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A study of cell behaviour on the surfaces of multifilament materials

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Since many fibres are very strong, they are considered to have useful potential for fibre reinforcement of orthopaedic and dental implant materials. Fibres exposed on the surface of composites may significantly influence the cellular response not only due to the chemistry but also due to the fibre size and shape. This study has concentrated on investigation of cellular responses to fibre-specific aspects of fibre-reinforced composites. Four multifilament materials with diameter less than 20 μm were used: Kevlar 29(K), silicon carbide(SiC), nylon 66(N), and polyethylene terephthalate(PET). Established cell line L929 fibroblasts were used as the cell model. Cell behaviour on the surfaces of fibres was examined using direct cell counting (after 3, 5, 8 h and 1, 2, 3 days), scanning electron microscopy (SEM) (after 2 h and 2 days), and fluorescent staining of F-actin, which was analysed by confocal laser scanning microscopy (CLSM) (after 2 h and 2 days). The results showed that fibroblasts adhered and grew very well on all fibre surfaces, although less cells were observed on PET from direct cell counting. Significant orientational behaviour of cells was found on all fibre surfaces from the SEM and CLSM analysis, independent of the bulk chemistry of the fibres.

1. Introduction

Since many fibrous materials are strong and display good mechanical properties they are considered to be candidates for the fibre reinforcement of orthopaedic and dental implant matrial. Fibre-reinforced composite materials (FRCM) can combine some of the physical and mechanical properties of both the fibre and matrix phases to produce a material with superior properties. Through the correct choice of fibre and matrix, it is possible to produce a range of mechanical properties which cannot easily be achieved by conventional un-reinforced materials. The elastic moduli of the composite material intermediating between polymer and ceramic can be obtained, which is important as far as matching the properties of bone and teeth is concerned, and much better toughness often results from this combination, as well as many directional characteristics [1].

It is recognized that fibres may not only be used as the reinforcement material in order to improve the mechanical property of the bulk component, but can also be used for the construction of a material with a composite structure, which allows the design of a bulk component to provide engineering performance and a surface component to achieve an optimal biological tissue reaction. If the fibre had good biocompatibility and could induce cellular responses in a certain way, it could be designed to be exposed on the surface of implant material and devices, in a specific orientation, to improve the biocompatibility of a composite material for a specific application. This may enhance the appropriate host cell response and facilitate the wound healing process in body tissue.

It has been recognized recently that features of the surface topography of implant biomaterials, when measured in dimensions around a micrometre, may influence the host response by virtue of physical rather than chemical or mechanical mechanisms [2–5]. Surface topography of microgrooved and microporous textures are recognized to have significant effects on host cell responses under some circumstances.

The objective of this study was to investigate the cell behaviour on the surfaces of four multifilament materials in terms of cell adhesion, spreading, orientation and growth using the following three techniques: direct cell counting; scanning electron microscopy and fluorescent staining of F-actin.

2. Materials and methods

2.1. Materials

The four multifilament materials used in the study were Kevlar 29 (K), silicon carbide (SiC), polyethylene terephthalate (PET) and nylon 66 (N) (Goodfellow Metal Ltd, England). The diameters of the fibres are all less than 20 μm as shown in Table I. The materials were cut into the lengths required for the experiments before they were thoroughly washed ultrasonically in double distilled water. The samples of SiC were washed in both ethanol and double distilled water

TABLE I Physical characteristics of the four multifilament materials

Material	Diameter of fibres (μm)	Number of fibres in a strand
Kevlar 29	18	120
Silicon carbide	15	500
Polyethylene terephthalate	17	24
Nylon 66	19	10

since it was found in a previous study that SiC was shown to be cytotoxic without ethanol wash [22]. All samples were sterilized by autoclaving at 120 °C for 20 min and they were then put into 35×10 mm Petri dishes separately before cell seeding.

2.2. Cell line

The established cell line L929 (obtained for Flow lab, Irvine, Scoland) was used as the test cell model in this study, and maintained in growth medium 199 supplemented with 5% foetal calf serum, 50 Iu/ml penicillin and 50 μg/ml streptomycin. A routine subculture was used to maintain the cell line. The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. They came to confluence after one week's incubation and the monolayer was then harvested by trypsinization. The cell suspension was aliquot diluted before cell seeding.

