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Alignment of muscle precursor cells on the vertical edges of thick carbon nanotube films

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ABSTRACT

The development of scaffolds and templates is an essential aspect of tissue engineering. We show that thick (>0.5 mm) vertically aligned carbon nanotube films, made by chemical vapour deposition, can be used as biocompatible substrates for the directional alignment of mouse muscle cells where the cells grow on the exposed sides of the films. Ultra high resolution scanning electron microscopy reveals that the films themselves consist mostly of small diameter (10 nm) multi-wall carbon nanotubes of wavy morphology with some single wall carbon nanotubes. Our findings show that for this alignment to occur the nanotubes must be in pristine condition. Mechanical wiping of the films to create directional alignment is detrimental to directional bioactivity. Larger areas for study have been formed from a composite of multiply stacked narrow strips of nanotubes wipe-transferred onto elastomer supports. These composite substrates appear to show a useful degree of alignment of the cells.

1. Introduction

Organ transplantation is limited by the supply of available donors and has a high associated cost. There are also problems of rejection of the transplant by the recipient. This provides impetus for the development of the interdisciplinary field of Tissue Engineering. Ideally, tissue or whole organs would be made from cells taken from the patient, obviating the problem of rejection.

In nature, cells with a structural role require the Extra Cellular Matrix (ECM) for support during growth and differentiation. The ECM is an intricate composite of protein fibres (fibrillar collagens and elastins) with length scales of ten to several hundreds of nanometers. This intertwined mesh is covered with adhesive proteins such as laminin and fibronectin that provide binding sites for cells. Polysaccharides such as hyaluronic acid and heparin sulphate fill the gaps and act as growth factor depots. See reference [1] for a review. For skeletal muscle formation, several myoblasts fuse to form multinuclear, long, cylindrical myofibres. Individual skeletal muscles consist of many myofibres bundled together. Structural proteins (dystrophin-associated protein complex) form a mechanical link between the sarcoplasm of myofibres and the ECM [2].

Myoblasts in cell culture are usually maintained in the presence of high levels of serum in order to prevent differentiation. If culture conditions are changed to low-serum medium, myoblasts that are in close proximity fuse to form myotubes [3]. Functioning myofibres in vivo may be several millimetres in length, which is longer than the myotubes that are obtained by conventional culture on a two-dimensional surface. Scaffolds with directional nanostructures may be employed as substrates in an attempt to increase the alignment and size of myotubes.

Research into synthetic versions of the ECM has explored both 3D structures and 2D surfaces. In 2D we are able to adapt electronic microtechnology to create patterned surfaces as templates for cell growth. In 3D, textile technology can be adapted to spin synthetic fibrous material. Either way, it now seems essential that appropriate nanoscale features must be a part of the template or scaffold for cell growth to be supported. The shape of cells can be influenced by the fine details present on the substrate. Epithelial cells cultured on long groove structures have been shown to form an elongated shape [4].

Because fabricating surfaces with very fine grooves by lithographic means is expensive for therapeutic applications, researchers have sought to obtain similar means by using self-assembling nanoscale fibres such as carbon nanotubes [5]. These materials have diameters ranging from 1 nm to 100 nm and lengths from several microns to many centimetres. Carbon nanotubes are generally made by some variant of the chemical vapour deposition (CVD) process and can produce either films on a substrate or free floating fibres that can be

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spun into thread [6]. It has also been shown to be possible to draw out thin sheets of highly aligned multi-walled carbon nanotubes from CVD growth forests [7]. Such sheets were subsequently used to cultivate ovary cells with a high degree of alignment [8]. Multi-walled carbon nanotubes (MWNTs) have been used as matrix for the culture of a number of cell types including osteoblasts [9,10], fibroblasts [11] stem cells [12–14] and cardiomyocytes [15]. However, single-walled carbon nanotubes (SWNTs) have been shown to have improved in vivo biocompatibility compared with multi-wall CNTs [16] and *may* therefore be preferable for tissue engineering applications. However the in-vivo study is a very different and more complex environment than on synthetic nanotube substrates as we have here or other similar studies. Single-wall CNTs have been shown to support the growth of carcinoma cells, fibroblasts, neuronal cells [17,18] and osteoblasts [19].

In this paper, we report the observation of highly aligned mouse precursor muscle cells on highly aligned MWNTs of small diameter. Muscle cells are inherently linear and an artificial scaffold or template that encourages preferential alignment along one direction is desired.

