A publication of
The Williamsburg BioProcessing Foundation

Spring 2007
ISSN 1538-8786

BioProcessing JOURNAL

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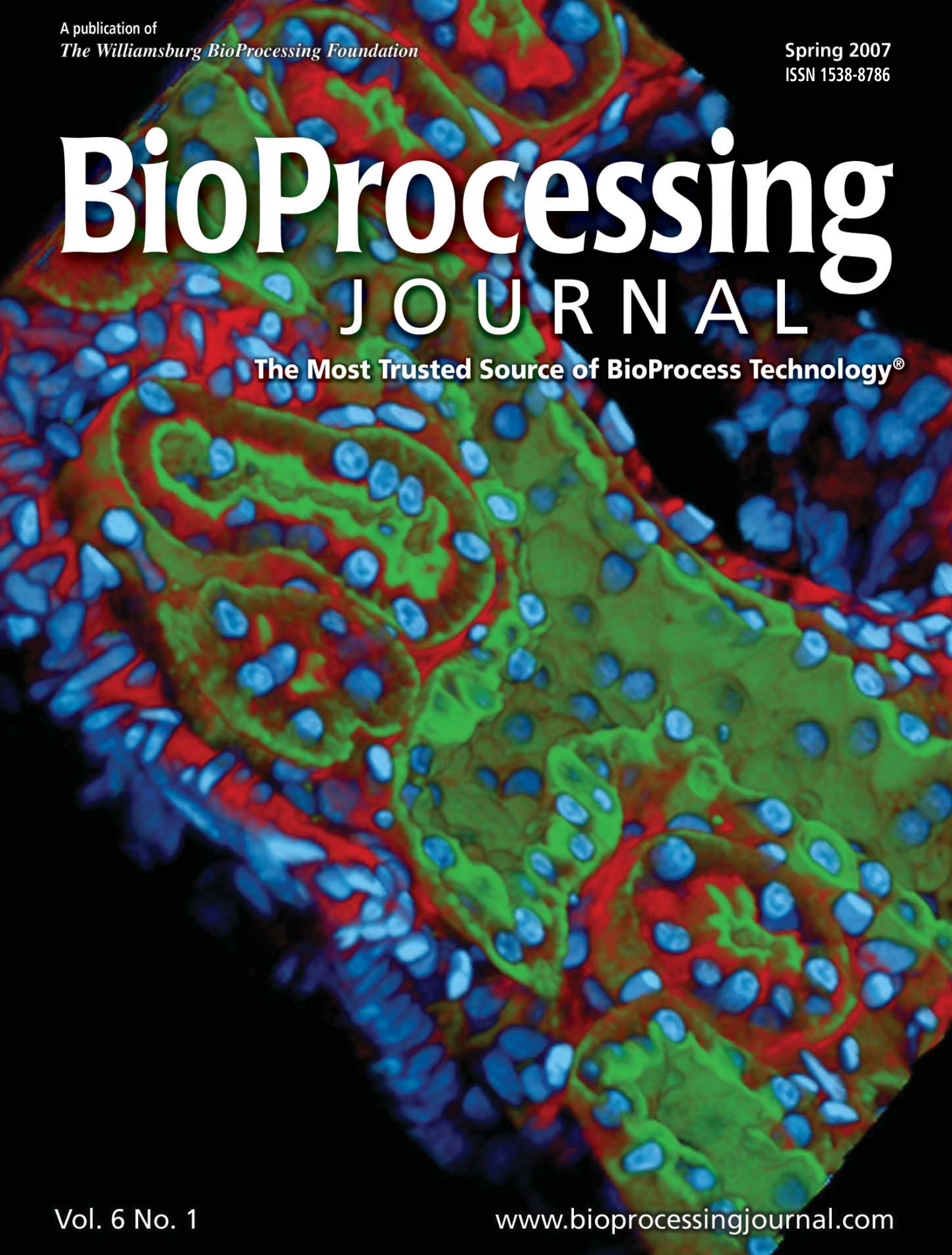

Vol. 6 No. 1

www.bioprocessingjournal.com

Bio-Electrospraying Primary Neonatal Cardiac Myocytes

By SEÁN P. BARRY, SUWAN N. JAYASINGHE*, DAVID S. LATCHMAN, and ANASTASIS STEPHANOU

Controlled cell deposition by way of micrometer-sized jets are increasingly becoming a fiercely pursued area of research. We recently uncovered the ability to jet living cells using one such jetting methodology, now referred to as bio-electrosprays. This technique has never been explored for processing living cells until now.

