Bio-protocols for directly forming active encapsulations containing living primary cells

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Electrosprays and electrospinning were recently pioneered for directly handling living cells. These recent discoveries are now widely referred to as "bio-electrosprays" and "cell electrospinning", which have been demonstrated as having tremendous applicability to the life sciences in regenerative and therapeutic medicine. In the current work, we report our developmental studies with these protocols as submerged entities with primary rabbit aorta smooth muscle cells for generating cell-bearing encapsulations, which demonstrate proof-of-concept for a plethora of biomedical applications. Cell viability of the post-treated cells was assessed in comparison with two controls by way of flow cytometry over a three week period, establishing a viable cellular population >70%. Hence these investigations demonstrate the ability to explore these electrified encapsulating approaches for directly forming biologically viable emulsions, which could potentially be exploited from mechanisms for cancer therapy, hormone, and diabetic treatment to applications with cosmetics. Therefore, these studies elucidate the strong implications these bio-protocols have to offer the life sciences.

Introduction

Encapsulated advanced materials (structural, functional and biological) presented as emulsions are playing a crucial role in our every day lives. General products such as shampoo, sun block creams and other assays having within them encapsulated nanomaterials and biological and non-biological molecules are used for a range of purposes upon their application. From a medical standpoint, bio-emulsions have great applicability in a range of pivotal roles within regenerative and therapeutic medicine. Encapsulations having within them biological molecules and matter (*e.g.* living cells), can be explored for applications ranging from tissue engineering to the regeneration of viable biological structures to advanced therapeutics for treating cancers, diabetes, and hormones to novel routes for combating a wide range of human pathogens.[1–7]

Microencapsulation is a vibrant field of research in which several scientists have demonstrated a wide range of approaches for forming controlled encapsulations containing a variety of materials. We could essentially categorically segregate the encapsulation techniques into two headings, namely jet and non-jet based. The non-jet based routes of encapsulation entail chemical routes.[8–10] These routes are most effective but are limited when the requirement extends to the encapsulation of sensitive biological matter, such as living primary cells. In the jet based category (this includes jets that are either contained within a channel or capillary/needle and those that are free jets) there are a wide range of protocols, which have made "jet based" micro-encapsulation a robust and economical approach to encapsulating a wide range of materials, in particular living cells. Within this category, a further division is made for specifically addressing channel and capillary/needle based methodologies.[11–13] In the former a range of fabrication approaches have been explored for creating fine channels and flowing networks. These fabrication approaches range from soft lithography techniques[14] to those formed by means of micro machining.[15] Several advanced materials have been jetted through such mechanisms for the formation of multi-dimensional/composition particles and encapsulations, and in some cases have been aided by UV irradiation.[16–20] However, in this article we will focus on capillary/needle jet based techniques in particular focussing on those possessing the ability to directly encapsulate living cells.

In this sub-category there are four different encapsulation protocols, namely, aerodynamically assisted jets/jetting (AAJ),[21,22] co-flow techniques (CF)[23,24] and those very recently discovered, bio-electrosprays (BES)[25,26] and cell electrospinning (CE).[27,28] Aerodynamically assisted jetting is a technique that is brought about by applying pressure to a chamber with respect to the surrounding atmosphere. A needle accommodating the flow of media is placed within the pressurised chamber, which has an exit orifice residing centrally below the exit of the needle. The differential pressure gives rise to a drawing effect on the flowing media, which forms a jet exiting through the base orifice subsequently fragmenting into droplets.[29,30] The technique has been investigated as an immersed entity, which has demonstrated its ability for forming wet forms on collection.[31] However, it was only very recently that this approach was investigated for jetting media having particulate systems, which demonstrated AAJ as a novel materials processing science as applied to the jetting of structural and functional materials.[32–35] More importantly we

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discovered that living primary cells could be directly jetted by means of AAJ.[36] Similarly in some respects, co-flow employs by-pass media flow within a bath of liquid for drawing out media flowing within a needle. Cells have been jetted through this route successfully for encapsulation. Although CF has handled cell suspensions at a cellular density higher than AAJ and demonstrated a viability of >90%, the actual percentage of viable cells has not been clearly distinguished from those that are undergoing apoptosis. In the case of the post-jetted cells by way of AAJ, which have been analysed using flow cytometry, a gold standard in cell viability assessment for the biomedical sciences, elucidated a cell viability of >70%. Having said that it should be noted that this viability assessment was carried out on cells that were jetted in open-air, ambient conditions and not in a submerged scenario.

Conversely, to both AAJ and CF, BES is a jetting phenomenon determined by high intensity electric fields. Here an applied voltage is subjected in the thousands of volts on the jetting needle(s) with respect to some ground electrode. The charged needle holds the flow of a cell suspension [or if in the concentric or coaxial configuration (one needle fitted within another finally having a common centre) could hold a medical grade polydimethylsiloxane (PDMS) medium which could be jetted simultaneously][37,38], which is accelerated, in this external electric field promoting the generation of a jet, subsequently undergoing well-known instabilities giving rise to the formation of cell-bearing droplets and residues (structures formed on deposition).[37–40] An extension of this protocol is CE, which took place in a coaxial device where the medium (medical grade PDMS having a viscosity of 12 500 mPa s) passed through the outer needle had two noticeable properties, namely high viscosity and a high viscoelasticity, which on entering the external electric field promoted the drawing out of a continuous composite thread. It is interesting to note that both BES and CE are capable of generating micro and nano sized droplets/threads[41–44] and residues, a feature not possessed by either AAJ or CF. Although primary living cells have been jetted by both BES and CE as encapsulated droplets and continuous threads, (the latter as a function of collection time generating living scaffolds to membranes) these have only been investigated as AAJ's have, namely in open-air environments and not as submerged entities. However, the non-biological versions of electrosprays and electrospinning have previously been explored for jetting and threading liquids[45,46] in a submerged configuration. Interestingly, recently electrosprays in a submerged arrangement was explored for jetting micro and nanomaterials as suspensions.[47] These studies demonstrated the ability to directly form emulsions that hold individual micrometre sized capsules containing either micro or nanomaterials.

The current work reports in-depth investigations carried out on both BES and CE as submerged entities for the direct formulation of biologically viable emulsions. The work elucidates the ability for directly forming biologically viable capsules containing primary living cells. This study discusses the mapping of operational guides to more importantly assessing the post-treated cellular viability over a time period of three weeks, employing flow cytometry. These studies are the first of their kind since the discovery of both bio-electrosprays and cell electrospinning, which show another dimension to their versatility having significance for several applications in the life sciences.