2. Experimental

2.1. Carbon nanotube growth

Carbon nanotubes (CNTs) were grown onto silicon substrates using an in-house built tube furnace system (850 °C) using BOC acetylene (10 sccm), Palladium membrane purified hydrogen (Johnson Matthey HS5 system, 300 sccm), getter purified helium (BOC rare gas purifier, 90 sccm) and as a growth promoter, water vapour. The latter was derived from a stainless steel bubbler held at 9.7 °C in a temperature controlled bath (Lauda) with a helium flow rate of 20 sccm. The silicon substrates ((Siltronix) 10 mm by 10 mm square) were solvent cleaned and then exposed to an air plasma discharge (home built microwave discharge chamber, 133 Pa, 150 W) and then vacuum coated through a shadow mask (6 mm square aperture) with aluminium (5 nm), oxidised in air at ambient for 30 min and then coated with 0.7 to 0.9 nm of iron as the catalyst. The film thicknesses were measured in-situ by an Inficon XTC/2 quartz crystal microbalance. The substrates were sintered at 850 °C to form discrete iron islands of some 1–10 nm in diameter (as revealed by high resolution scanning electron microscopy, SEM, see below) for 5 min in pure hydrogen before growth for 30 min. The substrates were inserted into the tube furnace on a fused silica shoe using a magnetic transfer device. This enabled the substrates to be rapidly heated to growth temperature to gain better control the sintering process. Typical film thicknesses were at least 0.5 mm, typically 1 mm and on occasion reached over 4 mm in extent, albeit over a very limited area indicating that the growth rate is very sensitive to local conditions. This procedure for growing thick carbon nanotube films has been developed in-house, based on the super-growth method of Hata et al. [20] for SWNTs and, as judged by our research, seems to work also for small diameter MWNTs.

Using a laser cut mask during the deposition of the catalyst layer, ten narrow width (0.25 mm) strips of vertically aligned CNTs were also created on silicon substrates. These were either mechanically pushed over flat using a horizontal pin or contact transferred to thin strips of silicone rubber (Sylgard 184, Dow Corning) coated with partly cured fresh silicone as an adhesive. The transfer was performed by gently sideways wiping the CNT strip off the silicon wafer. The latter mounted strips were transferred to a second substrate after rotation through 90° to provide a larger area CNT surface for cell growth.

Some unpatterned CNT pads were also wiped flat using either a Teflon™ (Polytetrafluoroethylene, PTFE) block or a clean circular glass plate. No other materials were considered as we did not wish to transfer surface layers to the CNTs, only to realign them and there were no other suitable large flat and very smooth substrates available. Float glass is known to be very smooth with local RMS roughness values of 0.4 to 0.6 nm [21]. We found that a special jig had to be employed to successfully wipe the CNT pads on glass: the friction was far too high to perform this task by hand. Even PTFE offered considerable resistance to sliding motion but this could be performed by hand. Therefore we modified a polishing tool (South Bay Technology model 910) to take a rigid box-section pillar screwed to the internal base plate and fitted with a vertical motion micrometer drive to bring the samples into gradual contact with the rotating glass at about 1 rpm.

The as grown nanotubes were characterised by an ultra high resolution SEM (FEI Verios 460L) operated at 2 kV and were imaged directly with no overcoating applied. Micro Raman spectroscopy was also used with 780 nm illumination (Renishaw 1000, with Olympus optical microscope).

2.2. Cell culture

The mouse myoblast cell line C2C12 [22,23] was grown to 80% confluency in Dulbecco's Minimal Essential Medium (DMEM) (Gibco, Life Technologies Ltd., Paisley, UK) supplemented with 20% decomplemented foetal bovine serum (Gibco) and antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin) (Gibco). Cells were trypsinised and resuspended in fresh culture medium at 100,000 cells/ml. The sterile SWNT mat was placed in a sterile Petri dish (90 mm × 16.2 mm, code: FB51510, Fisher Scientific Ltd., Loughborough, UK) and the minimum amount of C2C12 cell suspension was gently added to completely submerge the SWNT mat. Cell cultures were incubated at 37 °C in a humid air atmosphere containing 5% CO_2 (LEEC, Nottingham, UK). After 2 days of culture, cells on the SWNT mat were fixed with 1% formalin in PBS for 10 min, followed by gentle washing with distilled water. The samples were allowed to dry slowly in air during which time examination using a reflection microscope (Riechert PolyVar Met) fitted with Differential Interference Contrast (DIC or Normarski technique) was undertaken. Culture of C2C12 cells with SWNT mats was repeated on nine separate occasions.

3. Results & discussion

The morphology of the top of the as-grown carbon nanotube pad as seen by SEM shows a tangled mass of fibres forming into clumps around 200 nm across and with deeper 'pockets' some 200–300 nm across of unknown depth. There is clearly no obvious directional bias in the nanotubes that can be seen in Fig. 1a where the darker unfilled 'pockets' extending into the depth of the pad can be seen. Many nanotubes appear to loop along the plane of the surface rather than terminate there. The edges of the nanotube growth pad reveal a preferred alignment to the nanotubes, Fig. 1b, but also that the nanotubes are not straight but rather irregular but with a constant diameter of no more than 10 nm. The projected gap between the nanotubes varies from 2 to 150 nm and there is a high degree of entanglement with large changes in direction. Again, clumping together (or entanglement) leads to the formation of deep 'pockets' in the pad structure. Lower magnification images indicated that the length of these open 'pockets' is some 2 μm in extent and also that the edge morphology has the appearance of vertically aligned grooves, Fig. 1c.

During initial examination of the first trial it was observed that cells had indeed grown on top of the CNT forest but with no preferential alignment as shown in Fig. 2a. Here, the forest was still wet and the CNT were still vertically aligned. The cells were branched with thin, elongated projections, indicating that this substrate is suitable for cell growth. Similar highly branched cells with no preferential alignment were found to have grown on the adjacent silicon areas, Fig. 2b. As the forest slowly dried out, the entire structure collapsed inwards to reveal the edges of the CNT pad, the whole sample now being planar in nature, Fig. 3a. Here it could be seen that not only were there cells grown on the edge but that they were highly