Electrosprays charge media within a needle, subsequently imposing an external electric field to assist in drawing the media into a micro-jet. The resulting instabilities assist in jet break-up, forming cell-bearing droplets. These droplets containing viable cells could be deposited onto a wide variety of biological and non-biological substrates. In this article, we report our developmental studies into this jet approach with a view to the successful handling and deposition of primary neonatal cardiac myocytes.

Cells were found to beat spontaneously several hours after bio-electrospraying and the procedure had no adverse affect on viability, as assessed by annexin V/7AAD staining. Moreover, there was no activation of stress signalling pathways nor evidence for DNA damage, as assessed by γhistone 2AX (H2AX) activation. Bio-electrospraying was also found to be compatible with gene therapy using adenoviral transduction. Our studies pave the way for the development of a bio-patterning approach for the fabrication of biologically-viable cardiac tissues, as well as employing this route as a jet technique for gene therapy. These studies are the first to demonstrate the feasibility of mechanically constructing a cardiac tissue. They represent a significant step toward the future development of cardiac tissue-engineering and have widespread applications in regenerative and therapeutic medicine, to the advancement of miniaturised bio-components.

Introduction

Advances in the safe handling and deposition of primary living cells have tremendous implications in many fields of life science research. Hence, collaboration between physical and life scientists have escalated. They are jointly exploring the ability to deposit living cells in a controlled manner with applications from regenerative medicine, studies in developmental biology, to the development of biochips and biosensors.

An enormous need for tissue and organ repair and/or replacement exists today. Nowhere is this more evident than in cardiac medicine where artificially-engineered cardiac tissue and vessels could revolutionise cardiac surgery. There are several routes for tissue engineering which are non-jet based but these, although encouraging, have limitations on the developed tissue resolution generally placed in the several hundreds of micrometers (>>100 μm).[1-5]

Although these existing non-jet based routes are continuously undergoing development to produce increased resolution, they are still limited to several tens of micrometers at best. It is also important to note that some of these techniques are particular to specific cell types which limit these tissue development protocols.

Jet-based methodologies have been able to improve these structural resolutions on developed tissues to a few tens of micrometers with a wide range of cells. The fundamentals of ink-jet printing (IJP) [6,7] have inspired the pursuit of engineering living cells via jet technologies, but it has limits. The processable cell concentration in suspension is generally employed with needles of 30-60 μm in diameter. This limitation is brought about by the jetting technology, driven by a controlled thermal explosion or piezoelectricity. These two driving systems are used to squeeze a cellular suspension from a reservoir through a needle to form a droplet which is subsequently deposited. This technique has been rapidly developed in conjunction with controlled movement in the three axes for uncovering a unique, three-dimensional tissue engineering biotechnique.[8]

Several viable tissue constructs have been fabricated, but the needles used for jet processing a suspension has remained its primary limitation. Briefly, these fine needles generate high shear stresses within them, and the cells passing through these needles undergo signifi-

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cant stress, leading to cellular damage. For this reason, a cellular concentration limit exists with a chosen needle size from which a given cell-bearing droplet-to-residue size is achieved. Although IJP has its spatial limitations, it has not only initiated this jetting endeavour, but has set the standard by which other emerging methods for cell handling by jet techniques will be benchmarked.

Electrosprays, an electric field-driven jetting phenomena investigated for over a century[9,10] has been used as a route for forming aerosols, as well as the processing of materials most relevant to materials science and engineering.[11-15] Essentially, this jetting method works on the principle of charging a single or multi-phase medium within a conducting needle which is later exposed to an external electric field. This electric field is brought about by the application of an applied potential difference between the needle, accommodating the flow of media, and a ground electrode which is placed centrally and in-line with the exit of the needle. Remarkably, this technique, unlike IJP, does not have a relationship between its needle diameter and generated droplet and residue sizes, meaning large bore needles are used (generally sized in the several hundreds of micrometers) from which droplets and residues in the micro and nanometer range can be generated with ease.[16-19]

