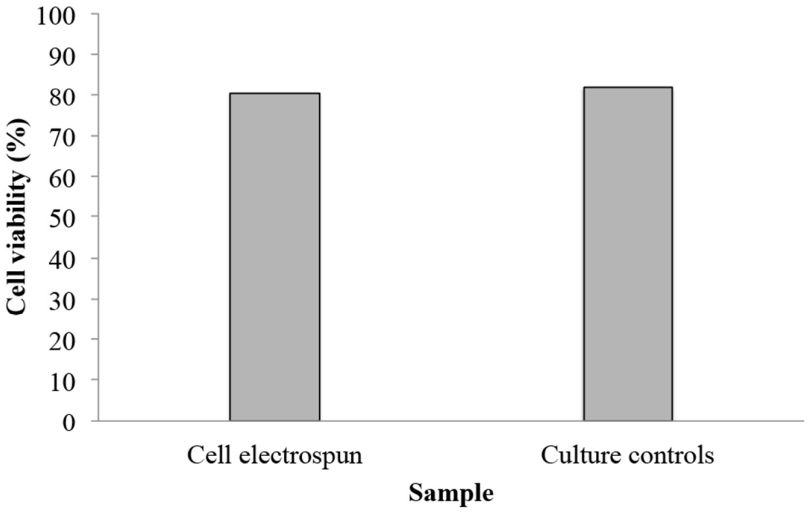

Figure 2. Flow analysis was carried out on several samples in the two groups for the assessment of cellular viability. These two groups contained samples of a) those cells that were cell electrospun, and b) those cellular samples that were culture controls which were not subjected to any processing. Cells from those cell electrospun samples were recovered/harvested as described and were labeled with Annexin V and PI as described and assessed for their cellular kinetics using flow cytometry for over 10000 events.

These results are consistent with our previous studies, which were carried out with a wide range of other cell types. [2-5] All the media explored in these studies for preparing cellular suspensions have been previously widely studied and used for in-vivo transferring of living cells into host model organisms through subcutaneous injection. [11] It should be noted that as previously stated although the applied voltage on the needles in these investigations are in the kV the applied current is extremely low, in fact in these studies we measured the current between the needles and the grounded electrode, and found it to be in the nanoamperes. This was carried out by connecting a Picoammeter system (9130 Picoammeter, RBD Instruments, Inc Oregon, United States) as seen in **figure 1A**, which measured the current throughout the cell electrospinning process. As previously stated the applied voltage does not directly have a negative effect on the cells as the current in these experiments are very low, if the current and the applied voltage was high the cells would undergo death as seen by those electroporation studies. [12] In the case of electroporation the current is in the 50mA range for an applied voltage of 230V, which is required for making the cellular membrane permeable so that desired molecules could be transported into the cells. This is a technology explored widely by genetics utilizing this technology to transfect cells during which a large population of cells are found to undergo death (>60 depending on cell type). The reader should also bear in mind that in cell electrospinning (as in electrospinning) the charge acquired by the media flowing within the coaxial needle system transfers to the skin of the fluid being drawn out into a cell-bearing

fiber. We assume this charge subsequently is lost exponentially to the surrounding atmosphere in the form of space charge. It is interesting to note that in a clinical setting high intensity electric fields have been explored and to date are still utilized for many applications, in particular for cutting tissues both internally and externally. This technology is widely referred to as electrosurgery or electrocutting. [13]

Since the general viability of the cardiac myocytes was not affected by the electrospinning procedure as shown above, we wanted to further investigate, whether the manipulation had affected the cardiac cytoarchitecture that is well described for these cells in culture. [8, 14] Cardiac myocytes with or without exposure to the cell electrospinning procedure were allowed to attach to cell culture dishes and subsequently stained for proteins that make up their contractile elements, the myofibrils, such as sarcomeric alpha-actinin, MyBP-C and myomesin together with phalloidin to visualize filamentous actin as well as for connexin-43, which constitutes gap junctions between the cardiac myocytes (**Figure 3**). No differences were observed, whether the cardiac myocytes had been cell electrospun or not. Intact myofibrils as shown by the cross-striated pattern of sarcomeric proteins such as sarcomeric alpha-actinin, MyBP-C and myomesin were observed throughout the cytoplasm of the cell electrospun cardiomyocytes (right hand column in Figure 3) and indistinguishable from those in control cardiomyocytes (left hand column). In addition, we could demonstrate the presence of gap junctions in cell electrospun as well as in control cardiomyocytes by staining for connexin-43 (arrows in Figure 3). This indicates that the cardiomyocytes are also electrically coupled. The integrity of the contractile elements and the electrical coupling and thus a lack of deleterious effect of the cell electrospinning procedure on the cardiac cytoarchitecture were also evident by the observation of beating cardiac myocytes under both conditions (see supplementary videos). We therefore conclude that the cell electrospinning procedure does not interfere with the integrity of the cardiac myocytes and that it can therefore be assumed

that they will maintain their differentiated status as evident by the possession of myofibrils and gap junctions also in the cell electrospun fibres/scaffolds.

