Platinum blue staining of cells grown in electrospun scaffolds

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Fibroblast cells grown in electrospun polymer scaffolds were stained with platinum blue, a heavy metal stain, and imaged using scanning electron microscopy. Good contrast on the cells was achieved compared with samples that were gold sputter coated. The cell morphology could be clearly observed, and the cells could be distinguished from the scaffold fibers. Here we optimized the required concentration of platinum blue for imaging cells grown in scaffolds and show that a higher concentration causes platinum aggregation. Overall, platinum blue is a useful stain for imaging cells because of its enhanced contrast using scanning electron microscopy (SEM). In the future it would be useful to investigate cell growth and morphology using three-dimensional imaging methods.

Porous biomaterials fabricated from polymers are used in a number of applications (1–3). These are commonly used to form scaffold structures that are widely used for tissue regeneration (4). These scaffolds provide wide open spaces and also act as structural supports, allowing cells to maintain their three-dimensional (3-D) architecture while growing in cell culture (5).

Observation of cells and/or scaffolds is achieved by many imaging techniques. A large number of studies have used the scanning electron microscope (SEM) for imaging (6). SEM sample preparation of cells is normally achieved after fixation of the sample in an aldehyde, followed by alcohol dehydration, drying using hexamethyldisilazane (HMDS) (6), or critical point drying (CPD) (7). Contrast from the samples is achieved using heavy metals that scatter electrons well and adsorb to biological matter easily. Sputter coating the sample with heavy elements such as gold (8) is generally used. An alternative method is to stain the sample using heavy metal compounds such as lead citrate or phosphotungstic acid. The most commonly used heavy metal stains for electron microscopy are uranyl acetate and osmium tetroxide (6). However, uranyl acetate is radioactive, and its use is severely restricted (9), whereas osmium tetroxide is a highly poisonous compound (10); thus, there is a need for safer and more easily obtainable heavy metal stains. Platinum blue is a safe alternative to these heavy metal stains and can be easily synthesized in the laboratory (11–13).

Recently, platinum blue has been used as a heavy metal stain for imaging cells using low-vacuum SEM (12,14) and atmospheric SEM (15). Platinum blue is a polymeric compound of deep blue, green, or purple in which platinum is coordinated with amide groups (16). The platinum blue complex used in this study was synthesized using the method of Hoffman and Bugge (13). It is a product of the reaction of dichlorobisacetonitrileplatinum(II) ($Pt(CH_3CN)_2Cl_2$) with silver sulfate. In this study, we imaged fibroblast cells after they were grown in electrospun poly(lactide-co-glycolide) (PLGA) scaffolds and stained with platinum blue. Poly(α-hydroxy esters) and their copolymers such as PLGA are a commonly investigated class of biomaterials used in

METHOD SUMMARY

Fibroblast cells grown in electrospun polymer scaffolds were stained with platinum blue, a heavy metal stain. The optimum platinum blue staining concentration for cells grown in scaffolds was obtained after performing scanning electron microscopy (SEM).

many applications, including scaffolds for tissue engineering. This is because they have biodegradable and bioreabsorbable properties, are approved by the US Food and Drug Administration and can be readily tailored to possess a range of material properties (17,18). The purpose of this study was to investigate and optimize heavy metal platinum blue staining of cells grown in scaffolds.

Material and methods

PLGA scaffold fabrication

PLGA scaffolds were prepared as described previously (8). Briefly, PLGA (Purasorb, 5004; Purac Biomaterials, Gorinchem, The Netherlands) had a monomer ratio of 50:50 lactide to glycolide, a weight-averaged molecular weight of 40,000–75,000. Solutions 12.5% (wt/wt) were made by dissolving in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma Aldrich, Gillingham, UK). These solutions were electrospun into fibers with diameters between 2 and 3 µm. The solutions were delivered at a constant feed rate of 0.8 mL/h and were electrospun vertically with an accelerating voltage of 11 kV supplied by a high-voltage DC power supply. Nonwoven fibrous scaffolds were collected on aluminum foil sheets that were wrapped around an earthed rotating collector 30 cm from the tip of the needle. The relative humidity was 60%, and the temperature was 30°C. The scaffolds were sterilized using ultraviolet light for four hours.