Experimental

Setting-up submerged BES and CE configurations

We have reported previously the equipment set-up employed for submerged electrosprays with a single needle device.[47] Briefly, the device explored in these investigations is similar to that set-up (Fig. 1) but has the addition of employing a concentric or coaxial needle system (Fig. 2). This device consists of two concentric needles separately held in resin and mounted together with three centralising precision screws. A similar device was explored in our concentric or coaxial work where we addressed and unveiled the issue with regards to attaining jet stability and continuity in BES[37,38] and for forming continuous cell-bearing threads to living scaffolds in CE respectively.[27,28] In these studies the inner needle held the flow of the cellular suspension while the outer needle held the flow of either a medical grade polydimethylsiloxane[27,48] (the PDMS explored in these investigations ranged in molecular weight from ~16 500–100 000 for viscosities ~1000–12 500 mPa s) or a similar grade polyethylene oxide (PEO) medium (in these studies we employed a PEO having a molecular weight of ~1 000 000 made soluble in 100% water at varied concentrations from ~1–5%).[49,50] It is important to note

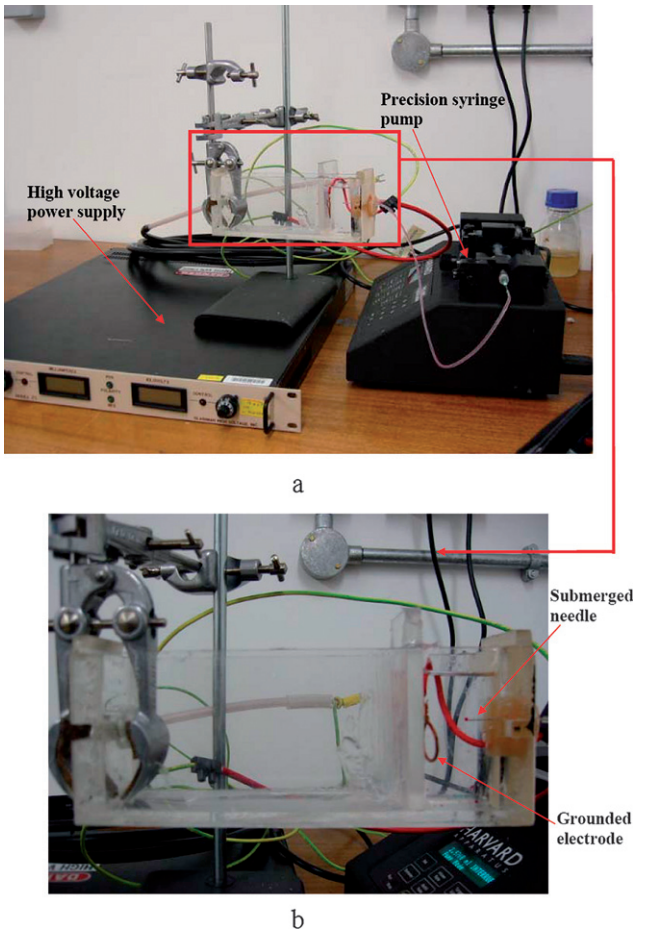


Fig. 1 Digital snapshots of (a) the submerged single needle apparatus and (b) the bath containing the submerged needle explored in these studies. The set-ups are not arranged in any particular experimental order.

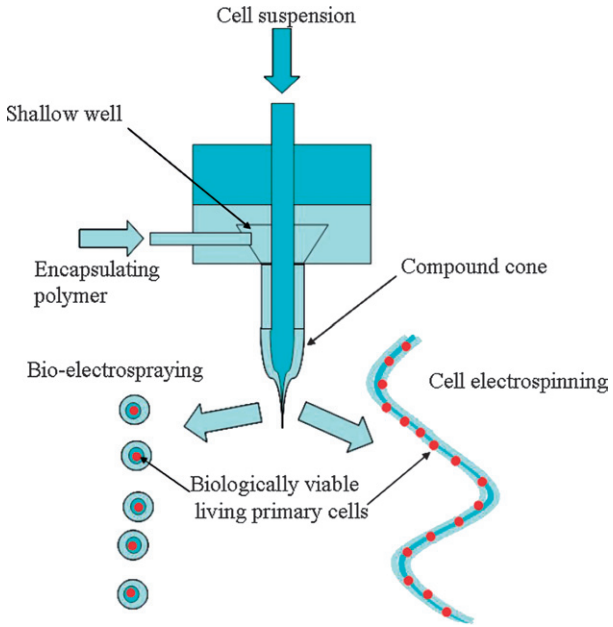


Fig. 2 A schematic representation of the submerged concentric or coaxial needle system investigated in these studies.

that in the coaxial needle configuration droplets were formed while jetting PDMS and PEO media in the outer needle having a viscosity of ~1000 mPa s and a PEO solution prepared with ~1–3% PEO in 100% water respectively. For continuous threading in this needle configuration the PDMS viscosity was increased to ~12 500 mPa s and the PEO concentration in water was increased to ~5%. Much like our previous work carried out with coaxial jetting and threading the outer needle flow needed to be higher than the flow rate of the inner needle medium for successful encapsulation of the living primary cells. The Perspex bath in which either of these needles were immersed had dimensions of ~6 × 2 × 3 inches in length, width, and height respectively. These jetting/threading needles were mounted to the baths by having a silicone adhesive applied on both surfaces, which provided the much needed sealing between the opening used for inserting the concentric needles into the bath, which was later fitted by means of positioning screws. The needles were connected to a high voltage power supply capable of consistently supplying either a positive or negative voltage up to 30 kV (FP-30, Glassman Europe Ltd, Tadley, UK) at a resolution of 0.1 kV. This power supply had a maximum output current of ~4 mA. The needle inputs were connected to syringes containing the respective media by way of medical grade silicone tubing. The flow rate of either media to the needles had two methods of control, firstly through the flow setting directly *via* the precision syringe pumps (PHD 4400, HARVARD Apparatus Ltd, Edenbridge, UK) and secondly by the variation in the selected syringe cross sectional area in combination with the settings of the syringe pump. The media used within the Perspex bath throughout these investigations where jetting/threading was carried out was a vegetable oil (sunflower oil having a viscosity of ~30 mPa s and a molecular weight of ~60 respectively), the properties of which have been reported previously.[51] Throughout these studies we explored only a ring shaped ground electrode, which was always kept in-line at a distance of ~15 mm from the exit of the needle system. The ring ground electrode had dimensions of ~14 mm in inner diameter with an outer of ~16 mm and was made of copper.

Preparation of the primary cell suspensions

The cellular suspensions explored in these studies contained primary living cells, namely rabbit aorta smooth muscle cells (RASMCs, gifted in-kind by Dr Stephen Hart, Institute of Child Health, UCL, London). The RASMCs were prepared using the explant method[52] and were identified by staining with an anti-smooth muscle actin antibody (Dako Ltd, UK). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% foetal calf serum (Life Technologies, UK) and 1% penicillin–streptomycin.

Cell suspension preparation was carried out by washing the respective cells with phosphate buffered saline (PBS, Sigma Aldrich, UK) followed by the addition of 0.5% trypsin–EDTA (Invitrogen, UK) at approximately 2 ml per 75 cm^2 of cellular monolayer. The cells were incubated in the trypsin–EDTA for 10 min at 37 °C and 5% CO_2. The loosened primary cells were collected and centrifuged at 1000 g for 5 min forming a cellular pellet. Subsequently, the supernatant was removed and the pellet resuspended in growth medium. Cell counts and densities were established by way of a haemocytometer. On preparing the cell suspensions their electrical conductivity, viscosity, surface tension, relative permittivity, and density were estimated as previously described and were found to be similar to those previously reported.[25,26,37,38] The prepared ready to jet/thread primary cell suspensions were at a cellular concentration of 10^7 cells ml^{-1}.