2.3. Cell growth counting

Since the number of cells that can attach to the fibres will be related to the surface area of materials, the length of the specimens to be used was estimated to provide relatively equal surface areas of the materials. The materials were cut into 2.5–3 mm lengths before washing. The cell suspension was diluted 3.5 times (about 3.5×10^5 cells/ml) using growth medium 199. 3 ml of cell suspension was seeded into each Petri dish with the material samples and then they were maintained in the incubator for the required times.

The number of cells attached to the materials was measured by harvesting with trypsin and counting in a Coulter Counter (Model "7", Coulter Electronics Ltd, England). The number of cells attached to the surfaces of the four multifilament materials were counted after 2, 5 and 8 h and after 1, 2 and 3 days in culture. The experiments were performed in triplicate. At the required time, the samples were removed from the Petri dishes and washed with phosphate-buffered saline (PBS) twice in clean dishes before the cells were harvested by trypsinization in 1 ml trypsin at 37 °C. After 30–60 min incubation, in order to be certain that almost all the cells had detached from the surfaces of the fibrous materials, the samples were washed and diluted in an isotonic solution and counted in the Coulter Counter. At each observation point, an average of three samples of each material were made.

2.4. Scanning electron microscopy

Cell morphology, spreading, orientation and growth on the surfaces of fibres were evaluated using one of the most common qualitative techniques, scanning electron microscopy (SEM). The samples of fibres were adhered separately to sterilized microscope coverslips 13 mm in diameter (Chance Propper Ltd, England) by "Silastic" medical adhesive (Dow Corning Co. USA) and incubated with L929 fibroblasts in Petri dishes for 2 h and 2 days.

At the required time, the samples were removed from the incubator and washed immediately in PBS twice before they were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min, rinsed in 0.1 M phosphate buffer twice for 20 min, then dehydrated in graded methanol: 70%, 90% and 100% for 1.5 h, twice for each concentration. Then the specimens were critical-point dried. Finally, the prepared samples were sputter-coated with gold and examined in a JSM-35C scanning electron microscopy (JEOL, Japan).

2.5. Fluorescent staining of F-actin

The microfilament structure of the cytoskeleton of cells attached to the fibres was examined using confocal laser scanning microscoy (CLSM) through fluorescent staining of F-actin. The autoclaved glass coverslip was again used as the surface to which the multifilament materials were adhered with "Silastic" medical adhesive. The monolayered cell suspension was diluted three times and 3 ml of cell suspension was seeded into each Petri dish with the material samples and maintained in the incubator for 2 h and 2 days.

At the required time the samples were removed from the Petri dishes, washed with PBS twice, and fixed in 3.7% formaldehyde solution in PBS for 10 min at room temperature. Then they were further washed in PBS twice before being transferred to clean Petri dishes, in which they were fixed with a solution of -20 °C acetone for 3–5 min. All the samples were air-dried. 200 μl of PBS solution, which was evaporated and dissolved with 5 μl of the fluorescent stock solution of rhodamine conjugated phalloidin (Molecular Probes, Inc. USA), were placed on each coverslip with the samples for 20 min at room temperature. This was followed by washing quickly twice with PBS. Finally, the coverslips were mounted with the cell side down in a 1 : 1 solution of PBS and glycerol. The edge of the coverslip was sealed with glycerol gelatin. All the specimens were stored in the dark at 4 °C in a refrigerator before examination by a CLSM microscope (Noran Instruments, Inc. Germany).

3. Results

3.1. Cell growth counting

Direct cell counting presented a preliminary approach to study the cell adhesion and growth quantitatively on the multifilament materials. The results showed that good cell adhesion and growth was present on the four fibrous materials, although fewer cells were

observed on the PET samples in comparison with the other three materials within the observation time. Table II and Fig. 1 present the Coulter Counter data of the number of cells attached to the fibres after the first few hours following cell seeding, and the cell growth for the next 3 days. It can be seen that SiC had the highest cell attachment and growth, followed by nylon 66 and Kevlar; the least cell adhesion and growth was found on PET. Nevertheless, the number of cells on PET was still shown to increase with time.

An interesting observation in this study was that the fibroblasts attached to the Petri dishes were more rapidly released following trypsinization than those on the multifilament materials. Usually, it takes only 2–5 min to harvest cells from a tissue culture dish using trypsin, however, 30–60 min was required to harvest cells attached to fibres.