In 2005, bio-electrosprays (BES) were uncovered as possessing the ability to safely jet living cells without compromising their viability.[20,21] Our studies with bio-electrosprays have shown that cellular size limits the spatial resolution in the generated cell-bearing droplets, which is contrary to IJP. We have demonstrated the ability to process, by way of bio-electrospraying, a highly concentrated cell suspension in stable jet conditions from which droplets, an order of magnitude smaller than those generated by ink jets, have been achieved.[22, 23]

In this article, we will show (as a first example) the ability to jet-deposit primary neonatal cardiac myocytes at a cellular concentration of one million cells per ml. This was achieved without compromising their viability in any way, neither was there activation of stress pathways nor evidence for DNA damage. In the purview of these investigations, we also demonstrate the ability to use this technique as a methodology for delivering transduced cardiac myocytes without perturbing their viability. These results not only further establish bio-electrosprays as a competing cell engineering approach, but also show great promise as a regeneration-to-therapeutic medicinal route in cardiac medicine. These results have far reaching implications to the widespread field of life sciences.

Experimental

Formulation of the Primary Cell Suspension

Neonatal rat cardiac myocytes were isolated from the hearts of 1-3 day old Sprague Dawley rats. Hearts were removed and serially digested in ADS buffer (116 mM NaCl, 5.4 mM KCl, 20 mM HEPES, 0.8 mM NaH_2PO_4, 405.7 μM $MgSO_4$, 5.5 mM glucose, pH 7.35) supplemented with 0.1% collagenase. Fibroblasts were allowed to attach for one hour at 37°C and myocytes were plated on gelatin coated plates at a density of 6 x 10^5/ml in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml) and 15% fetal calf serum (FCS) (Invitrogen Corp., Carlsbad, Calif., USA).

After 24 hours, cardiac myocytes were seen to form a monolayer of spontaneously beating cells. Bio-electrospraying was performed within 24-48 hrs of culture. To prepare the cardiac myocytes for jetting, the cells were washed with PBS followed by the addition of 0.4% trypsin/EDTA (Invitrogen) at approximately 2 ml per 75 cm^2 of cellular monolayer. The cells were incubated in trypsin/EDTA for 10 min at 37°C and 5% CO_2. The loosened cells were collected and then pelleted by centrifugation at 300 xg for 5 min. The supernatant was removed and the pellet resuspended in growth medium. Soon after the cell suspension was prepared, it was characterised for its viscosity, electrical conductivity, surface tension, density and relative permittivity. The suspended primary cardiac myocytes were counted using a haemocytometer. The formulated, ready-to-jet, primary cardiac cell suspension was at a cellular concentration of 10^6 cells ml^{-1}.

Bio-Electrospraying Equipment Setup

The equipment setup used in these bio-electrospraying investigations are similar to those described previously[20,21] and consists of a conducting needle firmly held in an insulating resin mould having a ring-ground electrode directly below and inline with the exit of the needle. The inlet of the needle is connected to a syringe holding the cell suspension by way of medical grade silicone tubing. The syringe fits onto the cradle of a precision syringe pump capable of delivering consistent flow rates as low as 10–20 m^3s^{-1} (PHD 4400, Harvard Apparatus Ltd., Edenbridge, UK).

The conducting needle, held in insulation material, was connected to a high voltage power supply (FP-30, Glassman Europe Ltd., Tadley, UK) capable of delivering a positive/negative voltage of up to 30 kV with a maximum drawing current of 4 mA. The power supply has an increasing/decreasing power resolution of 0.1 kV. The counter electrode, placed below and inline with the exit of the needle, was grounded. The experimental setup was housed in a Class II laminar flow safety hood.

Cell-Bearing Droplet Collection and Microscopy

Analysis of the bio-electrosprayed cells was carried out following collection of the residues in sterile tissue culture dishes. Controls were collected by passing the cellular suspension through the jetting device without the application of a potential difference between the electrodes. Several repeated measurements for collected cell-bearing residues and controls were examined over a period of five days. The cell-bearing residues were optically examined using a Leica MZ12-5 microscope (Leica Microsystems, Ltd., Milton Keynes, UK) with a 1x magnification tube. Cell confluence in the controls and jetted samples were achieved at similar times, during which cell growth and phenotypic properties were photographically recorded at selected time-points post-jetting.