Generating operational parametric guides

In our previous work with submerged electrosprays, operational guides were generated for the single needle set-up. These studies demonstrated fenced regions where stable jetting took place generating the finest bubbles or capsules containing either the micro or nanomaterials.[47] In our work presented herein we studied both single and coaxial submerged needle configurations while jetting and threading primary cells in suspension. An operational parameter space of ~1–20 kV for ~10^{-20}–10^{-7} m^3 s^{-1} was traversed for the applied voltage to the flow rate in either needle respectively. During these operational parameter studies, we noted as in our work with coaxial needle devices in open ambient conditions that the outer needle flow rate of the polymer needed to have a greater flow rate than the flow rate of the cellular suspension that flowed within the inner needle. This ensured that successful encapsulation of the cells was consistently taking place. The guide was initiated by setting the flow rates of the inner and outer media to the lowest possible with the current equipment namely ~10^{-20} m^3 s^{-1} and later subjecting an applied voltage to those needles with respect to the ground electrode. We observed the jetting behaviour of the exiting media to the external electric field by means of a high-speed camera, which recorded and monitored the process in real time. The high-speed imaging equipment consisted of a Phantom V7.1 camera (Photo-Sonics International Ltd, Oxford, UK) capable of capturing 160 000 fps held in-line with a range of Nikon and Leica lenses (Nikon Macro NIKKOR 25–85 mm and Micro NIKKOR

105mm and Leica Mono Zoom 7, UK), fitted along with a light source (Solarc® Metal Halide, 60 W, Everest VIT Part No. SPL-600) for illumination. At the lowest set flow rate, on applying a voltage unstable conditions were observed hence, the flow rates were modified in either needle and the voltage was re-applied. This process was repeated throughout the entire operational parameter space of ~1–20 kV for ~10^{-20}–10^{-7} m^3 s^{-1}.

Collection and characterisation of cell-bearing residues

The equipment was set-up in a Class II safety hood and encapsulation was initiated at stable operational conditions. Several composite samples where collected for assessing cell viability. This was carried out by manually syringing out (extracting) the generated encapsulations from the bath in which they were generated and collecting them into several sterile flasks (containing fresh cell media) for analysis. Many samples for needle controls (those cell samples collected in the bath without the application of an applied voltage) and those that were generated as compound droplets and threads were collected for analysis. "Needle controls" referred to in the single needle are those cell samples collected in the bath of sunflower oil without the application of an applied potential difference to the needle with respect to the ring ground electrode. In the case of the coaxial needle the polymer (either PDMS or PEO) was made to flow though the outer needle with respect to the inner flow accommodating the cell suspension into the oil bath. As in the single needle scenario no potential difference was applied between the needles (coaxial configuration) and the ring ground electrode. Importantly the flow rates in either scenario were set to those identical flow rates at which cells were either jetted or threaded. For microscope examination, collected cells were studied in real time using calibrated images captured by the high-speed camera. The cell collection method used in these studies was most effective as a needle attached to the syringe predominantly only extracted the cells and cell media, in the case of the coaxial scenario these components were extracted leaving the encapsulating polymer as a residue within the bath. This was possible as the needle penetrated the interfacial media layer or membrane (sunflower oil–cell suspension, in the case of single needle encapsulation) to the polymeric shell (sunflower oil–PDMS/PEO–cell suspension) in the coaxial scenario. As our focus was to establish proof-of-concept of this encapsulation protocol (Fig. 3) cells were always extracted (as described), placed in fresh media and incubated for assessing cell viability at the 7 and 21 day time points post-treatment. All the media explored in these investigations were immiscible to one another and the jetted or threaded components (PDMS or PEO) did not in any way solidify post-jetting/threading. The collected residues were optically micrographed using either a Leica or a Nikkon Axiovert microscope, which imaged the cells soon after jetting/threading. For cell viability analysis, the collected cells were assessed by means of flow cytometry throughout a period of three weeks.

Assessing cellular viability

Our very early work with bio-electrosprays employed trypan blue staining of the post-treated cells for assessing cellular viability.[25,26] This protocol for assessing cellular viability could in effect be said to be a very "first approach". Subsequently, our work with BES, CE, and AAJ has employed flow cytometry, which is a gold standard for assessing cell viability in the biomedical sciences. Hence, we have explored this method in these studies for quantifying post-encapsulated cells over a time frame of three weeks. Briefly, FACScan is a flow cytometry technique and system[53] able to quantify cellular populations as four categories namely, alive, general cell death (necrotic cells), programmed cell death (apoptotic cells) and cellular debris. The

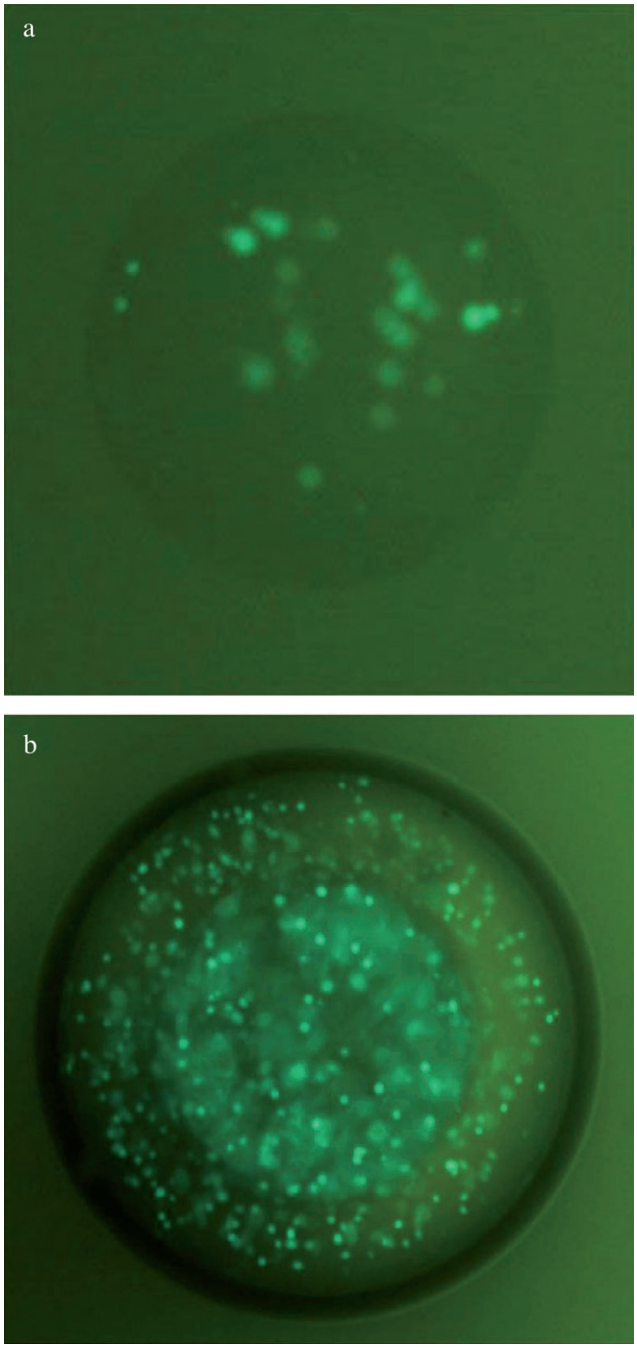


Fig. 3 Characteristic fluorescent optical micrographs depicting cell-bearing encapsulations generated by way of (a) single and (b) coaxial needle configuration.