A rotating collector drum was used to ensure a uniform distribution of the fibers over the collection area to achieve a consistent scaffold thickness. The goal was to achieve nonwoven scaffolds with a uniform thickness, instead of aligned fibers. The rotating revolution per minute is not necessary for random fibers. Fiber diameter, scaffold morphology and porosity analyses were performed by SEM (Phenom G2 Pro equipped with Fibermetric software, Phenom, Eindhoven, The Netherlands).

Cell culture

An immortalized human lung fibroblast cell line at passage 8 was cultured in Ham's F12 medium (Sigma-Aldrich, UK), supplemented with 10% fetal bovine serum (Sigma-Aldrich, UK), and 1% L-glutamine at 37°C in a 5% CO_2 incubator. The cells at a density of 3×10^4 were grown in the scaffold for 6 days. Cell culture medium was changed every two days. The CCD-37lu cell line was selected because it is a normal adherent fibroblast cell line.

SEM sample preparation

The scaffold containing cells were fixed using 2.5% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 2 hours. Samples were then washed three times with 0.1 M cacodylate buffer (pH 7.2). Dehydration of the samples was done using a series of ethanol solutions: 70%, 85%, and 100%. The scaffolds were dried using HMDS (Sigma Aldrich, UK) for 5 min. Sputter coating of the scaffolds was done using a Q150T ES sputter coater (Q150T ES; Quorum Technologies Ltd, East Grinstead, UK) where approximately 25 nm of gold was applied. Alternatively, staining of the scaffold samples was done using platinum blue at a concentration of

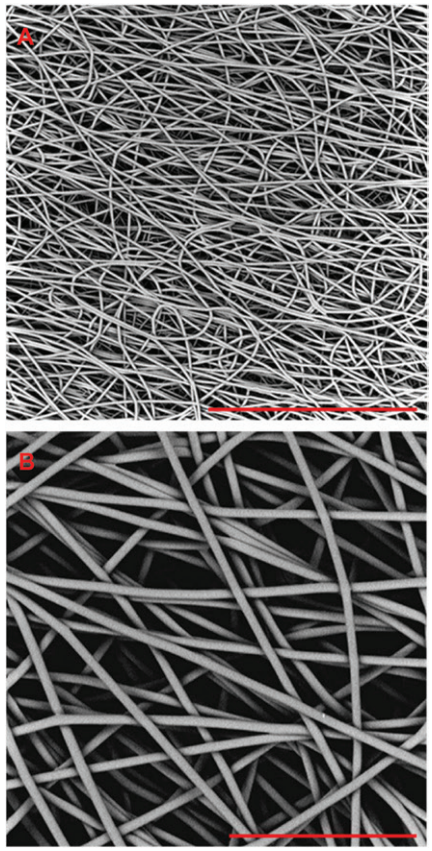

Figure 1. SEM images showing a PLGA 12.5 % wt scaffold. Magnification: (A) 600×, (B) 2000×. The scale bar for A is 220 µM and the scale bar for B is 60 µM.

