Electrostatic Stabilisation of Drop on Demand Bio-Ink through the Cationic Encapsulation of Cells

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Abstract
The ability to formulate bioprinting inks in which suspensions of cells and other biological materials can be maintained, without affecting biological response, is crucial in producing robust printing strategies for tissue fabrication. A piezoelectrically actuated drop-on-demand printing system has been used to deposit electrostatically stabilised cells from a human osteosarcoma cell line (U2OS). Experiments were intended to investigate the effectiveness of a polyelectrolyte cell encapsulant to maintain cell dispersion within a bio ink. Cells were coated with a number of thicknesses of a Cationic Poly-l-lysine (PLL) encapsulant and their ability to release studied over 7 days, with the thinner coatings proving to be more favourable. Printing of both coated and uncoated cells indicated the dispersion and printability of coated cells was significantly better than that of uncoated cells. Preliminary results suggest that electrostatic stabilisation of bio inks could provide a solution to cell aggregation, increasing viable printing time and decreasing poor yields due to orifice obstruction.

Introduction
Tissue engineering and regenerative medicine aim to promote the regeneration of tissues following damage or disease by the provision of scaffold/support materials incorporating appropriate cells and biological molecules. In order to deliver these aims, innovative delivery mechanisms and processes need to be developed. Additive layer manufacturing (ALM) is the collective term that describes production techniques which allow the construction of complex geometries through the assembly of 2.5D layers. The use of digitally controlled positioning systems to precisely deposit biological materials within scaffolds has been the topic of research for two decades. To this end, Bio plotting, the process of low temperature extrusion, has allowed researchers to extrude cell rich hydrogels in to predetermined 3D geomiteries[1]. While the low resolution grading of a number of materials is possible within bio plotting type systems, it adds significant complexity to the manufacturing system and results in lower productivity. Inkjet printing on the other hand is widely accepted within the graphics industry as a high throughput printing system capable of grading multiple materials at high resolution with little or no impact to productivity. Cells to be inkjet printed are suspended within a carrier fluid and as a mixture are referred to as a bio ink. The rheological properties of these inks and their impact on printing have been well documented[2] but the specific challenges associated with the flocculation of cell within bioinks has not been addressed. Brownian motion, a continuous time stochastic process, describes the random motion of particles through a liquid due to their continuous bombardment from the liquid's constitutes molecules[3]. Electrostatic stabilisation using polyelectrolyte coatings is a common method of stabilising colloidal pigment suspensions. Many of these coatings however, are cytotoxic and thus inappropriate for use within a bio-ink. A cationic polymer, Poly-L-Lyseen (PLL) is known to be both cell compatible[4] and an electrostatic stabiliser[5]. It was therefore hypothesised that the encapsulation of cells within PLL would aide dispersion, and increase the printability of a bio-ink.

2. Materials and Experimental procedure

2.1 Cells and Culture Conditions

U-2 OS (human derived osteosarcoma cell line) were obtained from the ATCC and cultured in 75cm^2 flasks (Coring-Costar, CLS3375) in low glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, D5546) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich, F9665), 5mM Glutamax (Invitrogen-Gibco, 35050) and 1% (v/v) penicillin/streptomycin (Invitrogen-Gibco, 15140). All cultures were maintained at 37˚C in a 5% CO_2 humidified incubator. Cells were detached every 3 days after they had reached 75-80% confluence. The media was aspirated from the confluent monolayer and washed with warm sterile 1X PBS. The monolayer was incubated with 0.25% w/v trypsin and 0.53 mMol/L EDTA (Sigma-Aldrich, 25300-054) for 2 minutes and followed by inactivation of the trypsin with growth medium. The cell suspension was centrifuged at 200g for 5 minutes. The pellet was resuspended in growth medium and cells were reseeded at 1:3 ratio