cellular populations identified, as alive, dead and debris are straight forward with the labelled cells. In the case of apoptotic cells the membrane phospholipid phospatidylserine (PS) is translocated from the inner to the outer layer of the plasma membrane. At the initial stages of apoptosis, the cell membrane remains intact, whilst at the moment of necrosis the cell membrane loses integrity and becomes leaky to the vital dye propidium iodide (PI). Staining with annexin V which has a high affinity for PS in conjunction with PI, allows the identification of live cells (annexin − PI −), early apoptotic cells (annexin + PI −), necrotic cells (annexin + PI +) and debris (annexin − PI +) to give a sensitive and accurate measurement of the dynamics of cell death.

Several single and fresh cell suspensions were prepared from each sample and were mixed with Annexin V FITC (final concentration 1 μg ml^{-1}) (Pharmingen, UK) and propidium iodide (PI) (final concentration 1 μg ml^{-1}) (Sigma, UK) in the presence of 1.8 mM calcium. Cells were incubated at room temperature for 15 min prior to quenching in calcium containing binding buffer (Pharmingen, UK) and were analysed immediately. A Dako Cytomation CyAn ADP flow cytometer was employed to collect data for >20 000 events. The excitation was at 488 nm using an argon laser, the FITC emission was collected with 525 ± 20 nm and PI with a 675 ± 20 nm band pass filter. The data was analysed using Summit 4.3 software (DakoCytomation, UK). Thus, in these studies several culture or untreated (CC), needle controls (NC: those passed through the needles without the application of an applied voltage) and encapsulated primary cell samples were passed through the FACScan apparatus to analyse the number of alive and dead cells through to cells which were undergoing programmed cell death to form debris. Culture controls (CC) are those cells that are not treated to either protocol. Using the above flow cytometry approach these samples where analysed at time frames of as collected (0 h), 168 and 504 h post-encapsulation respectively. In these experiments, each sample was represented at least five times for each time point. It should be noted that at the first time point the collected cells post-extraction from their encapsulations were immediately put through the flow cytometry cell assessment protocol in parallel with the assessment of both the needle and culture controls. During this time optical observation of these collected samples in conjunction with the controls were completely examined for assessing dead cell numbers which was comparable with the untreated/culture controls. Subsequently, prior to and leading to either changing media to the next cell assessment time point, all three samples were optically assessed to find an insignificant population of dead cells. In fact, the population of those cells that are necrotic, apoptotic and cell debris are comparatively similar to those of the culture control, which demonstrates that the encapsulation protocols have failed to show any detectable negative affects on the encapsulated cells.

Results and discussion

Submerged bio-electrospraying and cell electrospinning

The submerged single needle device was initially set-up in the Class II laminar flow safety cabinet, which initiated the encapsulation of the cellular suspension. We found that stable jetting of the cellular suspension took place at a flow rate of ~10^{-9} m^3 s^{-1} for an applied voltage of ~7 kV between the needle and ground electrode which was placed at ~15 mm centrally away from the needle exit. It should be noted that if an applied voltage >9 kV was applied to the directly jetting cell suspension (single needle system), discharging effects were present. At these conditions (~7 kV and ~10^{-9} m^3 s^{-1}) we observed the jetting process to be continuous, and formed cell-bearing droplets (Fig. 3a). Interestingly at an applied voltage of ~8 kV these cell-bearing droplets on generation were observed to undergo coulomb fission. We observed this event repeatedly on the generated droplets (Fig. 4, arrows indicate the encapsulation which evolves a jet which later generates a spray of droplets). The image sequence (Fig. 4a–g) shows that the generated droplet on losing contact with the liquid meniscus was seen to have a near-elliptical shape, which is not surprising. The elliptical morphology of the detaching droplets is owed as the charged low viscosity cell-bearing medium (viscosity: ~16 mPa s) is accelerated through the surrounding higher viscosity medium (sunflower oil: ~30 mPa s) towards the grounded electrode. However, as a function of time this elliptical droplet transformed from a near-spherical to

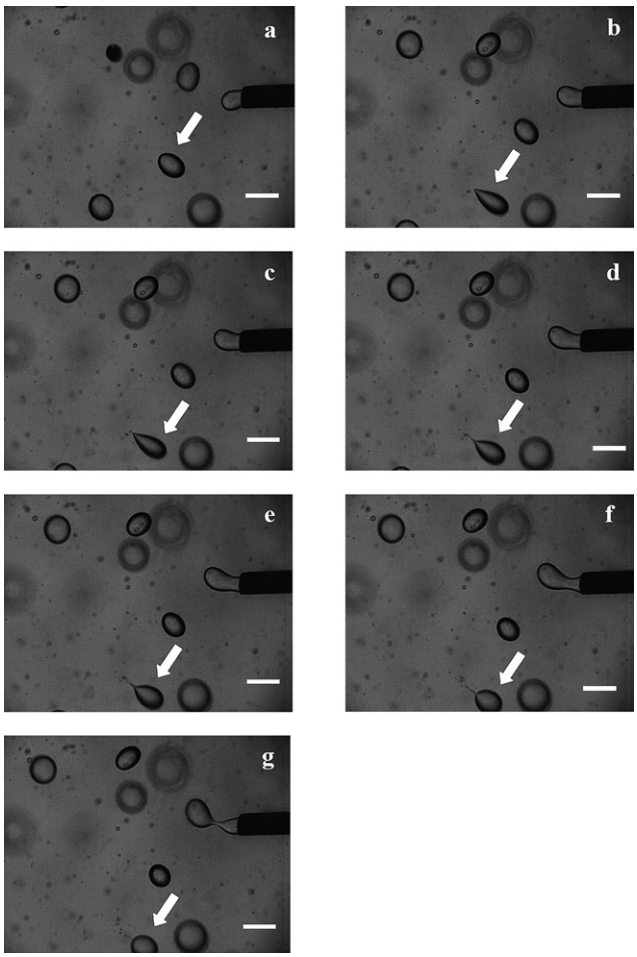


Fig. 4 A characteristic high-speed digital image sequence of Coulomb fission talking place while jetting the cell suspension in the single needle configuration. This scenario was repeatedly observed for set operating parameters of ~8 kV and ~10^{-9} m^3 s^{-1} for the applied voltage and flow rate respectively. The scale bars represent ~150 μm.