3.2. Scanning electron microscopy

It was observed during this study that L929 fibroblasts adhered and grew very well on all fibre surfaces after 2 h and after 2 days. However, different cellular responses were observed at the early period (2 h) on the multifilament materials. Most cells exhibited attachment and early spreading on the surfaces of fibres and appeared to be spherical in shape. This was seen most easily on the surfaces of nylon 66 (Fig. 2f). There were also some cells which appeared to be elongating and extending their lamellipodia processes along the long axis of the fibres. This was observed more clearly on the surfaces of Kevlar and PET (Fig. 2a, b and e). It was interesting to find that most cells on SiC samples appeared to be suspended between the fibres after 2 h (Fig. 2c and d). Lamellipodia of fibroblasts were observed at both ends adhering to the fibre surfaces. This phenomenon was not so clearly seen on the other materials.

After 2 days, more cells appeared on the surfaces of fibres and were well adhered to the materials. The cell morphology appeared more or less the same on all four multifilament materials (Fig. 3). Most cells appeared elongated in a spindle shape and aligned longitudinally along the long axis of the fibres, particularly on the surfaces of Kevlar 29, nylon 66 and PET. More cells were observed on the surfaces of SiC (Fig. 3b) than on the other materials. When the cells became crowded they appeared to be more spherical in shape and suspended in groups between the fibres. Nevertheless, there were still many cells which were elongated in shape and aligned on SiC surfaces.

TABLE II The number of cells attached to the surfaces of fibres at different time periods (× 10 000)

Time (h)	Nylon 66	SiC	Kevlar	PET
2	2.6	7.3	1.4	1.4
5	3.1	7.5	3.1	3.4
8	6.4	7.7	4.1	3.6
24	11.4	10.5	3.2	4.6
48	10.0	17.1	14.8	6.1
72	17.7	22.8	13.2	12.1

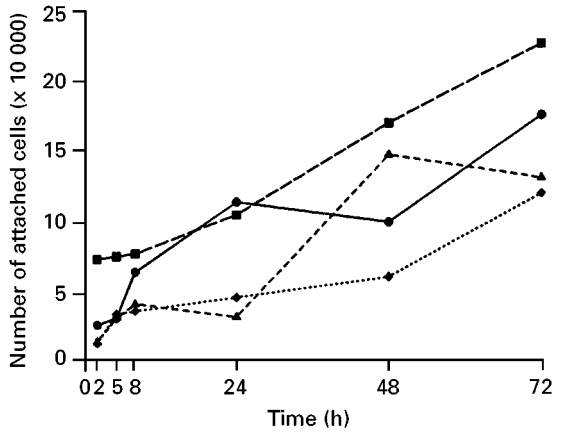

Figure 1 Direct cell counting of cell attachment and growth on the surfaces of multifilament materials over 3 days: ● Nylon 66; ■ SiC; ▲ Kevlar 29; ◆ PET.

3.3. Fluorescent staining of F-actin

Fig. 4 shows CLSM micrographs of fibroblast cells attached to the fibres. It was shown that L929 fibroblasts were spread randomly in various directions on the flat glass surfaces after 2 h. Stress fibres of microfilaments were seen clearly in the elongated cells (Fig. 4a arrow). Cells on the surface of fibres were observed to be elongated and aligned parallel with the long axis of fibres, even after 2 h (Fig. 4c and d). The actin meshwork and the actin-rich cortex area under the plasma membrane was clearly stained in all cells. After 2 days, the orientation of cells was more clearly shown on all fibres. No difference in cell morphology was observed among the fibre samples. The stress fibres could be defined in the cytoplasm of some cells in this group (Fig. 4e arrow) and their orientation was parallel to the cell body as well as the long axis of the fibres.

4. Discussion

It is known that materials to be implanted in the body must have intimate contact with the body tissue, providing a real, physical interface. For biomaterials and medical implants which are supposed to function within the human body for an appropriate length of time, it is necessary for them to permit the formation and retention of a mechanically stable interface. This requires, specifically, the stimulation of new tissue formation at the interface. In order to achieve optimum formation at the interface, consideration should be given to the factors which control cell behaviour at the surface of materials in general, the details of the specific application and the possibilities for control of these responses in order to optimize the biocompatibility of the materials and implants. Recent studies have demonstrated that small modifications in the composition and texture of the surfaces of materials can have major effects on the subsequent host–implant interaction, and can facilitate the wound healing process and tissue repair [4, 5].