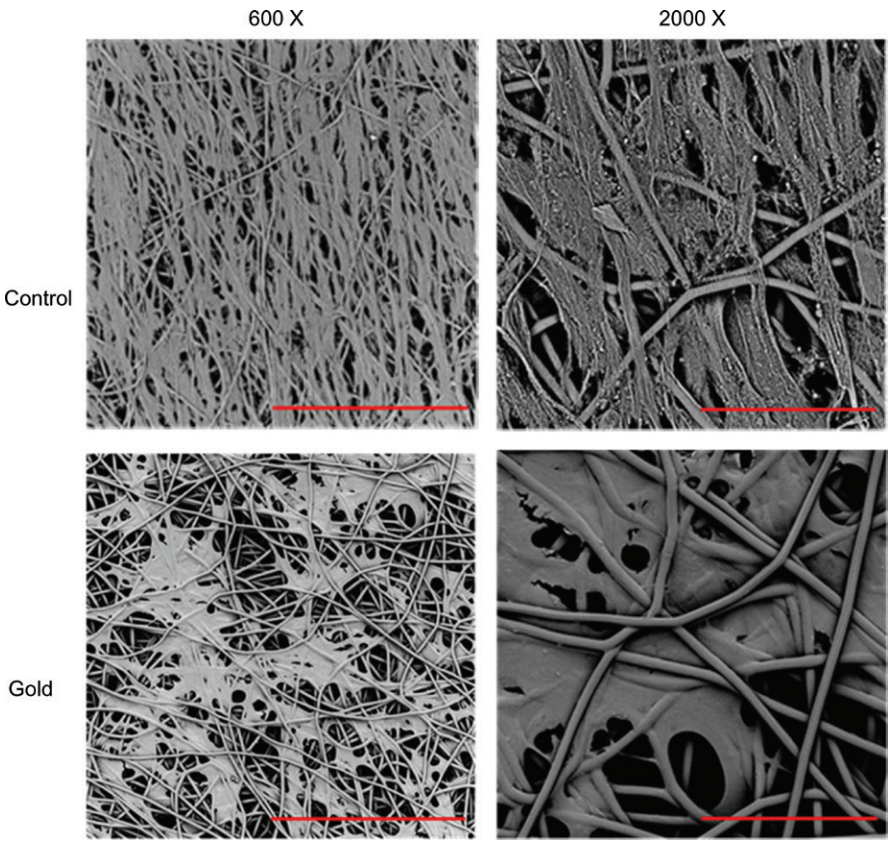

Figure 2. SEM images showing cells in scaffolds. Controls with no stain and after gold sputter coating. Scale bars are 220 μM for all 600× images and 60 μM for all 2000× images.

2.5 mM, 5 mM, or 7.5 mM for 30 min each at room temperature. Washes were performed with water twice for 5 min. Imaging was performed using a desktop scanning electron microscope (Phenom G2 Pro equipped with Fibermetric software; Phenom).

Synthesis of platinum blue

The platinum blue complex used in this study was synthesized using the method of Hoffman and Bugge (13). Potassium tetrachloroplatinate (2 g) was mixed with acetonitrile (3 mL) in 40 mL of water at room temperature. The reaction was left for 10 days after which yellow crystals were obtained. The liquid was decanted, and the crystals were air dried and weighed. The crystals were then mixed vigorously with the same amount of silver sulfate in a 5-fold volume of water until the blue color reached its maximum intensity after which a 10-fold volume of methanol was added to the solution. The solution was filtered followed by the addition of diethyl ether to the filtrate to precipitate the platinum blue. The platinum blue was then filtered out from the solution and air dried. As a powder or in water, platinum blue is stable for at least one month at room temperature.

Results and discussion

PLGA scaffolds were imaged prior to the growth of cells and before and after the staining steps. The relatively uniform, randomly orientated scaffolds displayed initial fiber diameters between 2 and 3 μm. Porosity analysis was performed using an SEM. This allows us to observe the spaces between the filaments permitting the 3D cell growth (Figure 1, A and B). A control sample where fibroblast cells were grown in the scaffold but not stained was imaged. Cells could be seen within the scaffold with the characteristic platelike morphology expected for fibroblasts. The cells in the control and gold sputter coated cells showed no significant contrast difference (by SEM) from the scaffold fibers that they were grown in (Figure 2). Three different concentrations of platinum blue were