a tear-drop like shaped droplet from which a fine jet evolved, subsequently seen to generate a fine spray of droplets, (Fig. 4, as indicated with the arrows) this event is well-known as "Coulomb fission" where the droplet has reached the Rayleigh limit.[54]

In the coaxial needle configuration, the cellular suspension was made to flow through the inner needle while the outer needle held the flow of a medical grade polymer (either PDMS or PEO), which was immiscible with the bath media. A large window existed for stable jetting and threading (for an applied voltage from ~10–13 kV for a flow rate regime of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) in this submerged coaxial configuration (Fig. 5). Stable continuous jetting and threading was seen to generate emulsions containing living cells. Fig. 5a demonstrates stable jetting conditions (at an applied voltage of ~10 kV for a flow rate of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively), which shows the generated near-monodispersed encapsulations. Similarly Fig. 5b shows by means of high-speed imaging the stable continuous threading (at an applied voltage of ~11 kV for a flow rate of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) process in action. Similar jets and threads were observed in the coaxial configuration when the outer media was switched from PDMS to PEO for those given operational parameter conditions. Closer examination of the coaxial jetting and threading process was seen to generate similar high magnification images of the compound cones and jets to threads (Fig. 6). These images clearly demonstrated the immiscibility of the cellular suspension with the encapsulating medical grade polymer along with the surrounding bath media. Having achieved stable jetting conditions while generating the finest possible cell-bearing droplets (in the case of BES, at ~10 kV for flow rates of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively: Fig. 3b), if the flow rate was increased (for a flow rate of $\sim10^{-8}$ and 10^{-7} m^3 s^{-1} in the inner and outer needles respectively) the process was seen to form larger cones and jet diameters to encouraging the jet break-up point further away from the needle exit, finally resulting in larger cell-bearing droplets. Conversely, if the applied voltage was increased (>10 kV) then we observed the cone area to jet diameter significantly reducing with the jet break-up point brought closer to the needle exit. Furthermore, if the applied voltage was increased beyond a critical voltage (>13 kV) for a given flow rate (flow rates of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) in either needle, stability was seen to diminish with the emergence of other well-known jetting modes appearing which are unstable and promoted the generation of a polydisperse distribution of cell-bearing encapsulations, an unattractive feature. While jetting in the compound and coaxial scenario at very high applied voltages (~12.5 kV) and low

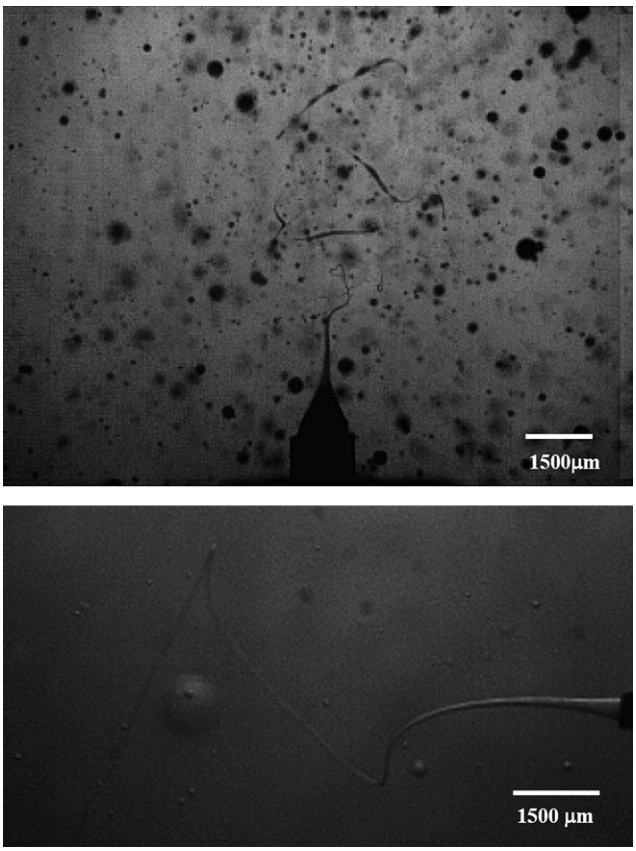


Fig. 5 Representative high-speed images depicting (a) stable coaxial cone-jet bio-electrospraying [at an applied voltage of ~10 kV for flow rates of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner (cell suspension) and outer (PDMS) needles respectively] and (b) stable and continuous coaxial cell electrospinning [at an applied voltage of ~11 kV for flow rates of $\sim10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner (cell suspension) and outer (PEO) needles respectively] as submerged entities. Similar stable scenarios were observed when the outer media were changed to either PDMS or PEO respectively.

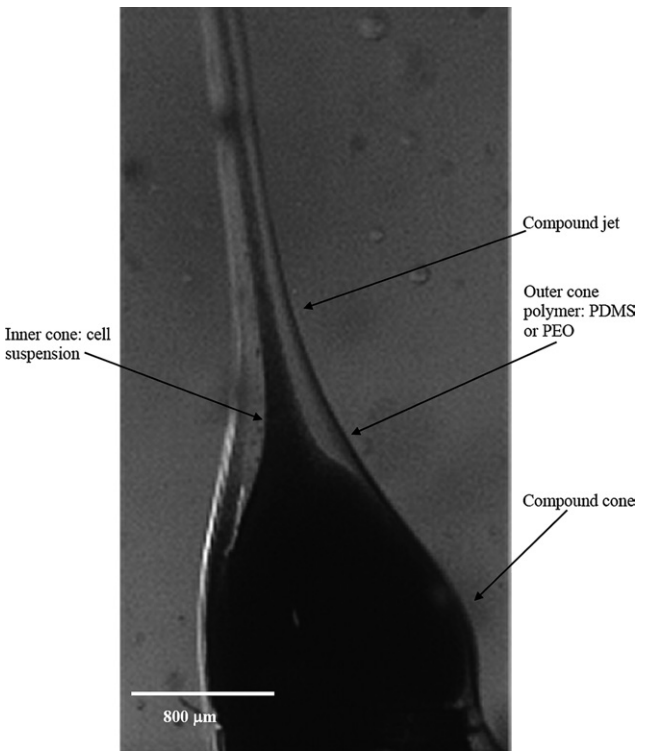


Fig. 6 Characteristic compound coaxial stable cone and jet formed during either submerged bio-electrospraying or cell electrospinning (for those operating parameter conditions listed for Fig. 5).

flow rates (flow rates of $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) we observed, as in the single needle, coulomb fission taking place on the generated cell-bearing droplets.