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Flow Cytometry Analysis For Cell Viability

Cells were removed with 0.4% trypsin solution (Invitrogen), centrifuged at 300 xg, washed with PBS and resuspended in 300 µl of annexin V binding buffer (0.1 M HEPES pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$) with 3 µl of annexin V-PE (BD Biosciences, Erembodegem, Belgium) and 5 µl 7AAD (1 mg/ml) (Sigma-Aldrich, St. Louis, Missouri, USA). Annexin V and 7AAD fluorescence was measured after 30 min using a flow cytometer (BD Biosciences) and 10,000 events were analysed. Viable cells were negative for both annexin V and 7AAD.

Western Blotting

Cells were lysed in RIPA buffer (0.75 M NaCl, 5% [v/v] NP40, 2.5% [w/v] deoxycholate, 0.5% [w/v] SDS, 0.25 M Tris-HCl pH 8.0, containing protease inhibitors). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, Illinois, USA). Twenty µg of protein was electrophoresed on polyacrylamide gels and transferred to Hybond-C nitrocellulose membranes (Amersham/GE Healthcare UK Ltd). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline and incubated overnight with antibodies against phospho-ERK1/2T204 (Santa Cruz), phospho-JNK1/2T138/Y185 (Upstate Cell Signaling/Millipore, Hampshire, UK) phospho-STAT1Y701 (Zymed/Invitrogen), phospho-H2AXS139 (Upstate) and GAPDH (Chemicon/Millipore, Hampshire, UK). HRP-linked secondary antibodies were from DAKO (Carpinteria, Calif., USA) and proteins were visualised by enhanced chemiluminescence (Amersham).

Adenoviral Transduction

GFP adenovirus was a kind gift from Professor Brian Foxwell (Imperial College, London). Adenovirus was propagated in 293 cells, purified over two cesium chloride gradients and viral titre was determined by plaque assay. Cardiac myocytes were removed in 0.4% trypsin solution, centrifuged at 300 xg for 5 min and resuspended in DMEM with 10% FBS. Cells were then infected in solution with adenovirus at a multiplicity of infection of 50, and immediately bio-electrosprayed.

Statistical Analysis

Data represents mean ± standard error of the mean (SEM). Statistical comparisons were performed with an analysis of variance followed by a Bonferroni post-test for multiple comparisons.

Results and Discussion

Prepared Cellular Suspension and Bio-Electrospraying

Many forms of pathology result in cell death in the myocardium. In recent years, much attention has been given to the possibility of regenerating the damaged cardiovascular system through the use of stem cells or cardiac precursors. An alternative strategy for cardiac regeneration is to directly deposit living cells onto three-dimensional scaffolds for use in cardiac surgery. To date however, it has not been demonstrated whether this strategy is possible in the context of primary cardiac myocytes. We therefore sought to examine the feasibility of this approach using bio-electrosprays.

The media properties of electrical conductivity, viscosity, surface tension, density, and relative permittivity of the medium with FCS and the specially formulated cardiac myocyte cellular suspension was characterised independently (Table 1). Of these media properties, electrical conductivity and viscosity are two parameters that govern jetting-to-jet stability. Previous electrospray literature suggests that a decrease in electrical conductivity and an increase in viscosity are more likely to promote and maintain jetting in stable conditions, which is most important if the requirement is to produce near one-sized, cell-bearing droplets and residues. In this study, we are primarily interested in identifying any changes that may have been brought about in the cardiac myocytes passed through this protocol. Hence, the production of a near mono distribution of cell-containing droplets and residues (to controlled jet deposition) is not paramount in these studies, but could be achieved as previously demonstrated with the use of a coaxial needle configuration.[22]

During our investigation, we traversed a wide operational space for a spectrum of applied voltage-to-flow rate permutations. Our intentions for doing so were to identify any conditions which would promote the formation of

Table 1.
Critical media properties of the cellular medium and the prepared cell suspension investigated in these studies.