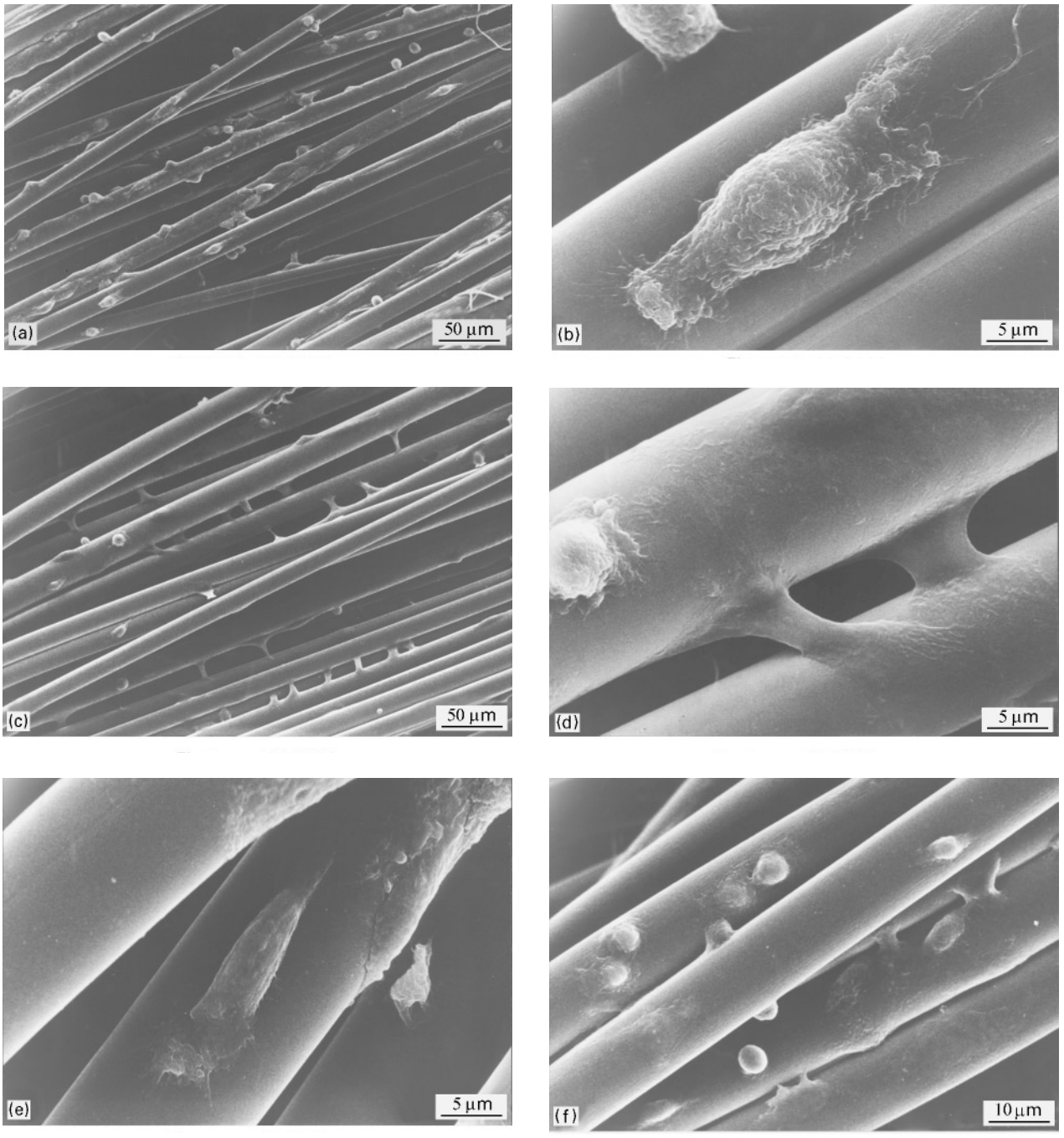

Figure 2 Cellular response of L929 fibroblasts to the multifilament materials after 2 h (a), (b) Kevlar 29. The cells attached and grew very well on fibres. Some of them showed obvious orientation and extended their lamellipodia processes along the fibres. (c), (d) SiC. Most cells appeared to be suspended between the fibres with extending processes adhering to the fibre surfaces on both ends. (e) PET. The cells began to extend their lamellipodia processes along the long axis of fibres. (f) Nylon 66. The cells showed early radial spreading on the fibres.