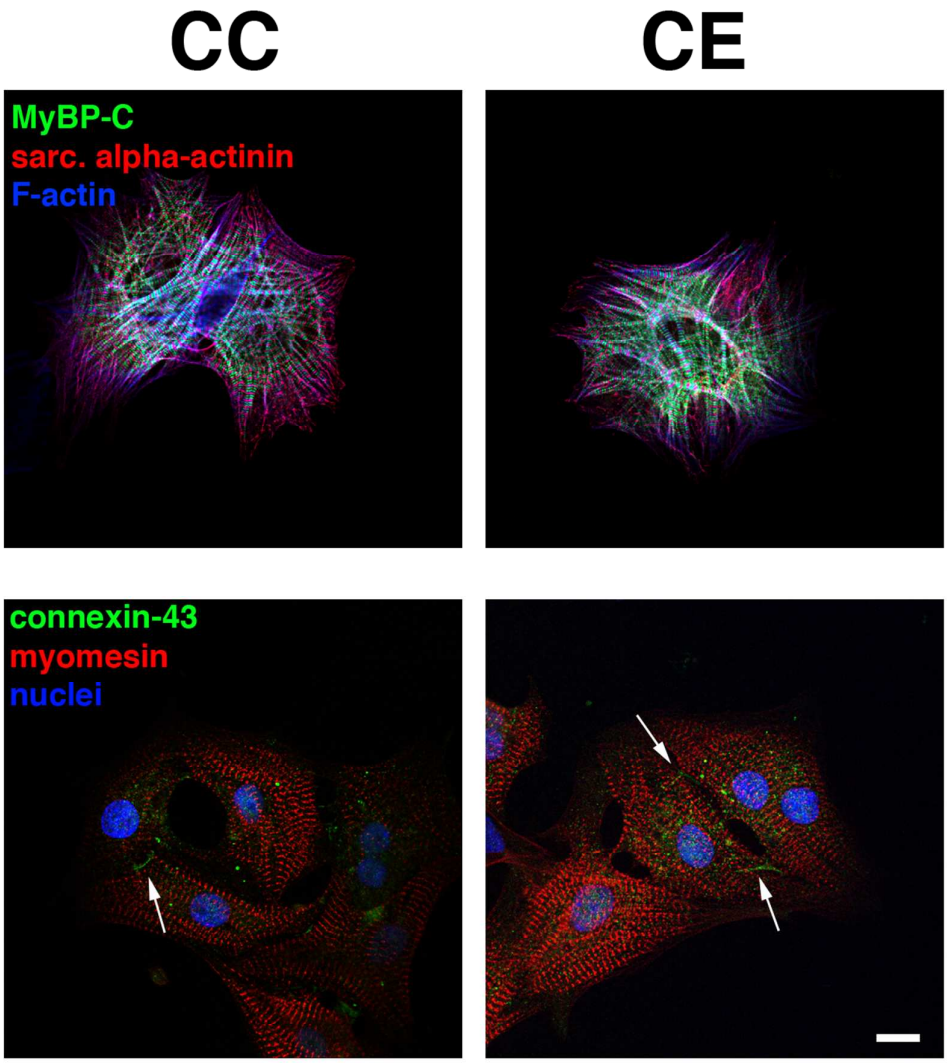

Figure 3: Confocal micrographs of neonatal rat cardiac myocytes that were plated with (CE – cell electrospun cells; right column) or without (CC – culture cells/controls, left column) undergoing the cell electrospinning procedure. Well-structured myofibrils are evident by staining for the A-band protein MyBP-C (green signal in top row), the Z-disc protein sarcomeric alpha-actinin (red signal in top row) and the M-band protein myomesin (red signal in bottom row) and no differences can be observed between the two treatments. In addition, gap junctions between neighbouring cardiac myocytes can be visualized by staining for connexin-43 (green signal in bottom row; arrows). Phalloidin was used to visualize the

filamentous actin cytoskeleton and DAPI for staining the nuclei (blue signal in top respectively bottom row). Scale bar represents 10μm and is applicable to all panles.

These investigative studies carried out on the direct processing of primary cardiac myocytes using cell electrospinning for forming living cardiac fibers and scaffolds demonstrate this concept not only as a promising approach to generating repair, replacement and rejuvenating tissues most useful for cardiac medicine and surgery, but also for forming functional three-dimensional cardiac models. The studies herein demonstrate for the first time the ability to directly cell electrospin cardiac cells into living fibers and scaffolds without causing any alterations on the cell's viability and on critical proteins found in cardiac cells most important for maintaining their function. These studies provide the confidence to pursue this platform technology for directly forming cardiac tissues, which could be generated in any three-dimensional architectural complexities demanded by native tissues for retaining their ability to function within a harsh mechanical and biological environment.

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Cell electrospinning living cardiac tissues for repairing, replacing damaged/diseased and/or ageing cardiac tissues. The investigative studies performed and presented in this communication demonstrates the ability for cell electrospinning to directly handle living primary cardiac myocytes from which living cardiac fibers and scaffolds are generated. This platform technology investigated in these studies holds great promise for cardiac medicine and surgery to diagnostics and bio-analysis of cardiac tissues at all states.

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