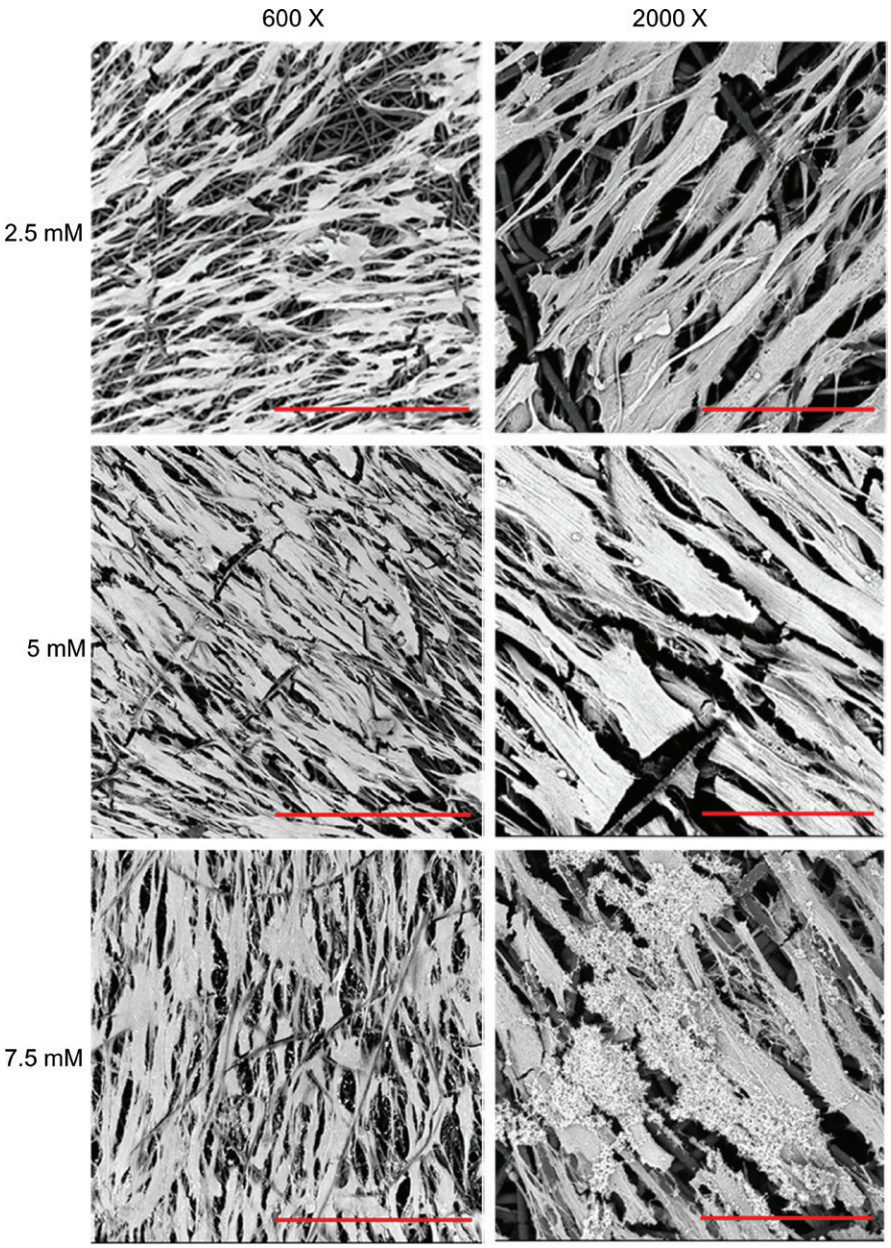

Figure 3. Scanning electron microscopy (SEM) images showing cells in scaffolds stained by platinum blue. Cells grown in scaffolds stained with different platinum blue concentrations. Scale bars are 220 μM for all 600× images and 60 μM for all 2000× images.

used to stain the cells in the scaffold. The concentrations investigated were 2.5 mM, 5 mM, and 7.5 mM. The cells were easily identified from all of the different concentrations used because platinum blue was found to stain only the cells but not the scaffold fibers. The immortalized human fibroblasts were observed to be attached and spindle shaped, displaying a typical elongated morphology (Figure 2). Both 2.5 mM and 5 mM platinum blue provided good contrast, but the 7.5 mM platinum blue showed aggregated clusters of the platinum on the surface of the cells. These aggregated clusters can been seen clearly in Figure 3. However, the clusters were not visible after 2.5 mM or 5 mM platinum staining (Figure 3).

Platinum blue is a useful stain for imaging cells grown in scaffolds, mainly because it is less toxic than other heavy metal stains. Other advantages are that staining with platinum blue takes only 30 min, and it gives sufficient contrast to the cells, allowing them to be distinguished from the scaffold fibers.

In the future, the stain should be further explored for growth of cells in scaffolds and imaging using three-dimensional techniques. Using SEM alone, it is not possible to determine the extent to which the stain penetrates the interior parts of the structure, as SEM is a surface imaging technique. We are optimistic that in the future, 3-D imaging methods will provide more information into stain penetration and growth of cells, including cellular morphology in scaffolds.

Author contributions

M.Y. designed the study and wrote the manuscript. M.Y. and A.L.G.M performed the experiment and performed the SEM imaging. A.K.E. synthesized the platinum stain. G.K.B. helped with manuscript preparation and images. R.M. supervised the electrospinning part of the project. All authors helped in amending the manuscript. M.Y., I.K.R., and E.B. supervised the study.

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Competing interests

The authors declare no competing interests.