Fibre-reinforced composite materials are particularly significant here, since the fibres at the surface can alter the surface morphology and can present morphological features which are able to take part in cell recognition and activation processes by virtue of their size and shape. The present study was undertaken in an attempt to examine the cell behaviour of the same L929 fibroblasts on a number of small-diameter fibres (less than 20 μm) which may be potentially useful for the fibre reinforcement of orthopaedic and dental implant material.

A consistent effect of the surface curvature on cell orientation behaviour was observed among all the multifilament materials in this group. This indicated that L929 fibroblasts were able to recognize and distinguish the surface curvature of small-diameter fibres from that of a flat substrate and thus exhibited an obvious alignment and orientation. As shown in Table I, the diameter of the four fibres were all less than 20 μm (SiC 15 μm; PET 17 μm; Kevlar 18 μm and nylon 19 μm). Orientational behaviour of cells occurred on all four multifilament materials tested and seemed to be independent of the bulk chemistry of fibres. This finding was in agreement with the report by Ricci *et al.* [6], who used a primary cell model of rat tendon fibroblasts to investigate the cell morphological characteristics on four synthetic fibres with small diameters (carbon/8 μm; dacron/11 μm;

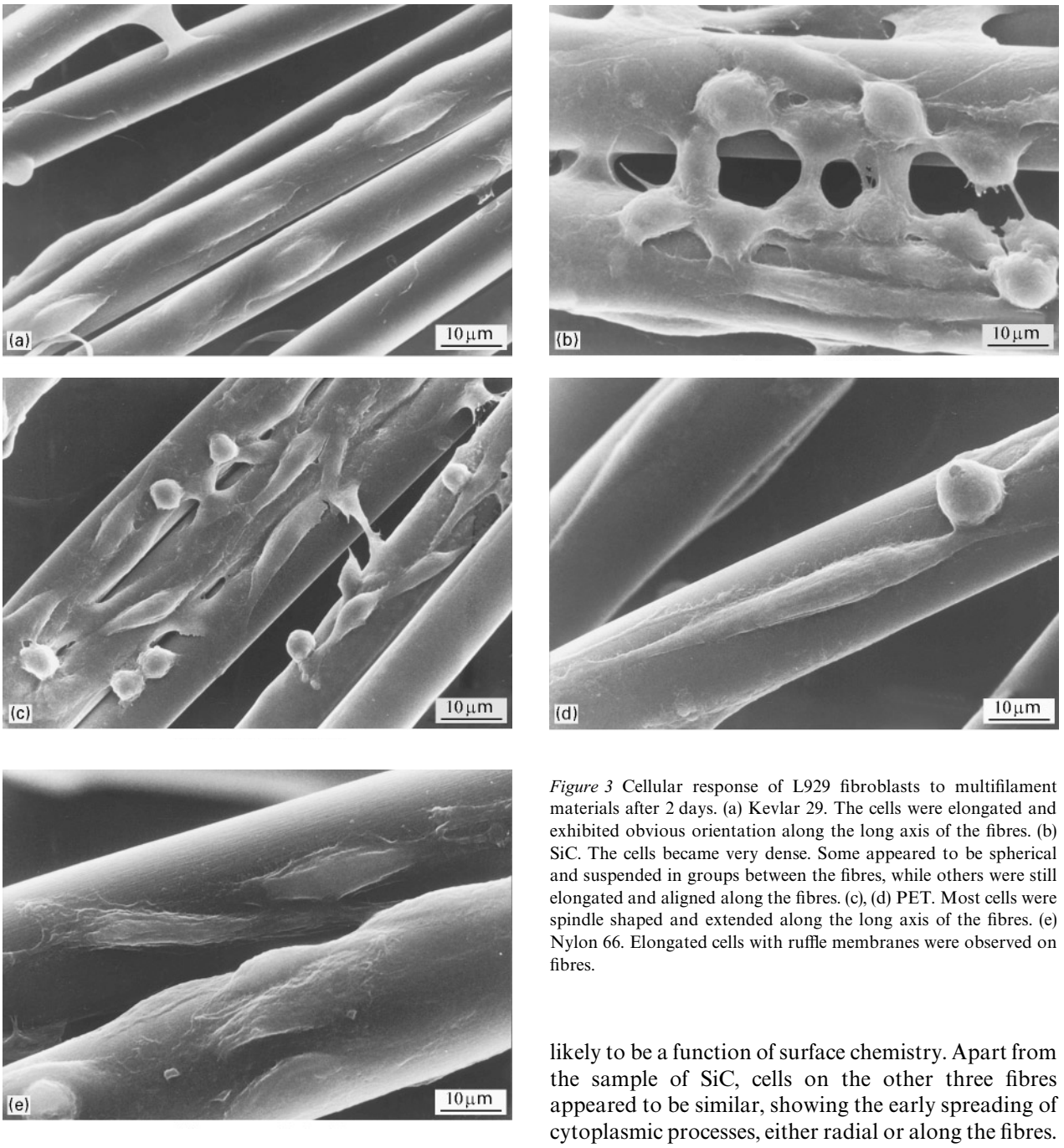

Figure 3 Cellular response of L929 fibroblasts to multifilament materials after 2 days. (a) Kevlar 29. The cells were elongated and exhibited obvious orientation along the long axis of the fibres. (b) SiC. The cells became very dense. Some appeared to be spherical and suspended in groups between the fibres, while others were still elongated and aligned along the fibres. (c), (d) PET. Most cells were spindle shaped and extended along the long axis of the fibres. (e) Nylon 66. Elongated cells with ruffle membranes were observed on fibres.