Sample	Electrical Conductivity (Sm^{-1})	Viscosity (mPa s)	Surface Tension (mNm^{-1})	Density (kgm^{-3})	Relative Permittivity
Cell media having foetal calf serum	$\sim10^{-2}$	~23	~54	~905	~33
Cardiac myocyte suspension	$\sim10^{-3}$	~24	~57	~934	~38

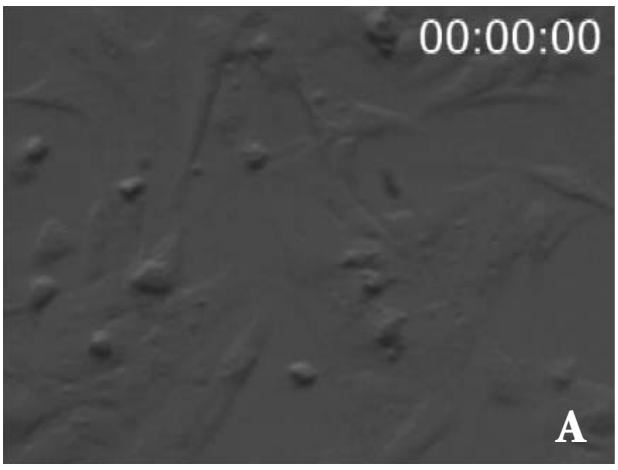
jet stability. Our observations confirmed that achieving jet stability with the bio-electrospray setup used in these investigations was impossible. This is directly due to the very high conductivity of the cellular suspension, together with the low viscosity. In particular, we point out that the electrical conductivity in these suspensions are at unprecedented levels due to the high ion concentrations necessary to maintain normal cellular physiology and metabolism. Nevertheless, if jet stability and cell bearing residue spatial resolution is required, our previous bio-electrospray setup could be explored.[22]

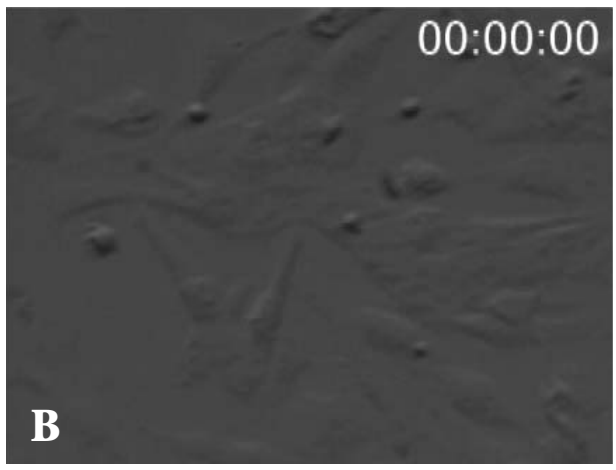
The estimated values of electrical conductivity and viscosity for the cell suspension are noticeably high and low, respectively. It was not surprising to observe the cellular suspension generating a polydisperse distribution of cell-containing droplets, as previously observed.[20,21] Jet continuity is not achieved as the cell suspension undergoes unstable bio-electrospraying with the hydrodynamic time (t_h) >> than the electrical relaxation time (t_e), where $t_h = \frac{LD^2}{Q}$ and $t_e = \frac{\beta\varepsilon_0}{K}$.[24] Our study investigated a wide parametric space of applied voltage-to-flow rate and demonstrated that it is virtually impossible to achieve stable jetting conditions in this single needle configuration.

Viability and Phenotype of the Cardiac Myocytes Subjected to Bio-Electrospraying

In order to ascertain if bio-electrospraying primary cardiac cells has any adverse effect on their phenotype or viability, cells were bio-electrosprayed, either left untreated or passed through the apparatus without applying voltage, and cultured for 12 hours. In all cases, the cells appeared entirely similar under light microscopy (Figure 1A) and were seen to beat spontaneously in culture, suggesting that bio-electrospraying did not adversely affect this characteristic phenotype of cardiac myocytes. Cell death was measured by annexin V and 7AAD staining, and demonstrated equal viability (77.4 ± 5.2%) in all three groups (Figures 1B and C).

While bio-electrospraying may not promote cell death, the possibility exists that it may induce stress-signaling pathways which might perturb normal cellular function. To explore this further, protein extracts from the three groups were examined by Western Blot for known markers of cellular stress. Many studies have demonstrated increased phosphorylation of the MAP kinases JNK and ERK following various cel-