2.2 Cell Coating and Bio-Ink Formulation

For encapsulation, U-2 OS were suspended in Hank's Balanced Salt Solution (HBSS) without phenol red in a concentration of 2x10^6 cells/ml. PLL-FTIC (fluorescein isothiocyanate) solution (1 mg/ml) was prepared in Hank's Balanced Salt Solution (HBSS) and filtered using 0.22 µm Whatmann sterile filter. Cationic PLL (Sigma) was first added in three different concentrations: a) 200 µg, b) 400 µg and c) 2000 µg of PLL-FTIC to a 1 mL suspension of U-2 OS of 2 million count. Cells were incubated with the PLL during 15-minute at 37 °C in a 5% CO2, 95% humidified air environment, followed by three washings steps with HBSS at 37 °C using centrifugation at 250g for 5 min to remove any excess or unadsorbed polyelectrolyte. Encapsulated cells were re-suspended in 1 ml of filtered HBSS media to form a bio-ink. The uncoated cells were detached from culture as indicated in the previous section and resuspended in filtered HBSS at a ratio of 2x10^6 cells/ml to form a second bio-ink.

2.3 Printing Experiments

Two channels of a Jetlab 4 (Microfab Inc., USA) single orifice piezoelectric printer were used to perform the printing experiments. Droplet ejection was achieved through the actuation of the piezoelectric material within the inkjet head, affected by the application of a potential difference. The impulse was applied in a simple trapezoidal wave form with a ramp up, dwell, ramp down, amplitude and drop frequency of 3µs, 17µs, 5µs, 60v and 5 kHz respectively. Droplet formation was confirmed prior to printing using a LED stroboscope which was phase shifted from a base frequency matching a factor of the print head frequency. The phase shift allows the tracking of a set of droplets over a given displacement. Aphelion (Microfab) image analysis software was used to calculate the mean velocity, diameter and therefore the volume of the ejected droplets. Following formulation of the two bio-inks, one containing PLL Coated and one containing Non-coated U2OS cells, and after a 15min transport from cell culture to the printing laboratory two 10µl sample droplets were taken directly from the micro tubes prior to printing. The bio-inks were printed from a 60µm diameter orifice, onto a transparent poly(methyl methacrylate) (PMMA) substrate, from a height of 1mm. 10 Deposits consisting of 50 droplets were deposited in a 1x10 array for each Bio-ink at 7 time points from 0 to 60mins in ten minute increments. Following deposition of the sample and at each 10min time point, the deposits were viewed through an inverted microscope (1OJ3, Olympus, Japan) and the number of cells recorded.

3.0 Results:

3.1 Cell Encapsulation

Verification of the cell coating was obtained by florescence microscopy where the FITC marker, present within the PLL shell, fluoresced green in the presents of the microscopes ultraviolet radiation source. Figure 1, provides a view of the shell structure at two days post-encapsulation, showing the adsorption of PLL onto the cell membrane and the formation of an encapsulating shell around the cells. The comparison of fluorescence images revealed a decrease of the coated cell diameter with an increase in PLL concentration during the coating process.

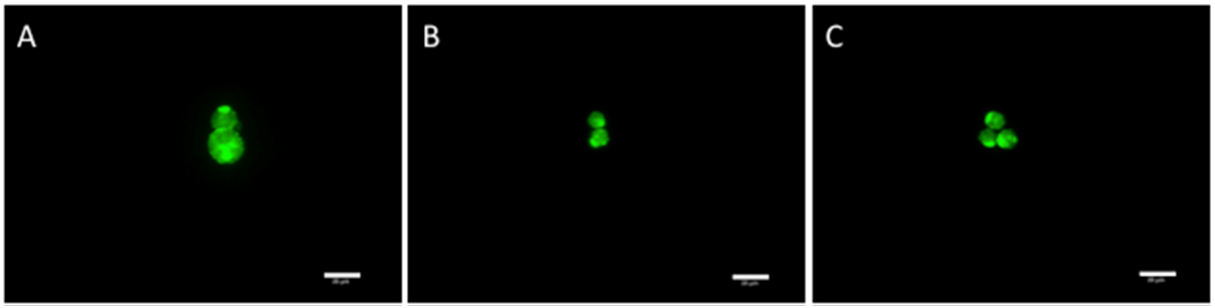

Figure 1. Fluorescence microscopy of encapsulated U2-OS cells at different concentrations of PLL: A) 200 µg/ml, B) 400 µg/ml and C) 2000 µg/ml (per volume of cell suspension), after incubation during 2 days. Scale bar: 25 µm.