polyethylene/20 μm; nylon/22, 52, 102 μm). They also found that cell orientational response was significantly influenced by the small diameter fibres (less than 20 μm). It was claimed by Christel [7] that the geometric configuration of the material had a greater effect than its chemistry on cell orientational behaviour. However, it was found in this study that the orientational behaviour of cells could be influenced by the cell population, as observed on the sample of SiC after 2 days; when the cell population became very dense many cells appeared to be spherical and suspended in groups between the fibres. The spherical appearance of cells in this situation may be due to the mechanism of contact inhibition [8], i.e. the tendency for two cells moving towards one another to cease movement on making contact.

The differences between specimens at the early stage of cell attachment (2 h) detected by the SEM study is likely to be a function of surface chemistry. Apart from the sample of SiC, cells on the other three fibres appeared to be similar, showing the early spreading of cytoplasmic processes, either radial or along the fibres. On the sample of SiC, however, it was interesting to find that many cells were suspended between the fibres rather than attached along the long axis (Fig. 2c and d). The same phenomenon was observed by Curtis and Varde [9], who reported that chicken heart fibroblast cells formed a sheet over the surfaces of silica fibres of 30 μm diameter. It is known thatcell adhesion and spreading are very complex processes and involve first of all the adsorption of serum proteins [10–12]. The surface properties of the materials may influence the type, amount and conformation of the protein that is adsorbed. Different material surface properties may give rise to differential amounts and conformations of protein adsorption, and also may further influence the secretion of protein by the cells into the extracellular matrix since, following cell contact with the substrate for a few hours, the cells assemble and actively secrete extracellular matrix proteins [10]. This may further change the surface of the substrate and enhance cell adhesion and spreading.