Similarly, on achieving stable continuous threading (at an applied voltage of ~11 kV for flow rates of $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) if the flow rate was increased (flow rates of $\sim 10^{-8}$ and 10^{-7} m^3 s^{-1} in the inner and outer needles respectively) in either needle this gave rise to the formation of threads with larger diameters (Fig. 7) owing to the increase in output media flow. On the other hand if the applied voltage was increased (>13.5 kV) for that given flow rate condition (flow rates of $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively) which promoted continuity and stability in the threading process with the PEO system, it was observed that multi-threads formed (Fig. 8) from the primary thread which has been reported previously in electrospinning studies carried out in open-air conditions.[55] Related instabilities resulting from elevated applied voltages giving rise to the emergence of several jets have also been observed and reported in electrospray studies.[27,56]

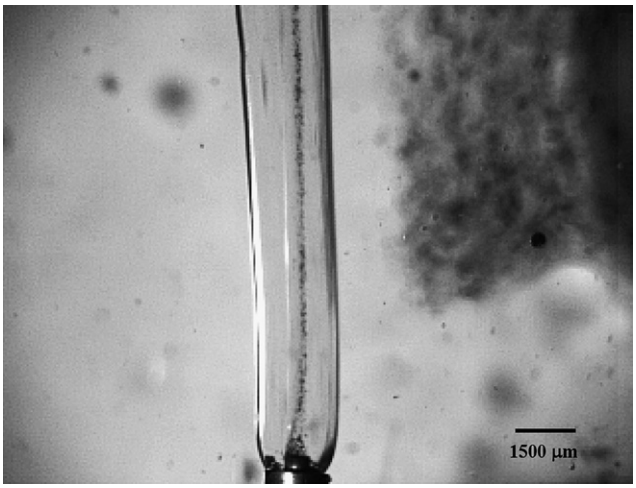


Fig. 7 An illustrative high-speed image depicting the effect of increasing the flow rate [$\sim 10^{-8}$ and 10^{-7} m^3 s^{-1} in the inner (cell suspension) and outer (PDMS) needles respectively for an applied voltage of ~11 kV] while continuous and stable coaxial cell electrospinning occurs.

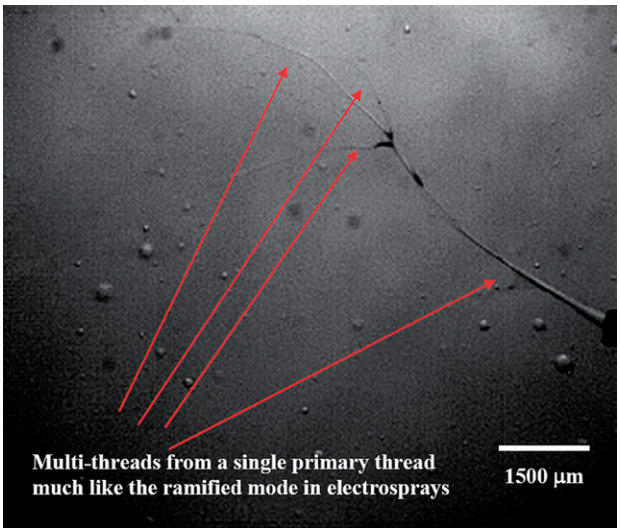
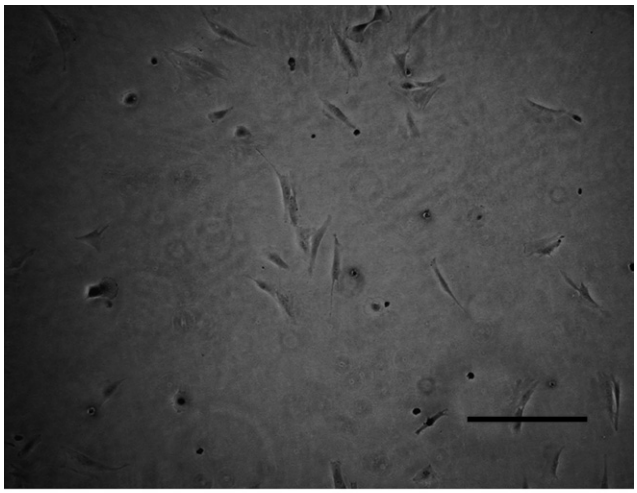


Fig. 8 A characteristic high-speed image illustrating the multiple threads formed from a single primary stable and continuous thread while cell electrospinning at an applied voltage of >13.5 kV for flow rates of $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner (cell suspension) and outer (PEO) needles respectively.

Ascertaining cellular viability

Analysis of cellular viability is paramount in such studies. Hence, viability can be assessed by several different routes; however, here as in our previous studies[28,39,40] we employ a viability



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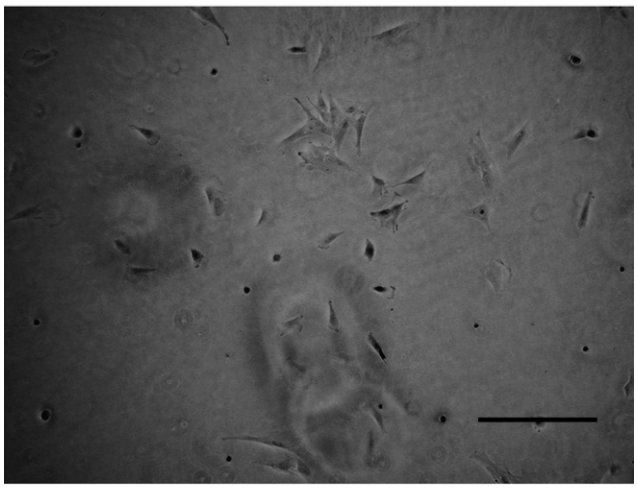

b

Fig. 9 Distinctive optical micrographs illustrating (a) needle control (which morphologically were similar to the culture controls) and (b) post-jetted (which were comparable to the threaded cells) cells at day 0. These cells are those which were extracted from cell-bearing encapsulations generated at applied voltage and flow rates of ~10 kV and $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively. The scale bar represents 200 μm.

assessment methodology well established in the life sciences. FACScan, a flow cytometry[53] approach where hydrodynamics are coupled with lasers for forming a single stream of labelled cells, which are later passed through an array of lasers for analysing multiple cellular characteristics. These lasers of known wavelength excite the labelled single cells at which time their response is detected in terms of their physical and chemical characteristics which are then translated into four cellular populations, namely alive, necrotic, apoptotic, and cellular debris. This robust technique quantifies and makes sensitive measurements on the dynamics of cellular death. We use this protocol over our previous cell assessment methods[25,26] as this technique is able to rapidly and clearly identify those cells that are undergoing apoptosis, giving an actual population of living/viable cells.

In these studies we FACScaned several samples of both controls [culture/untreated (CC) and those cells with polymer passed through the device without the application of an applied voltage (NC)] and encapsulated samples, which were assessed for their viability over a period of three weeks. For these studies several fresh aliquots (>60 samples) of cells from each sample were assessed by flow cytometry for each time point. During this cell assessment time frame, the cells were optically observed to assess their phenotype in comparison to their controls. Stable and continuous encapsulation of these primary cells was carried out at the selected parameter conditions, during which time they were collected into sterile flasks by means of manual extraction.