3.2 Printing

Prior to printing the 10µm sample taken from the micro tube reservoirs were viewed under a microscope to visually assess cell distribution. Figure 2, show the comparative cell distribution of the two bio-inks containing coated and non-coated cells. The bio-ink containing the non-coated cells showed significant cell agglomeration, whereas the coated cells are well dispersed throughout the imaged droplet.

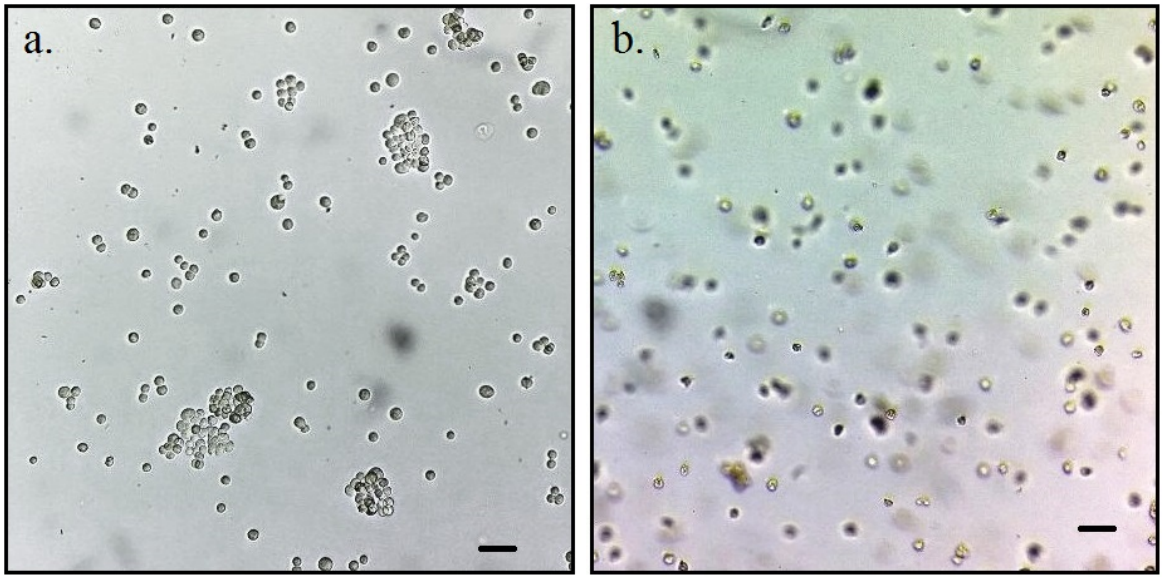

Figure 2. Optical microscope comparison of U2-OS cells in HBSS bioink suspension prior to printing experiments, a) non- coated cells, b) cells with a cationic Poly-l-lyseen coating

Figure 3, shows an example of droplet formation and one in a series of images from which drop diameter and velocity can be calculate. The voltage/time impulse variables for the bio inks were empirically derived to optimize droplet formation.

Figure 3. An optical micrograph taken with a stroboscope demonstrating optimised droplet formation.

The bio-inks were printed in 2 x 10 arrays with each point consisting of 50 droplets. The number of cells at each point was subsequently counted and the numbers recorded. Figure 4, shows the average number of cells over 10 points at each time point. The non-coated cells ceased printing after 10mins, and after showing initially very high cell per drop ratios. The coated cells demonstrated a relatively stable cell per drop ratio over the full printing period.