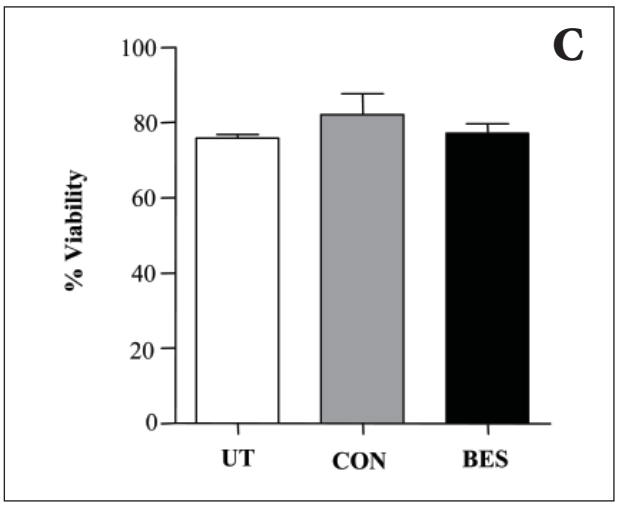


Figure 1. Representative micrographs of (A) control cells ("CON": passed through the bio-electrospray needle without the application of an electric field) and (B) post-bio-electrosprayed ("BES") cardiac myocytes. In both cases, the cardiac myocytes behaved as expected. (C) Cellular viability represented by cells negative for annexin V and 7AAD staining. The experiment was repeated twice and showed no statistically significant difference between the groups. UT: untreated cells; CON: control; and BES: bio-electrosprayed cardiac myocytes.

lular stresses in cardiac myocytes. We have previously shown oxidative stress-induced activation of STAT1 in these cells.[25,26] Bio-electrospraying did not induce phosphorylation of ERK1/2, JNK1/2 or STAT1, showing that general markers of cellular stress are not activated by the procedure (Figure 2A).

Following DNA damage such as chromosomal breakage, H2AX becomes phosphorylated at serine 139 (known as γH2AX). This facilitates recruitment of DNA repair factors, stalling of the cell cycle and in some cases, apoptosis.[27] As such, γH2AX serves as a marker for DNA damage. H2AX is phosphorylated in cardiac myocytes following treatment with various DNA-damaging agents (SPB, unpublished results). However, phosphorylation was not induced by bio-electrospraying, suggesting that the technique does not result in any detectable DNA damage (Figure 2A). Taken together, these results show that cardiac myocytes are viable and do not appear stressed following bio-electrospray, demonstrating proof of principle for use of this technique in tissue engineering for cardiac pathology.

Adenoviral Gene Delivery With Bio-Electrosprays

In future applications of this technique, it might be desirable to alter cardiac myocytes by gene therapy before bio-electrospraying. This would allow for reconstruction of the damaged myocardium while at the same time, introducing therapeutic factors to treat the underlying pathology. To address the feasibility of this process, we transduced cardiac myocytes in solution with a GFP adenovirus and immediately bio-electrosprayed the cells. As a control, cells were transduced and passed through the apparatus without applying voltage. After 12 hours, GFP expression was analysed by fluorescent microscopy and revealed 100% of cells expressing GFP in both groups (Figure 2B). This implies that it is indeed feasible to combine gene therapy with the bio-electrospraying process where gene transduction and bio-electrospraying can be carried out simultaneously.

Conclusions

In this study, we have elucidated that it is possible to deposit cardiac myocytes using the bio-electrospray process. This was achieved without causing any increase in cell death or activation of typical stress-signaling pathways. There was no evidence for activation of γH2AX, revealing the absence of any detectable DNA damage. More importantly, cardiac myocytes formed a spontaneously beating monolayer of cells several hours after bio-electrospraying, a prerequisite for the use of this cell type in cardiac tissue engineering.

While this study is a proof of principle of the applicability of this technology in cardiac medicine, it points the