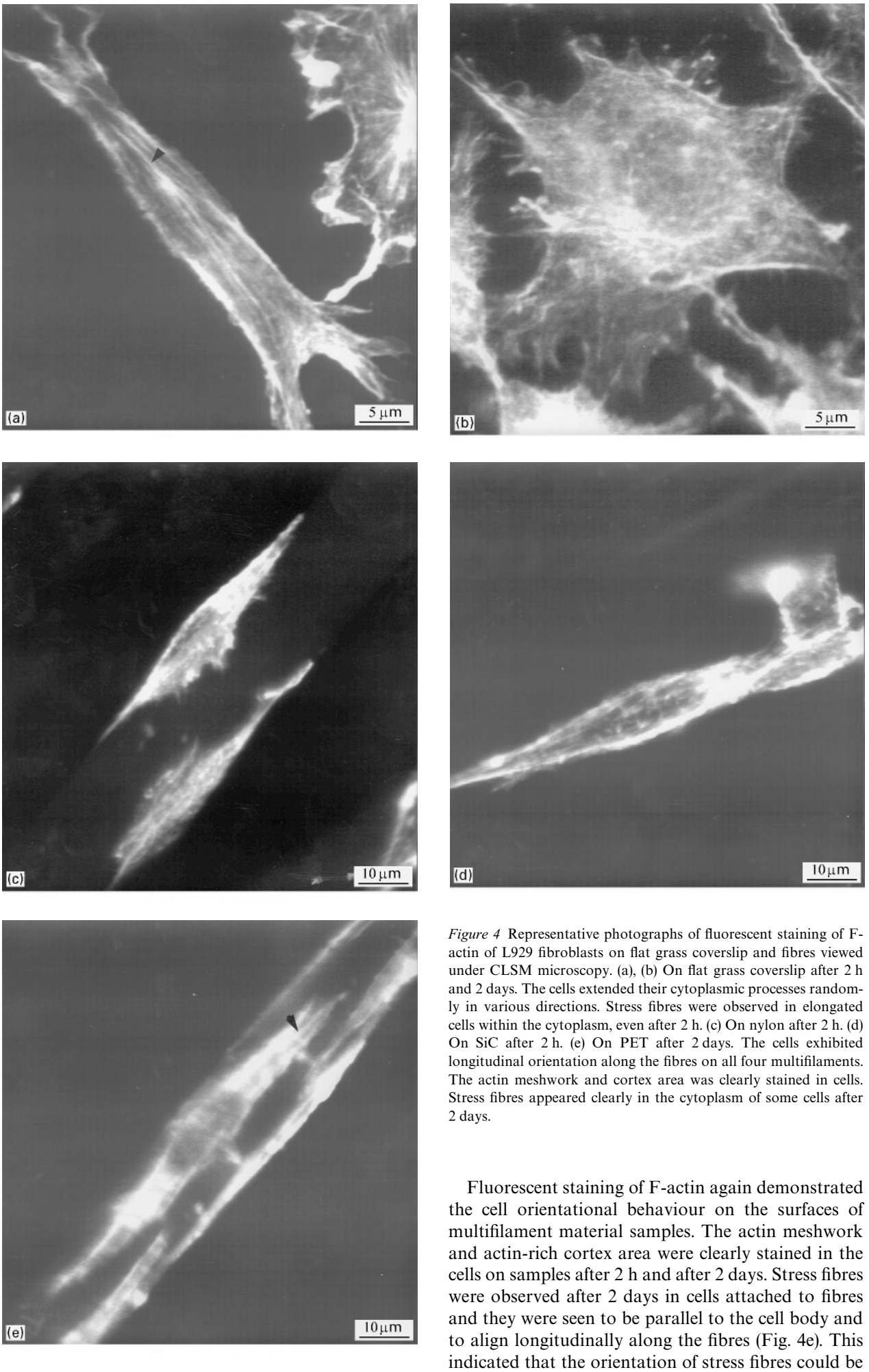

Figure 4 Representative photographs of fluorescent staining of F-actin of L929 fibroblasts on flat grass coverslip and fibres viewed under CLSM microscopy. (a), (b) On flat grass coverslip after 2 h and 2 days. The cells extended their cytoplasmic processes randomly in various directions. Stress fibres were observed in elongated cells within the cytoplasm, even after 2 h. (c) On nylon after 2 h. (d) On SiC after 2 h. (e) On PET after 2 days. The cells exhibited longitudinal orientation along the fibres on all four multifilaments. The actin meshwork and cortex area was clearly stained in cells. Stress fibres appeared clearly in the cytoplasm of some cells after 2 days.

Fluorescent staining of F-actin again demonstrated the cell orientational behaviour on the surfaces of multifilament material samples. The actin meshwork and actin-rich cortex area were clearly stained in the cells on samples after 2 h and after 2 days. Stress fibres were observed after 2 days in cells attached to fibres and they were seen to be parallel to the cell body and to align longitudinally along the fibres (Fig. 4e). This indicated that the orientation of stress fibres could be

a sign of cell orientation. However, the mechanism of this orientational behaviour on fibres is still not clear. A hypothesis concerning cell orientational behaviour on fibres proposed by Dunn and Health [13] was that, due to "the rigidity or inflexibility of the microfilament bundles" they could not function in a bent condition. A recent report on a study of the mechanism of cell orientation on microgrooved substrate revealed that the microtubule was the structure most responsible for cell orientational behaviour [14]. It remains an open question whether the same mechanism of cell orientation on microgrooved substrate can be applied to cells on the surfaces of fine fibres. In order to understand this, further study of the cytoskeleton network and focal contact of cells on the surfaces of small-diameter fibres is considered to be necessary.

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