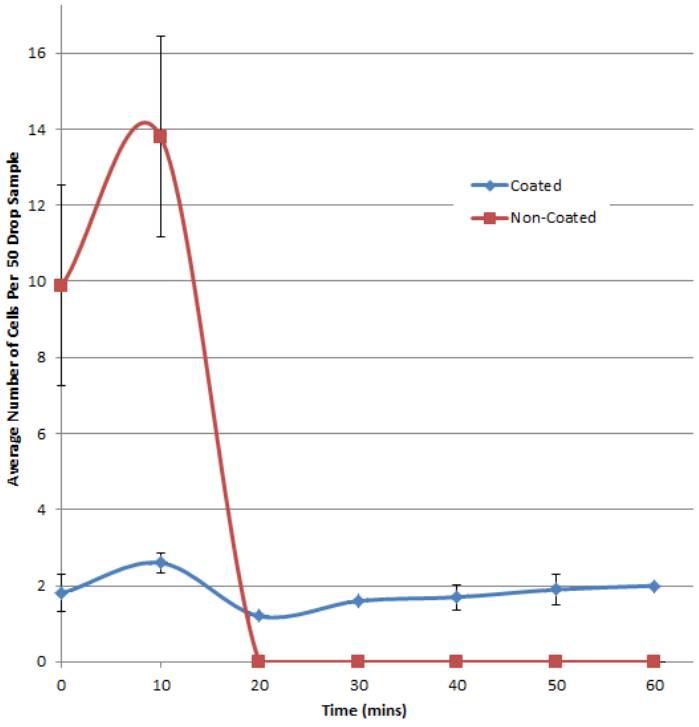

Figure 4. A plot showing the average number of cells per 50 drop sample over a 60min time frame.

4. Discussion

The cell encapsulation process showed that it was possible to effectively coat cells in PLL. The images of the coating, obtained by florescence microscopy, revealed a decrease of the coated cell diameter with an increase in PLL concentration during the coating process. This phenomenon could be attributed to an initial cytotoxic effect caused by the high polymer concentration and coating thickness. This increase could then be preventing the two way permeation of vital metabolic chemicals such as nutrients and waist. The pre-print contents of the bio-ink cartridges showed a significant difference between coated and non-coated cells, with non-coated showing significant flocculation. As flocculants were not visible during bio ink formulation it is believed that aggregation occurred in the 15min from formulation in the cell culture facility to arrival at the printing laboratory. Conversely, the coated cells showed good distribution through the drop and no evidence of flocculation. Uncoated cells were shown to block the orifice and stop printing after 10mins. The orifice blockage showed that a non-coated cell suspension would be unsuitable for prolonged printing. Conversely the coated cells showed a regular deposition of approximately 1 in 25 drops leading to the deposition of a cell. Although the cytotoxicity of the coating was not measured in this work, it is apparent that the U-2 OS cells coated in the lower concentration PLL survived, broke free and went on to proliferate after 7 days.

5. Conclusion

- It is possible to coat cells in a Cationic Polymer for use as an electrostatic stabiliser in bio-inks.
- Cells coated in a concentration of 200 μg/ml PLL will break free and proliferate after 7 days.
- The stability of bio-ink cell dispersions can be greatly enhanced by the use an electrostatic stabiliser, and will ultimately aid predictable printing.

6. References

[1] Kim G, Lee H, Kim Y, Kim S. Mineralized biomimetic collagen/alginate/silica composite scaffolds fabricated by a low-temperature bio-plotting process for hard tissue regeneration: fabrication, characterisation and in vitro cellular activities. J. Mater. Chem. B 2014;2; 5785-5798

[2] Derby. B, Inkjet Printing of Functional and Structural Materials: Fluid Property Requirements, Feature Stability, and Resolution. Annu. Rev. Mater. Res. 40:395-414

[3] Einstein A. Investigations on the Theory of, the Brownian Movement. Princeton University Press. 1989. Retrieved 10-06-2014 http://users.physik.fu-berlin.de/~kleinert/files/eins_brownian.pdf

[4] A.Diaspro et al Single living cell encapsulation in nano-organized polyelectrolyte shells. Langmuir 2002;18;5047-5050

[5] J.Kang et al (2012), Strong Response of Multilayer Polyelectrolyte Films to Cationic Surfactants. Langmuir 2012;28; 16751-16760