References

1. **Castillo-Dali, G., R. Velazquez-Cayon, M. A. Serrera-Figallo, A. Rodriguez-Gonzalez-Elipe, J. L. Gutierrez-Perez, and D. Torres-Lagares.** 2014. Importance of PLGA in scaffolds for guided bone regeneration: a focused review. J Oral Implantol. (In press.)
2. **Wang, Y., X.C. Liu, J. Zhao, X.R. Kong, R.F. Shi, X.B. Zhao, and F. Lu.** 2009. Degradable PLGA scaffolds with basic fibroblast growth factor: experimental studies in myocardial revascularization. Tex. Heart Inst. J. *36*:89-97.
3. **Yu, J., A.R. Lee, W.H. Lin, C.W. Lin, Y.K. Wu, and W.B. Tsai.** Electrospun PLGA fibers incorporated with functionalized biomolecules for cardiac tissue engineering. Tissue Eng. Part A. *20*:1896-1907.

4. **Hollister, S.J.** 2005. Porous scaffold design for tissue engineering. Nat. Mater. *4*:518-524.
5. **Deluzio, T.G.B., D.G. Seifu, and K. Mequanint.** 2013. 3D scaffolds in tissue engineering and regenerative medicine: beyond structural templates. Pharm Bioprocess. *1*:267-281.
6. **Lee, J.T.Y. and K.L. Chow.** 2012. SEM sample preparation for cells on 3D scaffolds by freeze-drying and HMDS. Scanning *34*:12-25.
7. **Jung, S.W., H.M. Joo, J.S. Park, and J.H. Lee.** 2010. Development of a rapid and effective method for preparing delicate dinoflagellates for scanning electron microscopy. J Appl Phycol. *22*:313-317.
8. **Millas, A.L.G., R. McKean, R. Stevens, M. Yusuf, J.V.W. Silveira, M.B. Puzzi, and E. Bittencourt.** 2014. Fabrication of electrospun scaffolds incorporating an Amazonian therapeutic oil from the Copaifera Sp. for wound care applications. J Biomater Tissue Eng. *4*:217-220.
9. **Nakakoshi, M., H. Nishioka, and E. Katayama.** 2011. New versatile staining reagents for biological transmission electron microscopy that substitute for uranyl acetate. J. Electron Microsc. (Tokyo) *60*:401-407.
10. **Brunot, F.R.** 1933. The toxicity of osmium tetroxide (osmic acid). J Ind Hyg. *15*:136-143.
11. **Arrizabalaga, P., P. Castan, and J.P. Laurent.** 1984. Intramolecular influence of a carboxylic function on platinum blue synthesis. A systematic study of complexes originating from acid amides. J Am Chem Soc. *106*:4814-4818.
12. **Inaga, S., T. Katsumoto, K. Tanaka, T. Kameie, H. Nakane, and T. Naguro.** 2007. Platinum blue as an alternative to uranyl acetate for staining in transmission electron microscopy. Arch. Histol. Cytol. *70*:43-49.
13. **Wanner, G. and H. Formanek.** 1995. Imaging of DNA in human and plant chromosomes by high-resolution scanning electron microscopy. Chromosome Res. *3*:368-374.
14. **Inaga, S., S. Hirashima, K. Tanaka, T. Katsumoto, T. Kameie, H. Nakane, and T. Naguro.** 2009. Low vacuum scanning electron microscopy for paraffin sections utilizing the differential stainability of cells and tissues with platinum blue. Arch. Histol. Cytol. *72*:101-106.
15. **Nishiyama, H., M. Suga, T. Ogura, Y. Maruyama, M. Koizumi, K. Mio, and C. Sato.** 2010. Reprint of: Atmospheric scanning electron microscope observes cells and tissues in open medium through silicon nitride film. J. Struct. Biol. *172*:191-202.
16. **Barton, J.K. and S.J. Lippard.** 1978. A crystalline platinum blue: its molecular structure, chemical reactivity, and possible relevance to the mode of action of antitumor platinum drugs. Ann. N. Y. Acad. Sci. *313*:686-687.
17. **Sander, E.A., A.M. Alb, E.A. Nauman, W.F. Reed, and K.C. Dee.** 2004. Solvent effects on the microstructure and properties of 75/25 poly (D, L-lactide-co-glycolide) tissue scaffolds. J. Biomed. Mater. Res. A *70*:506-513.
18. **MacNeil, S.** 2008. Biomaterials for tissue engineering of skin. Mater Today. *11*:26-35.

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