The PDMS encapsulated and collected primary cells in sterile flasks were immediately examined with their corresponding

Table 1 Cell viability as assessed by means of flow cytometry for a three week duration

Cell populations	Culture control (CC)[a] (%)			Needle controls (NC)[b] (%)			Jetted (%)			
Apoptotic (programmed cellular death)	0.59	1.92	0.08	2.01	5.07	0.13	0.89	5.78	0.03	R5[c]
Alive	92.29	76.98	71.22	82.96	80.4	70.35	86.52	64.31	71.3	R4[c]
Necrotic (general cell death)	0.99	3.08	4.72	3.59	4.65	5.21	1.7	4.97	5.37	R3[c]
Cell debris	6.13	18.02	23.97	11.44	9.87	24.23	10.9	24.94	23.3	R2[c]
Days	0	7	21	0	7	21	0	7	21	

[a] CC: culture or untreated controls. [b] NC: needle control (samples collected along with polymer at flow rates at which stable and continuous jetting/threading was achieved without the application of an applied voltage). [c] R5, R4, R3 and R2 represent apoptotic (programmed cell death), alive and necrotic (dead) cells, and cellular debris respectively as analysed by flow cytometry.

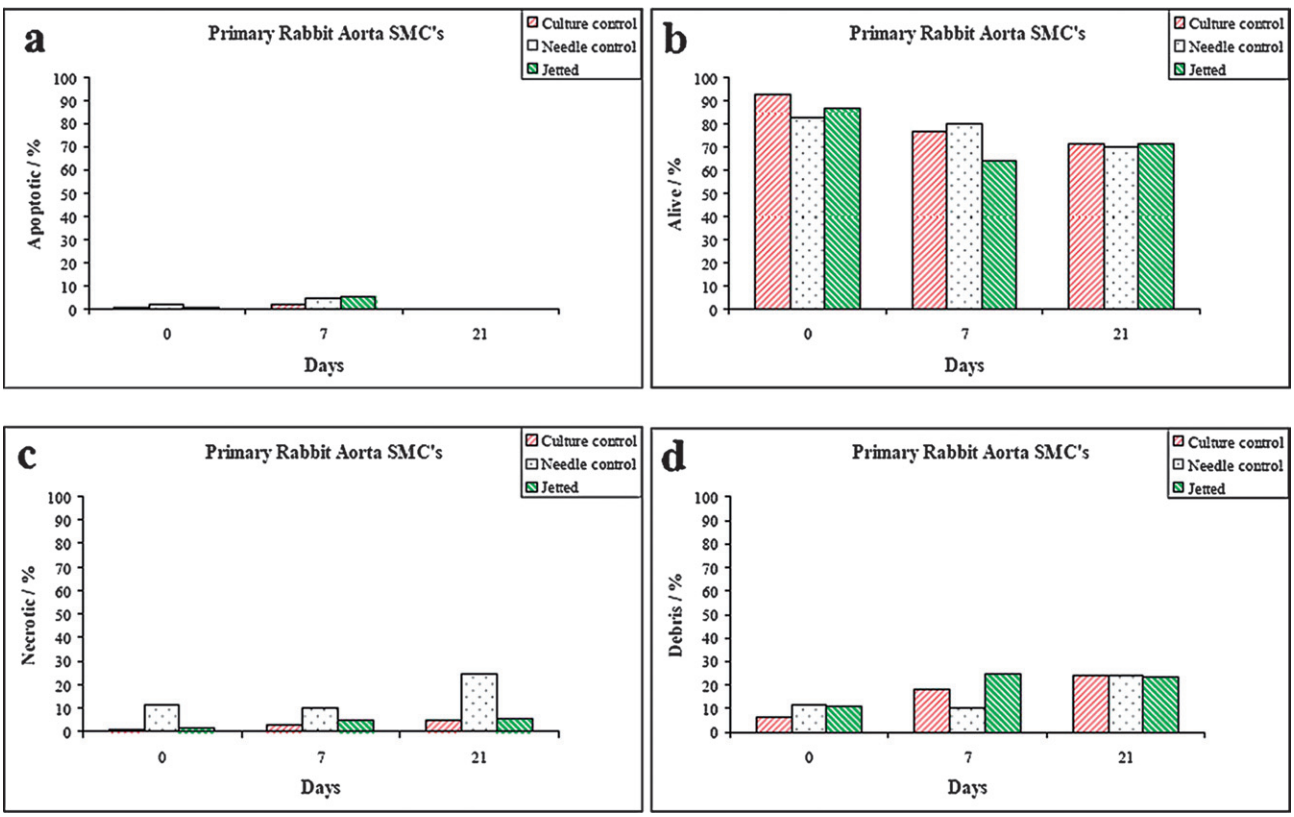

Fig. 10 Characteristic bar charts representing flow cytometry data on (a) apoptotic, (b) alive, (c) necrotic and (d) cell debris populations, in comparison with culture and needle controls to those jetted cells over the three week period of analysis. The jetted conditions were in PDMS having a viscosity of ~1000 mPa s at operational conditions of ~10 kV for the applied voltage for a flow rate combination of $\sim 10^{-9}$ and 10^{-8} $m^3 s^{-1}$ in the inner and outer needles respectively.

controls by means of flow cytometry. Optical observation of the cells' morphology soon after collection looked very similar to each other. Fig. 9a and b (cells which were encapsulated with PDMS) demonstrate representative optical micrographs of the needle control (which looked very similar to the culture control) and the jetted cells (which were morphologically comparable to the threaded cells) soon after extraction and collection respectively. We noticed that both the needle control and jetted/threaded cells did not exhibit any forms of cellular trauma. The controls and encapsulated cells' maintained their ability to adhere to the base of the flasks soon after collection demonstrating normal and expected characteristics of these cells. FACScan analysis of the controls and jetted samples on collection showed that a large population of cells were alive (Table 1 and Fig. 10). Similarly, we optically assessed both the jetted and controls at the 7 and 21 day time markers to find that the cells' morphologically looked very similar to each other (Fig. 11 and 12: cells encapsulated with PDMS). Similar viable cell populations were observed for those cells threaded with the PDMS having a viscosity of ~12 500 mPa s. Flow cytometry analysis illustrated that at these time points the viable populations of cells were >70% (Table 1 and Fig. 10). In these studies, several fresh repeated flow cytometry measurements were taken at each time point for each sample, which demonstrated insignificant discrepancies. Furthermore, these cell viability assessment studies analysed $>10^6$ cells for each sample by way of flow cytometry for that time point. All cellular samples reached cell confluence at approximately the same time. These results endorse these submerged electrified cell encapsulation protocols as versatile and economical routes for forming