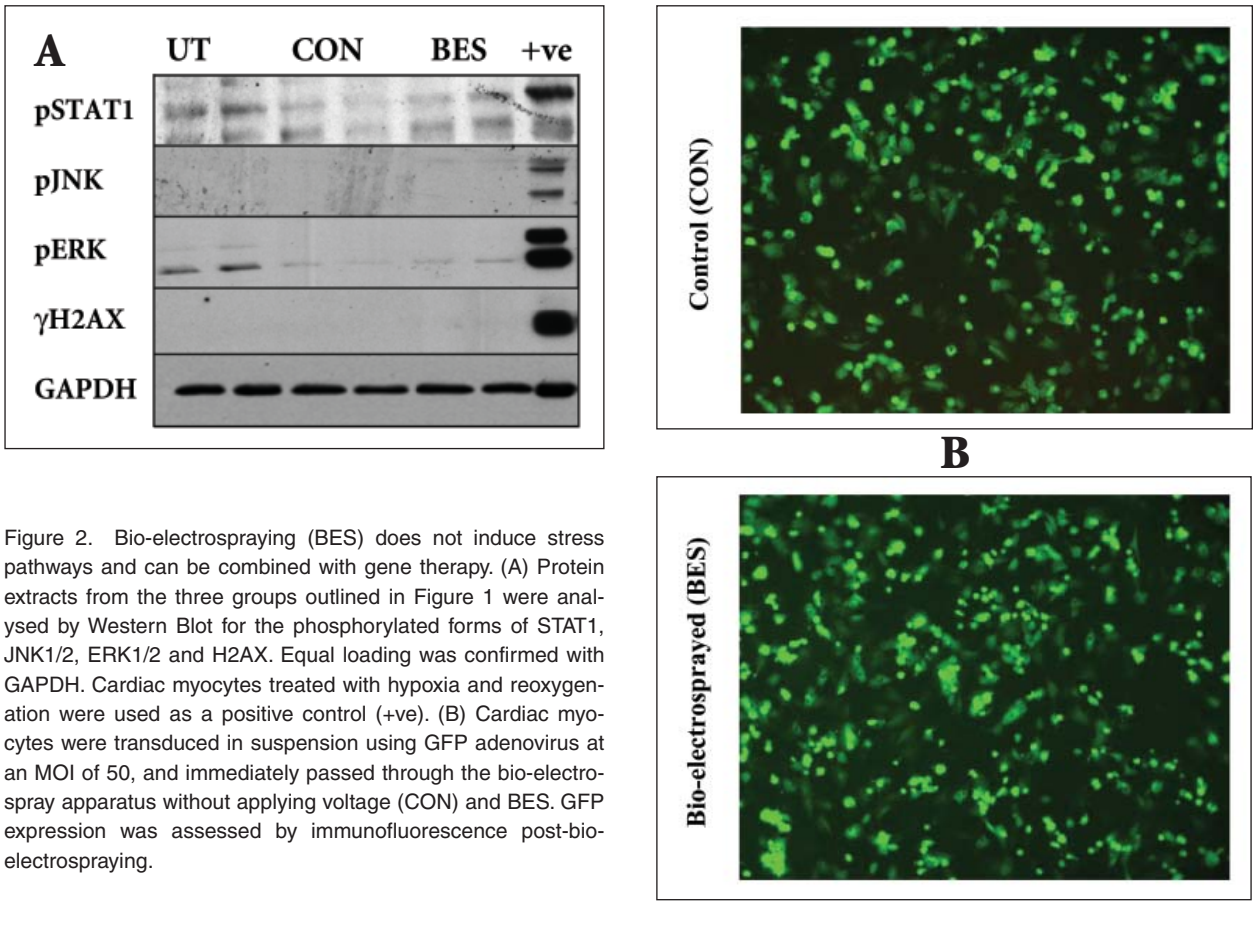


Figure 2. Bio-electrospraying (BES) does not induce stress pathways and can be combined with gene therapy. (A) Protein extracts from the three groups outlined in Figure 1 were analysed by Western Blot for the phosphorylated forms of STAT1, JNK1/2, ERK1/2 and H2AX. Equal loading was confirmed with GAPDH. Cardiac myocytes treated with hypoxia and reoxygenation were used as a positive control (+ve). (B) Cardiac myocytes were transduced in suspension using GFP adenovirus at an MOI of 50, and immediately passed through the bio-electrospray apparatus without applying voltage (CON) and BES. GFP expression was assessed by immunofluorescence post-bio-electrospraying.

way for future research into the development of cardiac tissue engineering, a process which is still in its infancy. There is no reason to doubt that other cardiac cell types such as smooth muscle and endothelial cells can also be successfully deposited in a similar fashion, thus opening up the possibility of creating *de novo* cardiac tissue containing all the requisite cell types.

The authors are currently studying in-detail the individual components of the cell, with the goal of understanding if there are any changes to the cellular makeup (through spectral karyotyping, etc.). Since this process can be successfully combined with a gene therapy approach, it allows the possibility of therapeutically altering cell types before deposition onto surfaces or generation of cardiac structures; an approach which may prove important if the technology is taken forward into any clinically relevant setting.

ACKNOWLEDGEMENTS

SPB is supported by a British Heart Foundation Ph.D. studentship. SNJ gratefully acknowledges The Royal Society of the UK for financially supporting these biophysical studies.

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