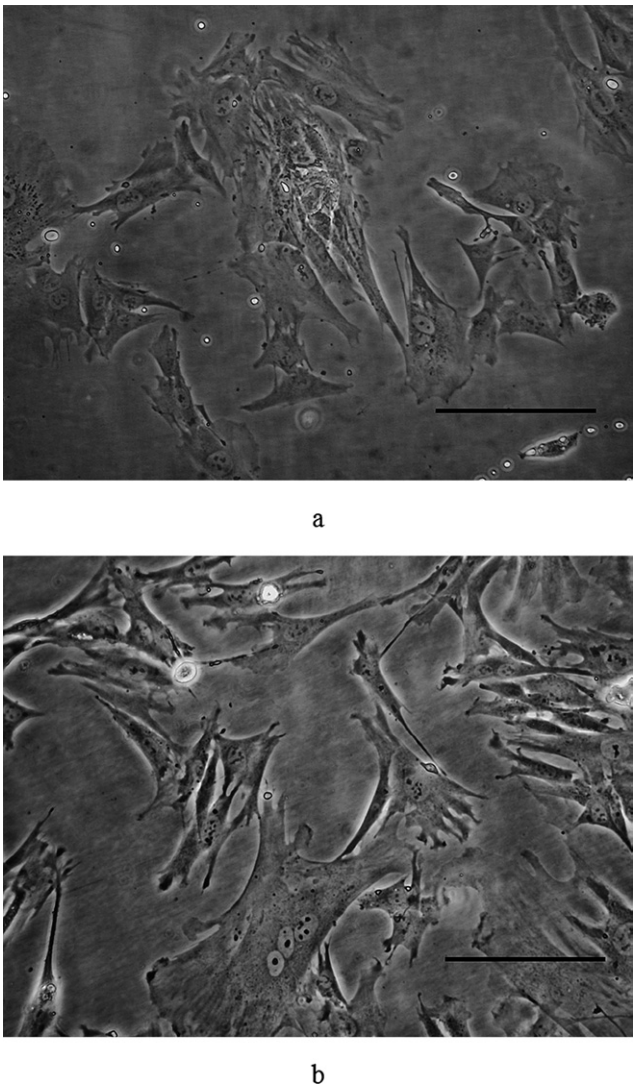

Fig. 11 Characteristic optical images depicting (a) needle control (which morphologically were similar to the culture controls) and (b) post-jetted (which were comparable to the threaded cells) cells at day 7 (168 h). These results demonstrate those samples extracted from PDMS (~1000 mPa s) shells, which were generated at those parameter conditions of ~10 kV and $\sim 10^{-9}$ and 10^{-8} m^3s^{-1} for the applied voltage and flow rates in the inner and outer needles respectively. The scale bar denotes 50 μm.

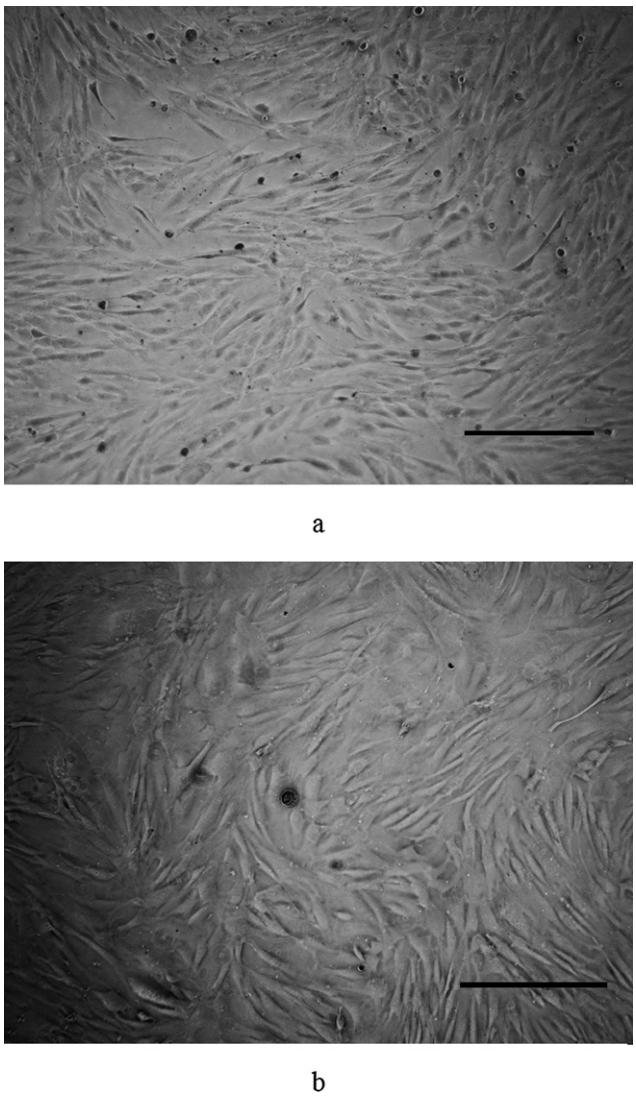

Fig. 12 Representative optical micrographs depicting (a) needle control (which morphologically were similar to the culture controls) and (b) post-jetted (which were comparable to the threaded cells) cells at day 21 (504 h). These micrographs depict those cells extracted from PDMS (~1000 mPa s) encapsulations formed at an applied voltage of ~10 kV for flow rates of $\sim 10^{-9}$ and 10^{-8} m^3 s^{-1} in the inner and outer needles respectively. The scale bar represents 1 mm.

biologically viable cellular emulsions. Cell assessment was also carried out on the cell-bearing droplets formed as a result of encapsulation by way of a single needle submerged device indicating a similar large population of viable cells comparable to those jetted and threaded by means of the coaxial device.

These studies directly demonstrate the ability to encapsulate a cellular suspension containing primary living cells as submerged entities, which elucidate a high viable population of cells over a long period of time. In the remit of these studies, we also encapsulated GFP (green fluorescent protein) expressing cells, which were viable post-treatment, implying that gene therapy could be coupled with these approaches for having a therapeutic element.[40] The authors are currently in the process of applying these bio-encapsulation approaches on more sensitive cell types (for example on neurons and stems cells *etc*.) for understanding whether these processes are having any effects. In parallel, those post-encapsulated cells will be investigated for studying their associated genomics and cytogenetics over a long period of time for understanding the effects (if any) that these submerged protocols may have brought on these cells at a sub-cellular level, which could have an effect on the cells' make-up.

Conclusions

Our studies reported herein on BES and CE as submerged entities have demonstrated a versatile and economical protocol for forming bio-emulsions (which could also be explored in advanced therapeutics) with primary living cells. The investigations elucidate a viable cell population of >70% post-encapsulation. These techniques are now undergoing investigation for assessing the cells' sub-cellular components and studying gene expression and karyotyping over both short and long time periods, for identifying if these protocols are having any affect on the cells' make-up. The authors are currently exploring this bio-emulsion preparation routes on a wide range of stem cells, which will bring out the therapeutic implications these processes, would have as applied to biology/medicine. In parallel, we hope to be able to control with accuracy the number of cells within an encapsulation, which would enhance this cell encapsulation protocol even further. The author's intentions are to take these jetting and threading biomedical protocols to a clinical setting.

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